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DOS AÑOS DE VIGILANCIA DE LA DINÁMICA CLONAL DE *Staphylococcus aureus* RESISTENTES A METICILINA AISLADOS EN UN HOSPITAL DE TERCER NIVEL EN QUITO-ECUADOR

Disertación previa a la obtención del título de Licenciada en Ciencias Biológicas

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Certifico que la Disertación de Licenciatura en Ciencias Biológicas de la Srta. Karen Adriana Loaiza Conza ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Dra. Iliana Alcocer Negrete

Directora de la Disertación

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“*Para ver el mundo en un grano de arena,*

y el cielo en una flor silvestre,

abarca el infinito en la palma de tu mano

y la eternidad en una hora.”

-William Blake (1757-1827)

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TABLA DE CONTENIDOS

LISTA DE ABREVIATURAS

Abreviatura	Significado
°C	Celsius degrees
CA-	Community-associated
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic acid
HA-	Hospital-associated
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
LIAN	LInkage Analysis
MLVA	Multi-Locus Variable-Number Tandem-Repeat Analysis
MLST	Multi-Locus Sequence Typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MSN	Minimum Spanning Network
NYJ	New York-Japan
PCR	Polymerase chain reaction
PVL	Panton Valentine Leukocidin
S. aureus	*Staphylococcus aureus*
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SLV	Single locus variant
SSTI	Skin and soft tissue infections
ST	Sequence type

Abreviatura	Significado
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
V-DICE	VNTR DIversity and Confidence Extractor
VNTR	Variable Number Tandem Repeats
WGS	Whole Genome Sequencing

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1. RESUMEN

El objetivo del presente estudio fue vigilar la dinámica clonal de dos años de aislados de *Staphylococcus aureus* resistentes a meticilina (SARM) en un hospital ecuatoriano de tercer nivel a través de un estudio con muestras colectadas desde Abril 2009 a Diciembre 2010. El genotipaje fue realizado con Multi-Locus Variable-Number Tandem-Repeat Analysis (MLVA), el tipaje con Staphylococcal Cassette Chromosome *mec* (SCC*mec*), y los genes (*lukS/F-PV*) de PVL (Panton Valentine Leukocidin) fueron detectados. A partir de 82 pacientes con infecciones por SARM, se analizaron 93 aislamientos. En general, al referirnos a los porcentajes más altos: 68 (73,1%) fueron USA300 (ST8) y variantes, 72 (77,4%) albergaron SCC*mec* atípicos, y 59 (63,4%) presentaron genes para PVL, incluyendo 44 (71,0%) de los 62 casos de infecciones en piel y tejidos blandos. Fenotípicamente, 30 (44,1%) de 68 aislados ST8 fueron sensibles a todos los antibióticos probados, exceptuando cefoxitina. Todos los 93 aislamientos fueron resistentes a cefoxitina y no se encontró niveles de resistencia para vancomicina o linezolid. La epidemiologia molecular revelo que los aislados ecuatorianos pertenecían a 2 grandes complejos clonales: CC8 y CC5. Con 22 de 31 genotipos representando al ST8, el CC8 es la población predominante en Ecuador. Sin embargo, la dinámica clonal no fue simple. El clon USA300 (ST8) y variantes circularon continuamente durante el período de 21 meses pero otros clones como el Brasileño (ST239), USA800/pediátrico (ST5), Ibérico (ST247), SLV de ST239 (ST241), Coreano (ST72) y Alemán del sur/Italiano (ST228) también estuvieron en circulación. La circulación esporádica de diferentes ST sugiere una alta diversidad de linajes junto con transferencia horizontal de genes apoyada por la variedad

encontrada en el SCC*mec.* Los autores creen que el surgimiento y la sustitución clonal de SARM están sucediendo más frecuentemente de lo que se había pensado anteriormente. Los resultados de este estudio complementan la investigación epidemiológica en la región dado que este estudio es el primer paso en la elucidación de las cepas de SARM predominantes de Ecuador y perfiles de resistencia.

Palabras clave: Ecuador, MLVA, SARM, PVL, SCC*mec*, ST8

2. ABSTRACT

This study aims to surveil the population dynamics in two-year methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in a tertiary Ecuadorian hospital through a study with samples collected from April 2009 to December 2010. Multi-Locus Variable-Number Tandem-Repeat Analysis (MLVA) genotyping and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing were performed and Panton Valentine Leukocidin (PVL) genes (*lukS/F-PV)* were detected. From 82 patients with MRSA infections, 93 isolates were analyzed. In general, when referring to the highest percentages: 68 (73.1%) were USA300 (ST8) and variants, 72 (77.4%) harbored atypical SCC*mec* patterns, and 59 (63.4%) presented PVL genes, including 44 (71.0%) of the 62 cases of skin and soft tissue infections. Phenotypically, 30 (44.1%) of 68 ST8 isolates were susceptible to all proven antibiotics, except cefoxitin. All 93 isolates were resistant to cefoxitin and no resistance levels were found for vancomycin or linezolid. Molecular epidemiology revealed Ecuadorian isolates belonged to 2 major clonal complexes: CC8 and CC5. With 22 of 31 genotypes accounting for ST8, CC8 is the predominant MRSA population in Ecuador. Nevertheless, clonal dynamics were not simple. USA300 (ST8) clone and variants circulated continuously during the 21 months period but other clones such as Brazilian (ST239), USA800/pediatric (ST5), Iberian (ST247), SLV of ST239 (ST241), Korean (ST72), and South-German/Italian (ST228) were also in circulation. Sporadic circulation of different ST suggests high linage diversity along with horizontal gene transfer supported by the variety found in SCC*mec*. The authors believe MRSA clonal emergence and substitution is happening more frequently than previously though. This results complement

epidemiological research in the region given that this study is the first step in the elucidation of Ecuador's predominant MRSA strains and resistance profiles.

Key Words: Ecuador, MLVA, MRSA, PVL, SCC*mec*, ST8

3. MANUSCRITO PARA PUBLICACIÓN

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TÍTULO

Two-year clonal dynamics surveillance of methicillin-resistant *Staphylococcus aureus* isolates in a tertiary hospital in Quito-Ecuador

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Two-year clonal dynamics surveillance of methicillin-resistant *Staphylococcus aureus* isolates in a tertiary hospital in Quito-Ecuador

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Running Head: Clonal dynamics of methicillin-resistant *S. aureus*

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Keywords: Ecuador, MLVA, MRSA, PVL, SCC*mec*, ST8

This study aims to surveil the population dynamics in two-year methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in a tertiary Ecuadorian hospital through a study with samples collected from April 2009 to December 2010. Multi-Locus Variable-Number Tandem-Repeat Analysis (MLVA) genotyping and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing were performed and Panton Valentine Leukocidin (PVL) genes (*lukS/F-PV)* were detected. From 82 patients with MRSA infections, 93 isolates were analyzed. In general, when referring to the highest percentages: 68 (73.1%) were USA300 (ST8) and variants, 72 (77.4%) harbored atypical SCC*mec* patterns, and 59 (63.4%) presented PVL genes, including 44 (71.0%) of the 62 cases of skin and soft tissue infections. Phenotypically, 30 (44.1%) of 68 ST8 isolates were susceptible to all proven antibiotics, except cefoxitin. All 93 isolates were resistant to cefoxitin and no resistance levels were found for vancomycin or linezolid. Molecular epidemiology revealed Ecuadorian isolates belonged to 2 major clonal complexes: CC8 and CC5. With 22 of 31 genotypes accounting for ST8, CC8 is the predominant MRSA population in Ecuador. Nevertheless, clonal dynamics were not simple. USA300 (ST8) clone and variants circulated continuously during the 21 months period but other clones such as Brazilian (ST239), USA800/pediatric (ST5), Iberian (ST247), SLV of ST239 (ST241), Korean (ST72), and South-German/Italian (ST228) were also in circulation. Sporadic circulation of different ST suggests high linage diversity along with horizontal gene transfer supported by the variety found in SCC*mec*. The authors believe MRSA clonal emergence and substitution is happening more frequently than previously though. Results complement epidemiological research in the region given that this study is the first step in the elucidation of Ecuador's predominant MRSA strains and resistance profiles.

Staphylococcus aureus is one of the world's clinical significant pathogens, and the subject of intensive investigations in matters of: Virulence, drug resistance phenotypes, genotypes, and population genetics (1). Investigations are constantly being focused on methicillin-resistant *Staphylococcus aureus* (MRSA) population structure; since an appropriate control of the MRSA problem requires a thorough understanding of the processes underlying the emergence and spread of its clones (2). The key step in emergence of MRSA clones is the acquisition of mobile genetic element Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (2, 3), which carries the *mecA* gene responsible for methicillin resistance (2, 4). MRSA is defined by the presence of SCC*mec* through SCC*mec* types being recognized by combinations of *mec* and *ccr* gene complexes (5). With at least 11 SCC*mec* types (I-XI) reported so far (6), SCC*mec* typing is considered more reliable than clinical risk factors and demographic data for molecular epidemiologic analysis (7). Yet, the development of a high-throughput molecular typing assay (8), based on binary combinations of the *mec* class and the *ccr* allotype, for rapid and simple strain characterization, as well as a rationalized unifying nomenclature (6, 9), based on the structure of SCC*mec* elements, was accomplished just recently.

Understanding the molecular epidemiology and evolution of MRSA offers many advantages for infection control (8). In *S. aureus,* infection outcome is generally determined by strain's genetic background, virulence factors, along with host response (10-12). Emerging MRSA clones appear to have acquired traits that render them more virulent or able to colonize better (12). Acquisition of Panton-Valentine Leukocidin (PVL) genes has been proposed as potentially contributing to the success of some MRSA linages as well as a significant virulence factor in particular types of infections (13, 14). Clinically, MRSA which harbor PVL are most

often associated with pyogenic skin and soft tissue infections (15) but can also cause life-threatening disease such as necrotizing pneumonia (16). PVL is a bicomponent leukocidin, encoded by two co-transcribed genes: *lukS-PV* and *lukF-PV* (*lukS/F-PV*) (17), that cause human neutrophil cell death (13) which are the first line of defense against staphylococcal infections (12). Even though some animal-model-based investigations have demonstrated PVL's pathogenicity along with an epidemiological association between PVL with MRSA lineages and clonal complexes (14), PVL's role as a virulence determinant is still under debate (12, 13) making screening for PVL and MRSA's genetic background a routine laboratory procedure.

Clonal analyzes are a trend research topic for they uncover bacteria evolution and diversification (18). For *S. aureus*, molecular typing tools enable a clonal dynamics approach to assess both the substitution phenomenon (diverse clonal groups becoming more or less prevalent in healthcare centers through time) and dissemination, while genotyping assays allow the tracing of outbreaks (19). In MRSA's evolutionary history, since the analysis of Multi-Locus Sequence Typing (MLST) data allowed the description and unification of MRSA clonal complexes (20), disclosing strain's genetic background has come to be necessary. Unfortunately MLST relatively high costs made the method not so accessible for most laboratories (21). In recent years, Multi-Locus Variable-Number Tandem-Repeat Analysis (MLVA) emerged as a more reasonably priced alternative. MLVA is a genotyping technique, based on the measurement of DNA fragment's length that enables epidemiological studies, follow-up of clonal complexes (CCs) evolution and identification of potential ancestors (1).

MRSA worldwide dissemination is due to a few successful clones (22) with a rather specific geographical pattern (23). In Latin America, since 1990, various epidemic clones have spread with most of the current circulating clones being related to the five major international clones: NYJ (New York/Japan), Pediatric, Brazilian, Iberian, and Hungarian (24). As for South America, north cone countries like Ecuador, Colombia, and Peru have reported a variety of epidemic MRSA clones and variants circulating. In Ecuador, the highly virulent USA300 lineage has been established as the almost exclusive CA-like (Community-Associated-like) clone with the Brazilian clone accounting as the HA-like (Hospital-Associated-like) clone (25). For Colombia, the Cordobes/Chilean clone is the predominant clone (24, 26), with the USA300 clone variant (25), variants of the Pediatric clone (24, 27-29), Brazilian clone (25, 30, 31), and Iberian clone also being reported (24). On the other hand, Peru has identified the HA-MRSA clone USA800 (24, 32). South cone countries like Chile, Brazil, and Argentina also report several clones. In Chile, the Cordobes/Chilean clone is the main clone but the Brazilian clone has also been reported in the country (24, 30). For Brazil, the Brazilian clone and variants persistently circulate through hospitals (24, 33) followed by other minor clones that have also been detected, in particular clones related to the NYJ (24, 34), Hungarian and Pediatric clone (24, 35). For Argentina, the Cordobes/Chilean clone and variants along with the Brazilian clone are the major clones (30, 31), coexisting with variants of the Pediatric clone (24).

The aforementioned highly epidemic MRSA clones have been described owning the following genotypes (11, 25). Brazilian clone: MRSA-ST239-III (an MRSA sequence type 239 clone bearing SCC*mec* type III), Chilean/Cordobes clone: MRSA-ST5-I, USA300 variant: MRSA-ST8-IVc [and its single-locus variant

(SLV) MRSA-ST923-IV], and MRSA-ST30-IVc clone that has recently been reported in other South America countries like Uruguay and Brazil (11, 25).

Even though information on MRSA epidemiology in Latin America has grown (36), data concerning Ecuador is still scarce. Factors as a biased population sample, and unrepresentative or affected data enforce a challenge in research. In spite of the defiance, Reyes et al. (25) and Cardenas et al.(19) revealed some information about Ecuador's MRSA lineages. Through their research preliminary information and preclusive laboratory assays, we could hypothesize that strains similar or related to the USA300/ST8 clone in Ecuador had not been found yet, along with strains related to other highly epidemic clones in South America. Consequently, our goal in the present study was to surveil the clonal dynamics of two-year MRSA isolates in a tertiary Ecuadorian hospital aiming to contribute with data for a better understanding of the changing characteristics and epidemiology of MRSA. Despite data being from one hospital, results will complement epidemiological research on the region given that this study is the first step in the elucidation of Ecuador's predominant MRSA strains and resistance profiles.

MATERIALS AND METHODS

Setting. Vozandes Hospital of Quito, Ecuador recovered *S. aureus* isolates from patients attending hospital clinical laboratory facilities. *S. aureus* isolates were identified by standard phenotypic methods (mannitol salt agar, catalase test, and coagulase test) at the species level, and checked for methicillin resistance (disk diffusion antibiogram with both oxacillin and cefoxitin). All MRSA isolates were saved for further investigations purposes. One or more isolates per patient were stored frozen at -20/-80^{o}C in the Vozandes Hospital bacterial collection. For each patient, demographic data was collected (e.g. name, age, and sex), as was date and site of isolation. In addition, susceptibility profile encompassing ten antibiotics (cefoxitin, gentamicin, tetracycline, trimethoprim/sulfamethoxazole, ciprofloxacin, erythromycin, rifampin, clindamycin, vancomycin, linezolid) was performed by the Kirby-Bauer Disk Diffusion Susceptibility Test according to the CLSI guidelines (37).

Study population. All MRSA isolates recovered between April 2009 and December 2010 were borrowed from the Vozandes Hospital bacterial collection. A total of ninety three samples isolated from various clinical infections: 62 (66.7%) skin and soft tissue, 9 (9.7%) tracheal-bronchial, 3 (3.2%) post-surgical wound, 8 (8.6%) blood, 5 (5.4%) bone marrow, and 6 (6.5%) others recovered from catheters and sterile liquid samples like pleural, synovial, and drainage fluid (supplementary data). DNA from strains (TrSa214, TrSa152, TrSa150, and TrSa134) genotyped by fragment analysis were used as controls for MLVA assay.) DNA from this control strains was kindly donated by Christine Pourcel from París-Sud University.

MLVA genotyping. MLVA was performed following the methodology of Pourcel et al. (1) through agarose gel electrophoresis of PCR product. Amplicons size was measured with the assistance of ImageLab Software V 4.0.1 (Bio-Rad Laboratories, Hercules, California), and compared with the MLVAbank for Bacterial Genotyping (http://mlva.u-psud.fr/ [*Staphylococcus aureus* database]). MLVA profiles were assigned the nearest sequence type (ST) of its neighbor strain on *Staphylococcus aureus* database with criteria of relatedness that could differ from zero to five VNTR. Population clonal structure was determined using a ten loci panel as a first-line simplified assay, posteriorly a subsample of ST8 related isolates was submitted to a second set of four additional loci to increase resolution (1). MLVA profiles are available for comparison in the MLVAbank for Bacterial Genotyping (http://mlva.u-psud.fr/mlvav4/genotyping/view.php?b=CBQCA_MRSA/ [CBQCA_MRSA database]).

SCC*mec* typing and PVL. *S. aureus* species, methicillin resistance, and PVL genes detection were achieved through amplification of *nuc*, *mecA*, and *lukS/F-PV* (co-presence) genes respectively by a multiplex-PCR assay described previously (38). SCC*mec* types were characterized using a PCR based methodology reported by Chen et al. (8) through an assay that consists of two multiplex panels, the combination of which results in two targets (*mec* class, *ccr*) for each SCC*mec* type.

Statistical analysis. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method was run using the categorical coefficient (Hamming's distance) using PAST Ver. 2.06 with bootstrap analysis run at 10000 iterations. A minimum spanning network (MSN) was generated using R Ver. 3.1.2 to allow the creation of clusters and visual relationships between the isolates. Simpson's

184 diversity index and Hunter-Gaston's diversity index were calculated using V-DICE
185 (VNTR DIversity and Confidence Extractor; http://www.hpa-
186 bioinformatics.org.uk/cgi-bin/DICI/DICI.pl, last accessed on August 2015. Null
187 hypothesis of linkage equilibrium was tested using LIAN (LInkage ANalysis) Ver.
188 3.7 with Monte-Carlo simulation run at 10000 iterations,
189 http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query, last accessed on
190 August 2015. Disclaimer. Clinical variables were not included in the analysis, this
191 being a limiting factor of this study in the interpretation of findings related to
192 mortality and clinical recovery.

RESULTS

Genetic relatedness with MLVA. In our local MRSA population, a total of 31 genotypes (Appendix 1) were observed among the 93 isolates (panel 10 loci). Likewise, 22 genotypes (panel 10 loci) and 31 genotypes (Appendix 2) (panel 14 loci) were observed among 68 ST8 subsample. Population relatedness was as following: 68 (73.1%) ST8, 13 (14.0%) ST239, 6 (7.5%) ST247, 3 (3.2%) ST5, 1 (1.1%) ST241, 1 (1.1%) ST72, and 1 (1.1%) ST228.

SCC*mec* typing, antibiotic susceptibilities and PVL. SCC*mec* typing of 93 isolates identified the presence of four types: 10 (8.8%) isolates harboring SCC*mec* type IVh, 5 (5.4%) type IV, 3 (3.2%) type III and 3 (3.2%) type IIIB. Interestingly, 72 (77.4%) isolates showed an atypical PCR pattern that could not be fitted into current SCC*mec* types, representing 18 different variants (Table 1). Antibiotic susceptibility profiles showed 22 resistance phenotypes (Table 3). Among 93 cefoxitin resistant isolates, 34 (36.6%) were resistant to tetracycline, 34 (36.6%) to erythromycin, 27 (29.0%) to ciprofloxacin, 22 (23.7%) to gentamicin, 22 (23.7%) to trimethoprim/sulfamethoxazole, 22 (23.7%) to clindamycin, and 21 (22.6%) to rifampin. No resistant isolates were registered for vancomycin and linezolid (Figure 5). Finally, virulence factor Panton Valentine Leukocidin (PVL) was detected in 59 (63.4%) isolates.

DISCUSSION

Epidemiological surveillance of MRSA is complex. Circulating MRSA clones vary through regions and countries (24) highlighting the need for highly discriminatory typing tools (2). In our first approximation to MRSA clonal dynamics, an MLVA approach was performed as a first line assay. By applying MLVA on 93 MRSA, 31 distinct genotypes were obtained with 10 loci. To increase resolution in our subsample, we further enhanced the assay through the addition of four additional loci as suggested by Pourcel et al. (1). A rise in genotypes was observed in the subsample, 22 genotypes obtained with 10 loci to 31 genotypes obtained with 14 loci, validated how the discriminatory rate of MLVA improved with the use of more loci. We don't believe this assures the technique needs the adding of more loci continuously. Rather, we agree with a thoughtful selection of loci and primers for the formation of a proper set that can efficiently assign and amplify every single strain in a population. For *S. aureus* genotyping, various loci have been proposed through the years (1, 21, 39) but, there is still no consensus on the set of VNTR markers. *S. aureus* intraspecies genetic variability is an attribute to be taken into consideration for VNTR marker selection if few loci are going to be considered. Occasions when VNTR amplification does not occur has been attributed to intraspecies genetic variability. In our population, there were 15 times in which a locus could not be amplified. It is not a disappointing percentage (1.2%) when 1302 amplifications were made but as the majority of these events occurred in locus SA0122, the use of this locus in a single assay is not advisable. We recommend the more discriminatory loci: SA0122, SA1132, SA0704, SA0311 and SA0266 for recent evolution event assessment, as well as for short scale epidemiological studies (Table 2).

Before MLVA, the best available techniques used to type *S. aureus* were Multi-Locus Sequence Typing (MLST) and Pulsed-Field Gel Electrophoresis (PFGE). These techniques, though innovative at the time of their conception, had difficulties that needed to be assessed. MLST highly conserved sequences have a slow evolution rate which causes low resolution in recent evolution investigations, also low resolution is not suitable for short-term epidemiological studies (1). PFGE allowed the constitution of shared databases but only at a national level (21). MLVA technology has overcome the above technical difficulties as it has proven to be as portable as MLST, and more discriminatory than PFGE and MLST (21). Nonetheless, for complete and accurate epidemiological information on bacterial strain diversity, genomics are required. The ultimate choice of technology at the moment is Whole Genome Sequencing (WGS). However WGS requirements (resources, infrastructure) make the technology not accessible for every study. MLVA is the best alternative for *S. aureus* genotyping while Next Generation Sequencing is not yet easily available, especially in developing countries. MLVA is a technique that can be performed in a laboratory with basic molecular biology equipment and in contrast to other fingerprinting methods, it allows the coding of results as strings of numbers which makes the resulting profiles highly reproducible and portable between laboratories and countries (1). In this study, MLVA technology provided an informative basis for monitoring our MRSA population. MLVA high reproducibility and comparability, along with available on-line genotyping databases [MLVAbank for Bacterial Genotyping (http://mlva.u-psud.fr/)] enabled the query and comparisons of our results. Additionally it allowed the placement of our genotypic profiles online (http://mlva.u-psud.fr/ [CBQCA_MRSA database]). By using MLVA, we did not only succeed in

genotyping our local population, but also contributed with valuable data that will enable MLVA usage in our region.

To surveil which clones were the most prevalent in our MRSA population, we performed a cluster of our profiles together with other international MRSA in the MLVAbank. Cluster analysis revealed our population had genetic relatedness with 7 different ST but as we analyzed ST correlation with SCC*mec* type, we observed ST did not harbor only one kind of SCC*mec*, therefore they could be better described as linages and clone variants. In Ecuador, the presence of linages such as USA300 (ST8) and Brazilian (ST239) has been previously reported (25). Now, in our study we are also reporting the presence of such linages. ST8 is the predominant linage (73.1%) while ST239 is present in a lower percentage (14.0%) of our population. Interestingly we found the occurrence of ST5, USA800 linage (3.2%). USA800 linage, also known as pediatric, has been previously described circulating in other north cone countries (24, 40) but not yet in Ecuador. Aside the highly epidemic linages in our region; we are also reporting the presence of other linages such as ST247, Iberian; ST241, a single locus variant of ST239; ST72, Korean; and ST228, South-German/Italian. It is important to notice that among these new circulating linages, Iberian linage (ST247) is present in a higher percentage (6.5%) than the USA800 linage (3.2%).

Loci data provides a series of possibilities for MRSA. Population structure of MRSA used in this study can be observed in both a dendrogram and a MSN. A dendrogram (Figure 1) was built to show the earlier evolutionary history while an MSN (Figure 3,Figure 4) was built to achieve an adequate representation of recent evolutionary events as recommended by Feil et al. (1, 18, 41). With 10 loci data ST of 2 isolates could not be assigned, however when we observed the MSN

(Figure 3) in which ST unrelated isolates were clustered near ST228 and ST5, we suspected these isolates belonged to CC5. To achieve the right assignment of these isolates, a higher resolution was needed. With 14 loci, ST unrelated isolates were clustered on ST5 (Figure 4). Interestingly, in the ST8 subsample, previously designated ST8 isolates changed its relatedness. From 68 ST8 isolates, 3 change to ST241, 1 to ST239, and 64 remained ST8. MSN shows a more realistic model of the way in which bacterial clones emerge and diversify to form clonal complexes (18). Clonal complex assignment for each ST was done by literature comparisons. Using cut value of 0.6, 3 clusters could be observed in the dendrogram (Figure 1) which are supposed to be equivalent to MLST clonal complexes but in our isolates ST were assigned to 2 clonal complexes: CC8 and CC5. Interestingly one isolate was position as an outer group which caused the formation of 3 complexes: ST72 isolate corresponded to CC8 but it did not cluster with it. This outlier may have been misplaced due to an unequal evolutionary rate, or it could have been that ST72 is in fact more related to CC5 than to CC8. We also notice MSN positioned ST72 (Figure 4) near both CC5 and CC8. Our dendrogram showed low bootstrap values for some branches which may indicate a founder effect, leading to an overall low phylogenetic diversity of this population, although some limited horizontal gene transfer could also have played a role in generating these low bootstrap values. Both concepts are consistent to what would be expected for bacteria emergence. Also as expected, output data of LIAN (Appendix 3) I_A^S =0.2179 show linkage disequilibrium with a $P< 1.00 \times 10^{-04}$, which fitted with what was currently known about population genetics of bacteria in general. This data assures us that homoplasy (the occurrence of genotypes that are identical by state

but not by descent) is low in our population and shows MRSA following a clonal model of mutations more than homology.

MRSA typing of our 93 isolates revealed 4 SCC*mec* types and several (18) atypical variants circulating (Table 1). This shows some discrepancies as for what is expected from the local spread of a clone, where most isolates within clones harbored an identical SCC*mec* type (2). We could cautiously suspect not local spread but local emergence along with distinct importations of related isolates, because even if local acquisition is happening, it may not explain all the variability observed. Atypical variants may be caused by the dynamic nature of SCC*mec* elements in which varibility could be attributed to the oriC envirion. SCC*mec* is part of oriC envirion, a region around the oriC where multiple exogenous genes are accumulated as a result of repeated horizontal gene transfer events, once again low bootstrap values in the dendrogram (Figure 1) could confirm this suspicion. The oriC has been given the function of being an extremely active diversifier of the chromosome which means oriC environ serves as the generator of diversity by accumulating foreign genes, deleting useless or hazardous ones, and event inverting large chromosomal fragments across the oriC (42). Another plausible explanation for SCC*mec* variability could be the use of Chen et al.(8) methodology. Chen's assay allows a high-throughput typing targeting the specific *mec* and ccr regions of the isolates in an individual way. This is a fundamental difference with previous typing schemes which have risk discriminatory power for convenience by targeting zones located within J regions.

In our atypical variants exist a pattern that has been observed in a similar study by Basset et al. (2) whom reported our pattern NT16 as U7. Other studies have also reported atypical SCC*mec* in MRSA, however these patterns are more

common in other staphylococcal species (2, 8, 43). Due to the presence of atypical variants with different combinations of *mec* and *ccr* complexes, we believed in using *ccr* and *mec* complexes as an adequate nomenclature of SCC*mec* element. Several nomenclatures have been proposed through the years but a consensus among all staphylococcal researchers has not been reached. Interestingly, 73 (78%) of our variants carried *ccrC*. We believe this may have various explanations. 1. It may be due to *ccr* recombinase being carried in other unknown mobile genetic element rather than being inside SCC*mec.* 2. The recombinase might be outside of SCC*mec* but inside SCC*Hg.* 3. It is part of SCC*mec.* Further studies will be needed to define if atypical variants should be considered new cassettes. But for epidemiological purposes, *mec* and *ccr* assignment may be enough (43). If we interpreted our atypical SCC*mec* patterns with the original definition of SCC*mec* type being defined by the binary combination of the *ccr*-gene complex and the class of *mec*-gene complex, we could reduce the reported variants to 2 (NT10, NT12). At the present, 11 SCC*mec* types are registered according to the IWG-SCC (http://www.sccmec.org/Pages/SCC_HomeEN.htmL, last accessed 15 March 2015) but they do not exclude the finding of more variants: “more types are expected to be found in the future”. In addition, there were 4 occasions in which the *mec* type could not be amplified. Another attention-grabbing point in our results was the absence of HA-cassettes (I and II) and recently described cassettes (VI-XI). Absence of these previously described SCC*mec* types could mean sampling limitations or even primer mutations, mutations in specific primer regions that did not allow us to genotype 93 isolates.

The percentage of resistance in 93 isolates remained the same though the years (Figure 5) because independent resistance levels for each antibiotic did not reveal

361 much information. To assess resistance in a more realistic manner we analyzed
362 resistance phenotypes. When we correlated them together with ST, relationships
363 were noticed. ST239 strains harbored highly resistant phenotypes while ST8
364 harbored highly susceptible phenotypes (Table 3).

365 More than half of our isolates 59 (63.4%) presented PVL genes, including
366 44 (71.0%) of the 62 cases of skin and soft tissue infection isolates. In South
367 America, the presence PVL has been reported among MRSA populations (24).
368 Our high rate of PVL positivity could be attributed to the majority of isolates being
369 from skin and soft tissue infections and being related to the USA300 clone (ST8).
370 Both skin and soft tissue (15) and ST8 have been associated with PVL positivity
371 (11). We found important to say that an increased in virulence does not imply a
372 bad clinical outcome. In early reports PVL was alleged it to be a deadly toxin,
373 nevertheless nowadays those characteristics have been refuted in several papers
374 (11, 14). What it most, in recent studies PVL has been related to favorable and
375 improved clinical outcomes. In what is most in our analysis of resistance
376 phenotypes together with PVL, a relationship virulence-resistance was noticed.
377 Highly resistant ST239 strains did not carry PVL while ST8 highly susceptible
378 strains carry PVL. Even though some exceptions can be observed, patterns are
379 much clearer.

380 In conclusion, this study aimed to understand the clonal dynamics of a 93
381 MRSA population thought the surveillance of clones and SCC*mec* types. CC8 is
382 Ecuador predominant population, with USA300/ST8 linage being in constant
383 circulation. Constant genetic change at a local or short scale could be suspect.
384 Clone emergence along with acquisition of SCC*mec* is happening. Although
385 USA300/ST8 continues to be the highly epidemic clone reported for the country,

386 new circulating linages: Iberian/ST247, ST241/SLV of ST239, Korean/ST72, and
387 South-German/Italian/ST228, suggest importations. Because of the relationship
388 between ST linages and resistance phenotypes, monitoring MRSA evolution and
389 distribution supports the use of effective measures against the most prevalent
390 clones in each local region. More than 50 years later eradication is still far ahead
391 us as MRSA continues to be one of the most common hospital pathogens
392 worldwide.

Availability of supporting data

The data set supporting the results of this article is included as an additional file. MLVA profiles are available for comparison in the MLVAbank for Bacterial Genotyping (http://mlva.u-psud.fr/ [CBQCA_MRSA database]).

Competing interests

All authors report no conflicts of interest relevant to this article.

Authors' contributions

All authors equally participated in designing the study, acquisition, analysis and interpretation of data. All authors read and approved the final manuscript.

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Tables.

Table 1. SCC*mec* patterns found in 93 MRSA.

Varianta	No. of isolates	ccr type(s)	mec class(es)b
III	3	3	A
IIIB	3	3,5	A
IV	5	2	B
IVh	10	2,5	B
NT1	2	2,3	A
NT2	3	2,3,4,5	A
NT3	2	2,3,5	A
NT4	1	2	A,B
NT5	3	2,3	A,B
NT6	1	2,5	A,B
NT7	1	3,5	A,B
NT8	8	2,3,5	A,B
NT9	4	2,3	B
NT10	1	3	B
NT11	5	3,5	B
NT12	2	5	B
NT13	1	1,2,5	B
NT14	2	2,3,4,5	B
NT15	31	2,3,5	B
NT16	1	2	-
NT17	3	2,3,5	-
NT18	1	3,5	-

[a], NT stands for Non-Typable.

[b], - no amplification could be obtained.

Table 2. Diversity indexes for VNTR data of 93 MRSA.

Locus	K[a]	max(pi)[b]	Simpson's diversity index		Hunter-Gaston's diversity index	
			Diversity Index[c]	Confidence Interval[d]	Diversity Index[c]	Confidence Interval[d]
SA0122/24_01	7	0.567	0.631	0.540 - 0.722	0.637	0.546 - 0.728
SA1425	9	0.567	0.618	0.530 - 0.706	0.625	0.537 - 0.713
SA1132/63_01	6	0.536	0.607	0.540 - 0.674	0.614	0.546 - 0.681
SA0704/67_01	6	0.598	0.586	0.495 - 0.676	0.592	0.501 - 0.682
SA0906	6	0.639	0.534	0.440 - 0.628	0.540	0.445 - 0.634
SA1213	5	0.680	0.485	0.387 - 0.584	0.491	0.392 - 0.589
SA0311	5	0.711	0.441	0.344 - 0.538	0.446	0.349 - 0.543
SA1756	3	0.763	0.366	0.274 - 0.459	0.370	0.278 - 0.463
SA0266/81_01	5	0.825	0.305	0.193 - 0.417	0.308	0.196 - 0.420
SA1194	4	0.887	0.208	0.103 - 0.313	0.210	0.105 - 0.315
SA1729	6	0.907	0.175	0.073 - 0.277	0.177	0.075 - 0.279
SA2039	4	0.907	0.172	0.073 - 0.270	0.173	0.075 - 0.271
SA1291	4	0.928	0.136	0.045 - 0.228	0.138	0.046 - 0.229
SA1866	2	0.990	0.020	0.000 - 0.060	0.021	0.000 - 0.060

[a], K = Number of different repeats present at this locus in this sample set.

[b], max(pi) = Fraction of samples that have the most frequent repeat number in this locus (range 0.0 to 1.0).

[c],Diversity Index (for VNTR data)= A measure of the variation of the number of repeats at each locus. Ranges from 0.0 (no diversity) to 1.0 (complete diversity).

[d], Confidence Interval = Precision of the Diversity Index, expressed as 95% upper & lower boundaries.

Table 3. Resistance phenotypes found in 93 MRSA.

Phenotype[a]	Antibiotic[b]							PVL	No. of isolates	CC8[c]				CC5[c]		
	GEN	TET	SXT	CIP	ERY	RIF	CLI			ST8	ST239	ST241	ST72	ST247	ST5	ST228
P1	R	R	R	R	R	R	R	+	1		1					
								-	17		12	4		1		
P2	R	R	R	R	R	R		-	1		1					
P3			R	R	R			-	1	1						
P4		R			R		R	+	1	1						
P5	R	R						-	2						2	
P6		R	R					+	1					1		
P7				R	R			+	3	3						
								-	1	1						
P8					R	R		+	1	1						
P9					R		R	+	2	2						
P10	R				R			+	1	1						
P11		R			R			+	2						1	1
P12					R			+	3	3						
P13		R		R				+	1	1						
P14				R			R	+	1	1						
P15				R				+	1	1						
P16		R						+	7	6				1		
								-	1	1						
P17			R					+	1	1						
P18						R		+	1	1						
P19		S/R						+	1	1						
P20			S/R					-	1	1						
P21	S/R	S/R		S/R		S/R	S/R	-	1	1						
P22								+	31	30				1		
								-	9	6			1	2		

[a], P stands for Phenotype.

[b], gentamicin (GEN), tetracycline (TET), trimethoprim-sulfamethoxazole (SXT), ciprofloxacin (CIP), erythromycin (ERY), rifampin (RIF), clindamycin (CLI) vancomycin (VAN) and linezolid (LZD) not shown.

S/R missing data or inconclusive results.

[c], CC stands for clonal complex.

Figures.

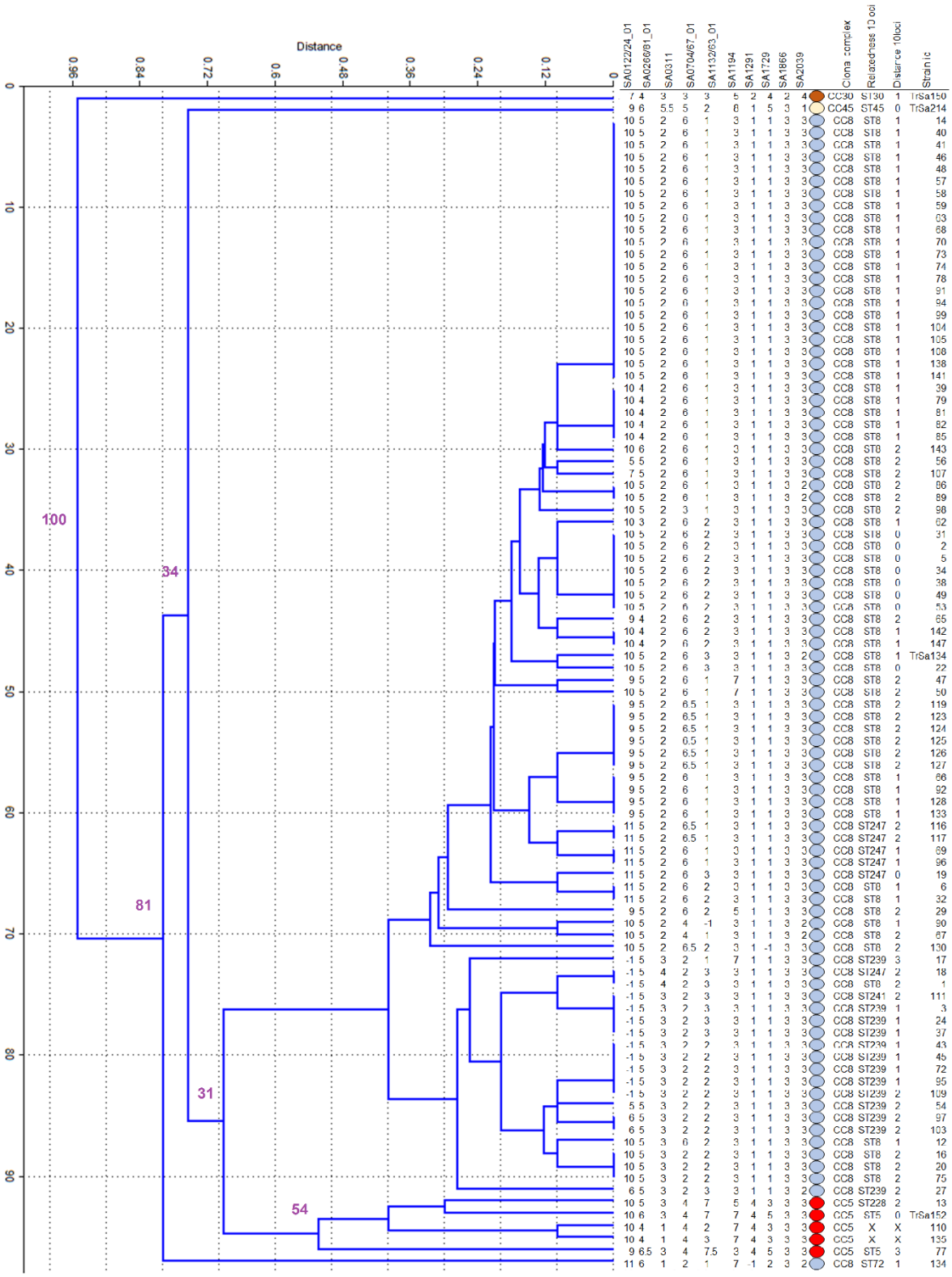

Figure 1. Unrooted dendogram with UPGMA for 10 loci data of 93 MRSA.
Coph. Corr.: 0.9534. Cut value at 0.6 corresponds to MLST clonal complex. Color code is done according to clonal complex whereas clustering is done for displayed loci data. Clonal complex, Relatedness, Distance, and Strain id are shown. Cluster bootstrap values are shown for main clusters.

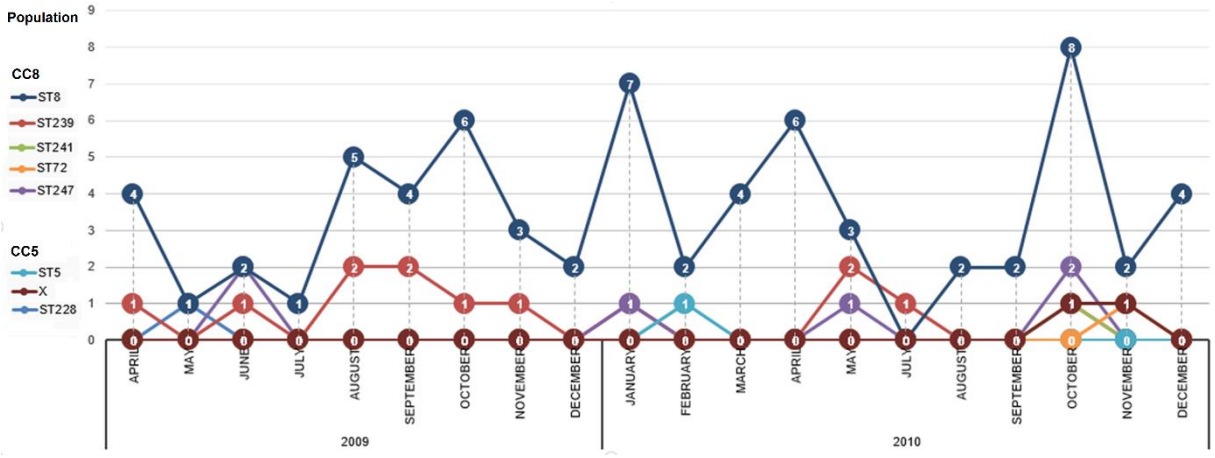

Figure 2. Two-year clonal dynamics of 93 MRSA.

Colors code is based on ST relatedness by MLVA. Each dot represents the occurrence of bacteria during the 21 month period.

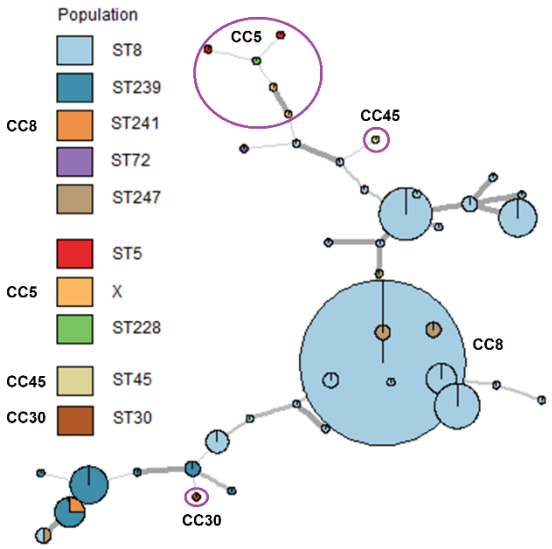

Figure 3. Population snapshot using minimum spanning network for 10 loci data of 93 MRSA.

Colors used are based on ST relatedness by MLVA. Each circle represents a genotype and its clones, and its size is proportional to the number of isolates assigned. A logarithmic scale was used when drawing branches. The thicker branches link genotypes differing by only one allele, and the thinner branches link genotypes differing by more than one allele.

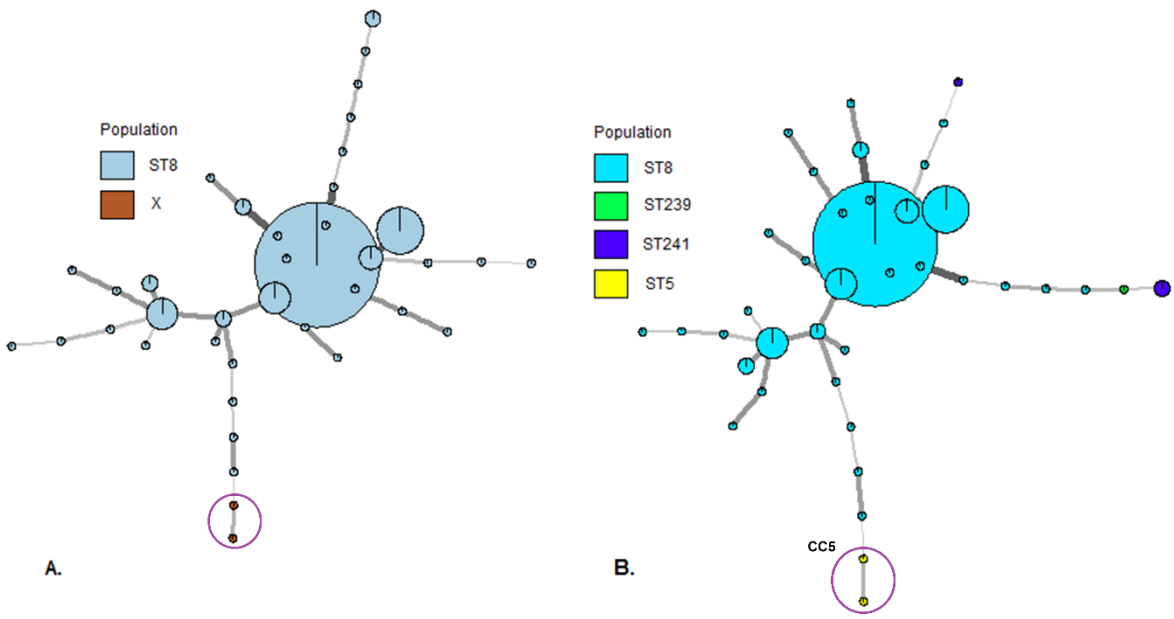

Figure 4. A. Minimum spanning network representation with 10 loci data for 68 ST8 MRSA isolates clustering. B. Minimum spanning network representation with 14 loci data for 68 ST8 MRSA isolates clustering.

Colors used are based on ST relatedness by MLVA. Each circle represents a genotype and its clones, and its size is proportional to the number of isolates assigned. A logarithmic scale was used when drawing branches. The thicker branches link genotypes differing by only one allele, and the thinner branches link genotypes differing by more than one allele.

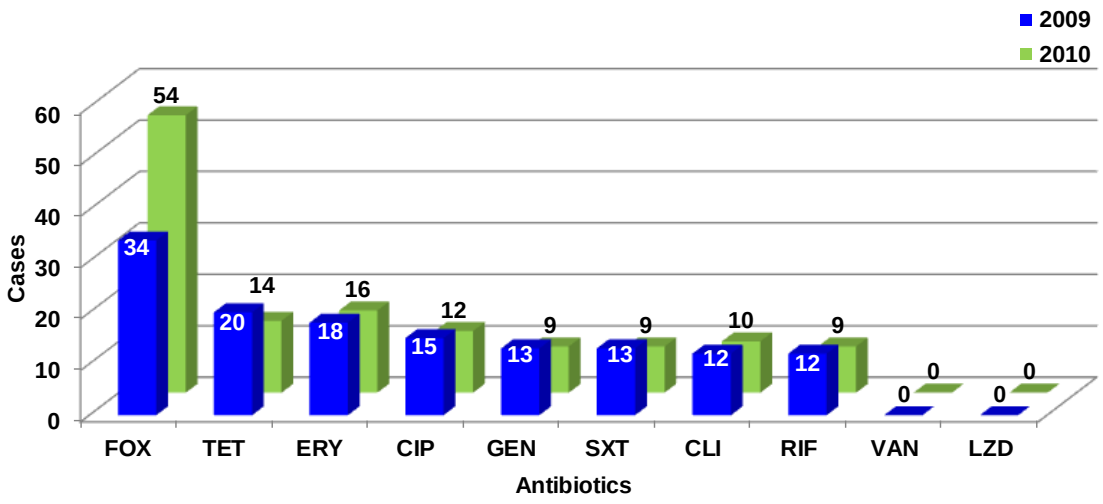

Figure 5. Two year antibiotic resistance levels of 93 MRSA.

Cefoxitin (FOX), tetracycline (TET), erythromycin (ERY), ciprofloxacin (CIP), Gentamicin (GEN), trimethoprim-sulfamethoxazole (SXT), clindamycin (CLI), rifampin (RIF), vancomycin (VAN) and linezolid (LZD).

1. APPENDIX

Appendix 1. Genotypes with 10 loci MLVA of 93 MRSA.

Relatedness[a]	Genotype[b]	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA1132/63_01	SA1194	SA1291	SA1729	SA1866	SA2039
ST239	G1	6	5	3	2	2	3	1	1	3	3
ST8	G2	10	5	2	6	1	3	1	1	3	3
ST8	G3	7	5	2	6	1	3	1	1	3	3
X	G4	10	4	1	4	2	7	4	3	3	3
ST247	G5	11	5	2	6.5	1	3	1	1	3	3
ST8	G6	9	5	2	6.5	1	3	1	1	3	3
ST8	G7	10	5	3	6	2	3	1	1	3	3
ST8	G8	9	5	2	6	1	3	1	1	3	3
ST228	G9	10	5	3	4	7	5	4	3	3	3
X	G10	10	4	1	4	3	7	4	3	3	3
ST8	G11	10	4	2	6	2	3	1	1	3	3
ST8	G12	10	6	2	6	1	3	1	1	3	3
ST8	G13	10	5	3	2	2	3	1	1	3	3
ST247	G14	11	5	2	6	3	3	1	1	3	3
ST8	G15	10	5	2	6	2	3	1	1	3	3
ST8	G16	10	5	2	6	3	3	1	1	3	3
ST239	G17	6	5	3	2	3	3	1	1	3	2
ST8	G18	9	5	2	6	2	5	1	1	3	3
ST8	G19	11	5	2	6	2	3	1	1	3	3
ST8	G20	10	4	2	6	1	3	1	1	3	3
ST8	G21	9	5	2	6	1	7	1	1	3	3
ST8	G22	10	5	2	6	1	7	1	1	3	3
ST239	G23	5	5	3	2	2	3	1	1	3	3
ST8	G24	5	5	2	6	1	3	1	1	3	3
ST8	G25	10	3	2	6	2	3	1	1	3	3
ST8	G26	9	4	2	6	2	3	1	1	3	3
ST8	G27	10	5	2	4	1	3	1	1	3	2
ST247	G28	11	5	2	6	1	3	1	1	3	3
ST5	G29	9	6.5	3	4	7.5	3	4	5	3	3
ST8	G30	10	5	2	6	1	3	1	1	3	2
ST8	G31	10	5	2	3	1	3	1	1	3	3
ST8	G32	10	5	2	6	3	3	1	1	3	2
ST30	G33	7	4	3	3	3	5	2	4	2	4
ST5	G34	10	6	3	4	7	7	4	5	3	3
ST45	G35	9	6	5.5	5	2	8	1	5	3	1

[a], X isolates that could not be related to any nearest ST

[b], G32, 33, 34, and 35 correspond to control strains

Appendix 2. Genotypes with 14 loci MLVA of ST subsample (68 MRSA).

Relatedness	Genotypea	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA0906	SA1132/63_01	SA1194	SA1213	SA1291	SA1425	SA1729	SA1756	SA1866	SA2039
ST8	G1	10	5	2	6	2	1	3	3	1	3	1	2	3	3
ST8	G2	7	5	2	6	2	1	3	3	1	3	1	2	3	3
ST8	G3	9	5	2	6.5	2	1	3	3	1	3	1	2	3	3
ST8	G4	10	5	3	6	3	2	3	3	1	3	1	2	3	3
ST8	G5	9	5	2	6	2	1	3	3	1	3	1	2	3	3
ST8	G6	10	4	2	6	2	2	3	3	1	3	1	2	3	3
ST8	G7	10	6	2	6	2	1	3	3	1	3	1	2	3	3
ST8	G8	10	5	2	6	2	2	3	3	1	3	1	2	3	3
ST8	G9	10	5	2	6	2	3	3	3	1	3	1	2	3	3
ST8	G10	9	5	2	6	1	2	5	3	1	4	1	2	3	3
ST8	G11	10	5	2	6	2	2	3	3	1	1	1	2	3	3
ST8	G12	11	5	2	6	2	2	3	3	1	3	1	2	3	3
ST8	G13	10	4	2	6	2	1	3	3	1	3	1	2	3	3
ST8	G14	9	5	2	6	2	1	7	3	1	3	1	2	3	3
ST8	G15	10	5	2	6	3	2	3	3	1	3	1	2	3	3
ST8	G16	10	5	2	6	2.5	1	7	3	1	3	1	2	3	3
ST8	G17	10	5	2	6	2	2	3	5.5	1	3.5	1	2	3	3
ST8	G18	5	5	2	6	2	1	3	3	1	3.5	1	2	3	3
ST8	G19	10	5	2	6	2	1	3	3	1	3.5	1	2	3	3
ST8	G20	10	3	2	6	2	2	3	3	1	3	1	2	3	3
ST8	G21	10	5	2	6	1	1	3	3	1	3	1	2	3	3
ST8	G22	9	4	2	6	2	2	3	3	1	3.5	1	2	3	3
ST8	G23	10	5	2	4	2	1	3	3	1	4	1	2	3	2
ST8	G24	10	5	2	6	2	1	3	3	1	4	1	2	3	3
ST8	G25	10	5	2	6	2	1	3	3	1	5	1	2	3	3
ST8	G26	10	4	2	6	1	1	3	3	1	3	1	2	3	3
ST8	G27	10	5	2	6	2	1	3	3	1	3	1	2	3	2
ST8	G28	10	5	2	6	2	1	3	3	1	3.5	1	2	3	2
ST8	G29	9	5	2	6	1	1	3	3	1	1	1	2	3	3
ST8	G30	10	5	2	6	3	1	3	3	1	3	1	2	3	3
ST8	G31	10	5	2	3	2	1	3	3	1	3	1	2	3	3
ST8	G32	10	5	2	6	2	3	3	3	1	3	1	2	3	2

[a], G32 correspond to control strain.

Appendix 3. LIAN Output data for 93 MRSA isolates

Summary Statistics	
V_D	5.2214
V_e	1.7635
I_A^S	0.2179

Testing Null Hypothesis (H_0: $V_D = V_e$)

Monte Carlo (10000 resamplings)	
Var(V_D)	0.0219
P	$< 1.00 \times 10^{-04}$
L	2.0248

Genetic Diversity

Mean genetic diversity (H): 0.3314 +/- 0.0709

Genetic diversity at individual loci ordered left to right:	
h_1	0.6372
h_2	0.3082
h_3	0.4457
h_4	0.5917
h_5	0.6136
h_6	0.2098
h_7	0.1377
h_8	0.1765
h_9	0.0206
h_{10}	0.1733

4. NORMAS PARA PUBLICACIÓN

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January 2015, Instructions to Authors, pages 1–21

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To protect the privacy of individuals mentioned in clinical studies, in case histories, and as sources of isolates, do not identify them by their initials, even as part of a strain designation. Change the initials to numerals or use randomly chosen letters. Do not give hospital unit numbers; if a designation is needed, use only the last two digits of the unit. (Note: established designations of some viruses and cell lines, although they consist of initials, are acceptable [e.g., JC virus, BK virus, and HeLa cells].)

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Authors of manuscripts which contain quantitative real-time PCR applications are encouraged to consult the article concerning minimum information for publication of quantitative real-time PCR experiments (the MIQE guidelines) by Bustin et al. (Clin. Chem. **55:**611–622, 2009) for guidance as to what information should be considered for inclusion in their submission.

Newly determined nucleotide and/or amino acid sequence data must be deposited and GenBank/ENA/DDBJ accession numbers must be included in the manuscript no later than the modification stage of the review process. It is expected that the sequence data will be released to the public no later than the publication (online posting) date of the accepted manuscript. The accession numbers should be included in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Short-Form papers. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/ENA/DDBJ accession number is not provided at the time of the review, authors should provide the annotated sequence data as supplemental material not for publication.

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Analyses should specify the database, and the date of each analysis should be indicated as, e.g., January 2015. If relevant, the version of the software used should be specified.

See "Presentation of Nucleic Acid Sequences" for nucleic acid sequence formatting instructions.

The URLs of the databases mentioned above are as follows: DNA Data Bank of Japan (DDBJ), http://www.ddbj.nig.ac.jp/; European Nucleotide Archive (ENA), http://www.ebi.ac.uk/ena/; and GenBank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/nucleotide.

Proper Use of Locus Tags as Systematic Identifiers for Genes

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4. **da Costa MS, Nobre MF, Rainey FA.** 2001. Genus I. Thermus Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605, p 404–414. *In* Boone DR, Castenholz RW, Garrity GM (ed), Bergey's manual of systematic bacteriology, 2nd ed, vol 1. Springer, New York, NY.
5. **Stratagene.** 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {*Use the company name as the author if none is provided for a company publication.*}
6. **Forman MS, Valsamakis A.** 2011. Specimen collection, transport, and processing: virology, p 1276–1288. *In* Versalovic J, Carroll KC, Jorgensen JH, Funke G, Landry ML, Warnock DW (ed), Manual of clinical microbiology, 10th ed, vol 2. ASM Press, Washington, DC.
7. **Fitzgerald G, Shaw D.** *In* Waters AE (ed), Clinical microbiology, in press. EFH Publishing Co, Boston, MA.* {*Chapter title is optional.*}
8. **García CO, Paira S, Burgos R, Molina J, Molina JF, Calvo C, Vega L, Jara LJ, García-Kutzbach A, Cuellar ML, Espinoza LR.** 1996. Detection of *Salmonella* DNA in synovial membrane and synovial fluid from Latin American patients using the polymerase chain reaction. Arthritis Rheum **39**(Suppl 9)**:**S185. {*Meeting abstract published in journal supplement.*}
9. **Carlson E.** 2013. Selective penicillin-binding protein imaging probes reveal substructure in bacterial cell division, p 59. Final Program 113th Gen Meet Am Soc Microbiol. American Society for Microbiology, Washington, DC. {*Abstract title is optional.*}
10. **Rotimi VO, Salako NO, Mohaddas EM, Philip LP.** 2005. Abstr 45th Intersci Conf Antimicrob Agents Chemother, abstr D-1658. {*Abstract title is optional.*}
11. **Green PN, Hood D, Dow CS.** 1984. Taxonomic status of some methylotrophic bacteria, p 251–254. *In* Crawford RL, Hanson RS (ed), Microbial growth on C_1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
12. **O'Malley DR.** 1998. Ph.D. thesis. University of California, Los Angeles, CA. {*Title is optional.*}
13. **Odell JC.** April 1970. Process for batch culturing. US patent 484,363,770. {*Include the name of the patented item/process if possible; the patent number is mandatory.*}
14. **Elder BL, Sharp SE.** 2003. Cumitech 39, Competency assessment in the clinical laboratory. Coordinating ed, Sharp SE. ASM Press, Washington, DC.

*A reference to an in-press ASM publication should state the control number (e.g., JCM00123-15) if it is a journal article or the name of the publication if it is a book.

Online-only references must provide essentially the same information that print references do. For online journal articles, posting or revision dates may replace the year of publication; a DOI (preferred) or URL is required for articles with nontraditional page numbers or electronic article identifiers.

1. **Bina XR, Taylor DL, Vikram A, Ante VM, Bina JE.** 2013. *Vibrio cholerae* ToxR downregulates virulence factor production in response to cyclo(Phe-Pro). mBio **4**(5)**:**e00366-13. doi:10.1128/mBio.00366-13.
2. **Winnick S, Lucas DO, Hartman AL, Toll D.** 2005. How do you improve compliance? Pediatrics **115:**e718–e724. doi:10.1542/peds.2004-1133.
3. **Dionne MS, Schneider DS.** 2002. Screening the fruitfly immune system. Genome Biol **3:**reviews1010-reviews1010.2. doi:10.1186/gb-2002-3-4-reviews1010.
4. **Giegé R, Springer M.** 2012. Aminoacyl-tRNA synthetases in the bacterial world. EcoSal Plus doi:10.1128/ecosalplus.4.2.1.

Note: a posting or accession date is required for any online reference that is periodically updated or changed.

Citations of ASM Accepts manuscripts should look like the following example.

Wang GG, Pasillas MP, Kamps MP. 15 May 2006. Persistent transactivation by Meis1 replaces Hox function in myeloid leukemogenesis models: evidence for co-occupancy of Meis1-Pbx and Hox-Pbx complexes on promoters of leukemia-associated genes. Mol Cell Biol doi:10.1128/MCB.00586-06.

Other journals may use different styles for their publish-ahead-of-print manuscripts, but citation entries must include the following information: author name(s), posting date, title, journal title, and volume and page numbers and/or DOI. The following is an example:

Zhou FX, Merianos HJ, Brunger AT, Engelman DM. 13 February 2001. Polar residues drive association of

polyleucine transmembrane helices. Proc Natl Acad Sci U S A doi:10.1073/pnas.041593698.

(ii) References cited in the text. References that should be cited in the text include

- Unpublished data
- Manuscripts submitted for publication
- Unpublished conference presentations (e.g., a report or poster that has not appeared in published conference proceedings)
- Personal communications
- Patent applications and patents pending
- Computer software, databases, and websites

These references should be made parenthetically in the text as follows:

. . . similar results (R. B. Layton and C. C. Weathers, unpublished data).
. . . system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).
. . . as described previously (M. G. Gordon and F. L. Rattner, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {*For nonpublished abstracts and posters, etc.*}
. . . this new process (V. R. Smoll, 20 June 1999, Australian Patent Office). {*For non-U.S. patent applications, give the date of publication of the application.*}
. . . available in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html).
. . . using ABC software (version 2.2; Department of Microbiology, State University [http://www.state.micro.edu]).

URLs for companies that produce any of the products mentioned in your study or for products being sold may not be included in the article. However, company URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.

(iii) Citations in abstracts. Because the abstract must be able to stand apart from the article, references cited in it should be clear without recourse to the References section. Use an abbreviated form of citation, omitting the article title, as follows.

(P. S. Satheshkumar, A. S. Weisberg, and B. Moss, J Virol 87:10700–10709, 2013, doi:10.1128/JVI.01258-13)
(J. H. Coggin, Jr., p. 93–114, *in* D. O. Fleming and D. L. Hunt, ed., *Biological Safety. Principles and Practices*, 4th ed., 2006)
". . . in a recent report by D. A. Hopwood [mBio 4(5): e00612-13, 2013, doi:10.1128/mBio00612-13] . . . ."

This style should also be used for Addenda in Proof.

(iv) References related to supplemental material. If references must be cited in the supplemental material, list them in a **separate** References section within the supplemental material and cite them by those numbers; do not simply include citations of numbers from the reference list of the associated article. If the same reference(s) is to be cited in both the article itself and the supplemental material, then that reference would be listed in both References sections.

Short-Form Papers

The Short-Form format is intended for the presentation of brief observations that do not warrant full-length papers. However, Short-Form papers should contain firm data; observations alone are not acceptable. Submit Short-Form papers in the same way as full-length papers. They receive the same review, they are not published more rapidly than full-length papers, and they are not considered preliminary communications.

The title, running title (not to exceed 54 characters and spaces), byline, and correspondent footnote should be prepared as for a full-length paper. Each Short-Form paper must have an abstract of no more than 50 words. Do not use section headings in the body of the Short Form; combine methods, results, and discussion in a single section. Paragraph lead-ins are permissible. The text should be kept to a minimum and if possible should not exceed 1,000 words; the number of figures and tables should also be kept to a minimum. Materials and methods should be described in the text, not in figure legends or table footnotes. Present acknowledgments as in full-length papers. The References section is identical to that of full-length papers.

Minireviews

Minireviews are expected to be focused discussions of defined topics relevant to clinical microbiologists. In general, they are to be submitted only following invitation by the editor in chief of JCM. Unsolicited Minireviews are discouraged. A topical outline should be provided to the editor in chief for approval prior to submission of the completed Minireview manuscript in the eJP online manuscript submission and peer review system.

Minireviews are not expected to be comprehensive reviews of the literature but rather focused discussions of specific topics. A standard title page should be provided. This is followed by an abstract of 100 words or less and then the text of the Minireview, which should not exceed 12 double-spaced manuscript pages in length, exclusive of tables, figures, photographs, and references. Up to three tables, figures, or photographs, total, may be included. References should be limited to no more than 30. Minireviews will be reviewed by two JCM editors, with the aim of expedited processing. In general, it is hoped that, barring the necessity of major revisions, accepted Minireviews will appear in print within 3 months of their submission and online ahead of print 6 to 8 weeks earlier.

Author bio. A short biographical sketch and photograph of the **one** author most responsible for the minireview should be submitted along with the initial version of the manuscript. These will be published at the end of the article.

- The text limit is 150 words and should include WHO you are (your name), WHERE you received your education, WHAT positions you have held and at WHICH

institutions, WHERE you are now (your current institution), WHY you have this interest, and HOW LONG you have been in this area, as well as a brief review of your scholarly interests and record of publication. In addition, please list pertinent significant awards you have received.
- The photo should be a recent black-and-white head shot of passport size. It will be reduced to approximately 1.125 inches wide by 1.375 inches high. The photo must meet the production criteria for regular figures and should be checked for production quality by using Rapid Inspector, provided at the following URL: http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp.
- To submit, upload the text and photo with your manuscript in the submission and review system. Include the biographical text immediately **after** the References section of your manuscript, in the same file. It should be labeled with the heading "Biosketch." Upload the head shot photograph in the submission system as a "Minireview Bio Photo"; **include the author's name or enough of it for identification in the photo's file name.**

Contact the scientific editor if you have questions about what to write. Contact the production editor if you have questions about submitting your files.

Commentaries

Commentaries are invited communications concerning topics relevant to the readership of JCM and are intended to engender discussion. Reviews of the literature, methods and other how-to papers, and responses targeted at a specific published paper are not appropriate. Commentaries are subject to review.

The length may not exceed four printed pages, and the format is like that of a Minireview (see above) except that the abstract is limited to 75 words.

Point-Counterpoint

Point-Counterpoint is a feature of JCM in which two experts present opposing views on a contemporary issue in the laboratory diagnosis of infectious diseases. This feature will be the lead article in the issue of JCM in which it appears. Participation as an author of a Point-Counterpoint feature is by invitation only.

A JCM editor will write a brief introductory piece of approximately 200 words outlining why a specific issue is important and then present the issue in the form of a question. The two experts will then each write a commentary, no more than 1,000 words in length, in which they present evidence in support of either the pro or con view. One table or one figure may be included. Since these discussions will be evidence based, authors may also cite up to 10 references. Unpublished or in-press data which reflect the current practice in their laboratory may be used but should not be the sole basis for their position.

Authors should send commentaries directly back to the JCM editor within 30 days of receipt of the introductory statement. Following receipt of both the pro and con commentaries, the editor will review the submissions and may return them to the author(s) with comments and/or suggested revisions. If revisions are required, the author(s) will have 14 days to craft a revised commentary, which will be sent directly back to the editor. Upon receipt of final commentaries, the JCM editor will write a brief summary consisting of no more than six one-sentence bullet points, outlining where the experts agree (no more than three points) and disagree (no more than three points). The JCM editor will then upload the introduction, both commentaries, and the summary in eJP.

Case Reports

A Case Report must include an abstract of no more than 50 words. The text starts with presentation of the case under the section heading "Case Report"; there is no introductory text before the Case Report heading. After the case is presented, the rest of the text follows in a separate section after a ruled line to separate the sections. No separate head is used for this short discussion section, but paragraph lead-ins are permitted. The total number of tables and figures (combined) must not exceed 3. For an example of a correctly formatted Case Report, see J Clin Microbiol **39:**1678–1679, 2001.

Photo Quiz

A Photo Quiz submission should present the findings of some relevant, interesting, and new observation pertinent to the practice of clinical microbiology in which a photograph is particularly useful in conveying important information **and** where the observation can serve as the basis for both a question and an answer. The photograph may be of a micrograph, some other laboratory material, a clinical lesion, or the results of an imaging study.

A Photo Quiz consists of two parts: (i) a case presentation featuring a photograph depicting some unusual and/or informative finding in clinical microbiology and (ii) an answer to the quiz. The case presentation and the answer must be submitted as two separate articles. Note that authors and affiliations are listed below the title.

Photo Quiz case presentation. The text in the Photo Quiz case presentation should be limited to 200 to 300 words. The header for the case presentation should read "Photo Quiz."

Please include a photograph about 39 picas (6.5 inches) wide and 28 picas (4.625 inches) high. Since photos appearing with published Photo Quizzes appear on the cover of the journal, a high-resolution TIFF or EPS file is preferred. A short legend for the photo must be provided, and the photo must be cited in the case presentation. Refer to a recently published Photo Quiz for correct formatting.

Answer to Photo Quiz. The text of the answer to the Photo Quiz should also be limited to 200 to 300 words. The header to the answer should read "Answer to Photo Quiz." Four to six references may be cited at the end of the Photo Quiz answer.

Submission. The Photo Quiz case presentation should be submitted in the "Photo Quiz" manuscript category. The Photo Quiz answer should be submitted in the "Photo Quiz Answer" manuscript category.

Letters to the Editor

Two types of Letters to the Editor may be submitted. The first type (Comment Letter) is intended for comments on final, typeset articles published in the journal (not on accepted manuscripts posted online) and must cite published references to support the writer's argument. The second type (New-Data Letter) may report new, concise findings that are not appropriate for publication as full-length papers or Short-Form papers.

Letters may be **no more than 500 words long and must be typed double spaced.** Refer to a recently published Letter for correct formatting. Note that authors and affiliations are listed below the title.

All Letters to the Editor must be submitted electronically, and the type of Letter (New Data or Comment) must be selected from the drop-down list in the submission form. For Letters commenting on published articles, the cover letter should state the volume and issue in which the article was published, the title of the article, and the last name of the first author. In the Abstract section of the submission form, put "Not Applicable." Letters to the Editor do not have abstracts. Both types of Letter must have a title, which must appear on the manuscript and on the submission form. Figures and tables should be kept to a minimum.

If the Letter is related to a published article, it will be sent to the editor who handled the article in question. If the editor believes that publication is warranted, he/she will solicit a reply from the corresponding author of the article and give approval for publication.

New-Data Letters will be assigned to an editor according to subject matter and will be reviewed by that editor and/or a reviewer.

Please note that some indexing/abstracting services do not include Letters to the Editor in their databases.

Fast-Track Communications

The Fast-Track route is intended for accelerated review of short communications that are of significant interest to clinical microbiologists. Manuscripts are limited to 750 words, one figure, one table, and 10 or fewer references. The format should be the same as that of a New-Data Letter (see "Letters to the Editor," above). Fast-Track articles should be submitted via the eJP online manuscript submission and peer review system.

A Fast-Track submission is subject to approval as such by the editor in chief. If approved for the Fast-Track route, the manuscript will be assigned to an appropriate JCM editor and reviewed, according to the same standards applied for traditional manuscripts, within 1 week. If accepted, the manuscript will be scheduled for the next available issue and edited. An acceptance letter and copyright agreement will be mailed to the corresponding author. Proofs will be made available electronically as for regular articles.

A Fast-Track submission that is not approved for the Fast-Track route will be handled as a New-Data Letter according to normal procedures.

Errata

The Erratum section provides a means of correcting errors that occurred during the writing, typing, editing, or publication (e.g., a misspelling, a dropped word or line, or mislabeling in a figure) of a published article. Submit Errata via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Erratum as a Microsoft Word file. Please see a recent issue for correct formatting.

Author Corrections

The Author Correction section provides a means of correcting errors of omission (e.g., author names or citations) and errors of a scientific nature that do not alter the overall basic results or conclusions of a published article (e.g., an incorrect unit of measurement or order of magnitude used throughout, contamination of one of numerous cultures, or misidentification of a mutant strain, causing erroneous data for only a [noncritical] portion of the study). Note that the addition of new data is not permitted.

For corrections of a scientific nature or issues involving authorship, including contributions and use or ownership of data and/or materials, all disputing parties must agree, in writing, to publication of the Correction. For omission of an author's name, letters must be signed by the authors of the article and the author whose name was omitted. The editor who handled the article will be consulted if necessary.

Submit an Author Correction via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). Select Author Correction as the manuscript type. In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Author Correction as a Microsoft Word file. Please see a recent issue for correct formatting. Signed letters of agreement must be supplied as supplemental material not for publication (scanned PDF files).

Retractions

Retractions are reserved for major errors or breaches of ethics that, for example, may call into question the source of the data or the validity of the results and conclusions of an article. Submit Retractions via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Retraction as a Microsoft Word file. Letters of agreement signed by all of the authors must be supplied as supplemental material not for publication (scanned PDF files). The Retraction will be assigned to the editor in chief of the journal, and the editor who handled the paper and the chairperson of the ASM Journals Board will be consulted. If all parties agree to the publication and content of the Retraction, it will be sent to the Journals Department for publication.

ILLUSTRATIONS AND TABLES

Illustrations

Image manipulation. Digital images submitted for publication may be inspected by ASM production specialists for any

manipulations or electronic enhancements that may be considered to be the result of scientific misconduct based on the guidelines provided below. Any images/data found to contain manipulations of concern will be referred to the editor in chief, and authors may then be requested to provide their primary data for comparison with the submitted image file. Investigation of the concerns may delay publication and may result in revocation of acceptance and/or additional action by ASM.

Linear adjustments to contrast, brightness, and/or color are generally acceptable, as long as the measures taken are necessary to view elements that are already present in the data and the adjustments are applied to the entire image and not just specific areas. Unacceptable adjustments to images include, but are not limited to, the removal or deletion, concealment, duplication (copying and pasting), addition, selective enhancement, or repositioning of elements within the image.

Nonlinear adjustments made to images, such as changes to gamma settings, should be fully disclosed in the figure legends at the time of submission. In addition, images created by compiling multiple files, including noncontiguous portions of the same image, should clearly distinguish that these multiple files are not a single image. This can be done by "tooling," or inserting thin lines, between the individual images.

File types and formats. Illustrations may be continuous-tone images, line drawings, or composites. Color graphics may be submitted, but the cost of printing in color must be borne by the author. Suggestions about how to reduce costs and ensure accurate color reproduction are given below.

On initial submission, figures may be uploaded as individual PDF files or combined and uploaded as a single PDF file. Place each legend in the text file, as well as on the same page with the figure to assist review. At the modification stage, production-quality digital files must be provided. The legends will be copy-edited and typeset for final publication and should not be included as part of the figure itself at this stage. All graphics submitted with modified manuscripts must be bitmap, grayscale, or in the RGB (preferred) or CMYK color mode. See "Color illustrations." Halftone images (those with various densities or shades) must be grayscale, not bitmap. JCM accepts TIFF or EPS files but discourages PowerPoint for either black-and-white or color images.

For instructions on creating acceptable EPS and TIFF files, refer to the Cadmus digital art website, http://art.cadmus.com/da/index.jsp. PowerPoint requires users to pay close attention to the fonts used in their images (see the section on fonts below). If instructions for fonts are not followed exactly, images prepared for publication are subject to missing characters, improperly converted characters, or shifting/obscuring of elements or text in the figure. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp. Note that, due to page composition system requirements, you must verify that your PowerPoint files can be converted to PDF without any errors.

We strongly recommend that before returning their modified manuscripts, authors check the acceptability of their digital images for production by running their files through Rapid Inspector, a tool provided at the following URL: http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp. Rapid Inspector is an easy-to-use, Web-based application that identifies file characteristics that may render the image unusable for production. Please note when using Rapid Inspector to check PowerPoint files that there is a known bug in the application that can occasionally fail PowerPoint Presentation (.pptx) files, even though the files meet all required production criteria. If you experience this bug, the issue can be corrected by saving the PowerPoint files as an older version, PowerPoint 97-2004 Presentation (.ppt), during the Save As process (use the drop-down format menu and select this format). Once you save your files as .ppt, they will pass Rapid Inspector if all required production criteria have been met.

If you have additional questions about using the Rapid Inspector preflighting tool, please send an e-mail inquiry to helpdesk.digitalartsupport@cenveo.com.

Minimum resolution. It is extremely important that a high enough resolution is used. All separate images that you import into a figure file must be at the correct resolution before they are placed. (For instance, placing a 72-dpi image in a 300-dpi EPS file will not result in the placed image meeting the minimum requirements for file resolution.) Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality will not be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:

- 300 dpi for grayscale and color
- 600 dpi for combination art (lettering and images)
- 1,200 dpi for line art

Size. All graphics **should be submitted at their intended publication size;** that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration. White space must be cropped from the image, and excess space between panel labels and the image must be eliminated.

- Maximum width for a 1-column figure: 20.6 picas (ca. 8.7 cm)
- Maximum width for a 2-column figure: 42 picas (ca. 17.8 cm)
- Minimum width for a 2-column figure: 26 picas (11.1 cm)
- Maximum height for a standard figure: 54.7 picas (ca. 23.2 cm)
- Maximum height for an oversized figure (no running title): 57.4 picas (ca. 24.3 cm)

Contrast. Illustrations must contain sufficient contrast to be viewed easily on a monitor or on the printed page.

Labeling and assembly. All final lettering and labeling must be incorporated into the figures. On initial submission, illustrations should be provided as PDF files, with the legends in the text file and with a legend beneath each image to assist review. At the modification stage, production-quality digital figure

files (without legends) must be provided. Put the figure number well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.

Fonts. To avoid font problems, set all type in one of the following fonts: Arial, Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. Courier may be used but should be limited to nucleotide or amino acid sequences, where a nonproportional (monospace) font is required. All fonts other than these must be converted to paths (or outlines) in the application with which they were created. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp.

Color illustrations. Color costs must be borne by the author. See "Publication Fees." All figures submitted in color will be processed as color. Adherence to the following guidelines will help to minimize costs and to ensure color reproduction that is as accurate as possible.

The final online version is considered the version of record for JCM and all other ASM journals. To maximize online reproduction, color illustrations should be supplied in the RGB color mode as either (i) RGB TIFF images with a resolution of at least 300 pixels per inch (raster files, consisting of pixels) or (ii) Illustrator-compatible EPS files with RGB color elements (vector files, consisting of lines, fonts, fills, and images). CMYK files are also accepted. Other than in color space, CMYK files must meet the same production criteria as RGB files. The RGB color space is the native color space of computer monitors and of most of the equipment and software used to capture scientific data, and it can display a wider range of colors (especially bright fluorescent hues) than the CMYK (cyan, magenta, yellow, black) color space used by print devices that put ink (or toner) on paper. For the print version (and reprints), ASM's print provider will automatically create CMYK versions of color illustrations from the supplied RGB versions. Color in the print journal may not match that in the online journal of record because of the smaller range of colors capable of being reproduced by CMYK inks on a printing press. For additional information on RGB versus CMYK color, refer to the Cadmus digital art site, http://art.cadmus.com/da/guidelines_rgb.jsp.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. All elements, including letters, numbers, and symbols, must be easily readable, and both axes of a graph must be labeled. Keep in mind that the journal is published both in print and online and that the same electronic files submitted by the authors are used to produce both.

When creating line art, please use the following guidelines:

(i) **All art must be submitted at its intended publication size.** For acceptable dimensions, see "Size," above.

(ii) **Avoid using screens (i.e., shading) in line art.** It can be difficult and time-consuming to reproduce these images without moiré patterns. Various pattern backgrounds are preferable to screens as long as the patterns are not imported from another application. If you must use images containing screens,

(a) Generate the image at line screens of 85 lines per inch or less.

(b) When applying multiple shades of gray, differentiate the gray levels by at least 20%.

(c) Never use levels of gray below 5% or above 95%, as they are likely to fade out or become totally black when output.

(iii) Use thick, solid lines that are no finer than 1 point in thickness.

(iv) No type should be smaller than 6 points at the final publication size.

(v) Avoid layering type directly over shaded or textured areas.

(vi) Avoid the use of reversed type (white lettering on a black background).

(vii) Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.

(viii) If colors are used, avoid using similar shades of the same color and avoid very light colors.

In figure ordinate and abscissa scales (as well as table column headings), avoid the ambiguous use of numbers with exponents. Usually, it is preferable to use the appropriate Système International d'Unités (SI) symbols (μ for 10^{-6}, m for 10^{-3}, k for 10^{3}, and M for 10^{6}, etc.). Thus, a representation of 20,000 cpm on a figure ordinate should be made by the number 20 accompanied by the label kcpm. A complete listing of SI symbols can be found in the International Union of Pure and Applied Chemistry (IUPAC) publication *Quantities, Units and Symbols in Physical Chemistry*, 3rd ed. (RSC Publishing, Cambridge, United Kingdom, 2011); an abbreviated list is available at http://old.iupac.org/reports/1993/homann/index.html.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral of the ordinate should be "2" and the label should be "10^{4} cells per ml" (not "cells per ml $\times$ 10^{-4}"). Likewise, an enzyme activity of 0.06 U/ml might be shown as 6 accompanied by the label 10^{-2} U/ml. The preferred designation is 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Long nucleic acid sequences must be presented as figures in the following format to conserve space. Print the sequence in lines of approximately 100 to 120 nucleotides in a nonproportional (monospace) font that is easily legible when published with a line length of 6 inches (ca. 15.2 cm). If possible, lines of nucleic acid sequence should be further subdivided into blocks of 10 or 20 nucleotides by spaces within the sequence or by marks above it. Uppercase and lowercase letters may be used to designate the exon-intron structure or transcribed regions, etc., if the lowercase letters remain legible at a 6-inch (ca. 15.2-cm) line length. Number the sequence line by line; place numerals representing the first base of each line to the left of the lines. Minimize spacing between lines of sequence, leaving room only for annotation of the sequence. Annotation may include boldface, underlining, brackets, and boxes, etc. Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the single-letter amino acid symbols. Comparisons of multiple nucleic acid sequences should conform as nearly as possible to the same format.

Figure Legends

On initial submission, each legend should be placed in the text file *and* be incorporated into the image file beneath the figure to assist review.

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be reported in a legend only if the discussion is very brief (one or two sentences). Define all symbols used in the figure and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. The preferred format for regular tables is Microsoft Word; however, WordPerfect and Acrobat PDF are also acceptable. Note that a straight Excel file is not currently an acceptable format. Excel files must be either embedded in a Word or WordPerfect document or converted to PDF before being uploaded.

Tables should be formatted as follows. Arrange the data so that **columns of like material read down, not across.** The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the "Abbreviations" section of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more-extensive table "legends" are not. Footnotes should not include detailed descriptions of the experiment. Tables must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 1 is an example of a well-constructed table.

TABLE 1 Distribution of protein and ATPase in fractions of dialyzed membranes[a]

Membrane	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
E1 treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

[a] Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively.

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (CAS; http://www.cas.org/) and its indexes. *The Merck Index*, 15th ed. (RSC Books, Cambridge, UK, 2013), is also an excellent source. For biochemical terminology, including abbreviations and symbols, consult *Biochemical Nomenclature and Related Documents* (Portland Press, London, United Kingdom, 1992) available at http://www.chem.qmul.ac.uk/iupac/bibliog/white.html, and the instructions to authors of the *Journal of Biological Chemistry* and the *Archives of Biochemistry and Biophysics*.

Do not express molecular weight in daltons; molecular weight is a unitless ratio. Molecular mass is expressed in daltons.

For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry (IUB) as described in *Enzyme Nomenclature* (Academic Press, Inc., New York, NY, 1992) and its supplements and at http://www.chem.qmul.ac.uk/iubmb/enzyme/. If a nonrecommended name is used, place the proper (trivial) name in parentheses at first use in the abstract and text. Use the EC number when one has been assigned. Authors of papers describing enzymological studies should review the standards of the STRENDA Commission for information required for adequate description of experimental conditions and for reporting enzyme activity data (http://www.beilstein-institut.de/en/projects/strenda/guidelines).

For nomenclature of restriction enzymes, DNA methyltransferases, homing endonucleases, and their genes, refer to the article by Roberts et al. (Nucleic Acids Res **31:**1805–1812, 2003).

Drugs

Whenever possible, use generic names of drugs; the use of trade names is not permitted.

Nomenclature of Microorganisms

Binary names, consisting of a generic name and a specific epithet (e.g., *Escherichia coli*), must be used for all microorganisms. Names of categories at or above the genus level may be used alone, but specific and subspecific epithets may not. A

specific epithet must be preceded by a generic name, written out in full the first time it is used in a paper. Thereafter, the generic name should be abbreviated to the initial capital letter (e.g., *E. coli*), provided there can be no confusion with other genera used in the paper. Names of all taxa (kingdoms, phyla, classes, orders, families, genera, species, and subspecies) are printed in italics and should be italicized in the manuscript; strain designations and numbers are not. Vernacular (common) names should be in lowercase roman type (e.g., streptococcus, brucella). For *Salmonella*, genus, species, and subspecies names should be rendered in standard form: *Salmonella enterica* at first use, *S. enterica* thereafter; *Salmonella enterica* subsp. *arizonae* at first use, *S. enterica* subsp. *arizonae* thereafter. Names of serovars should be in roman type with the first letter capitalized: *Salmonella enterica* serovar Typhimurium. After the first use, the serovar may also be given without a species name: *Salmonella* Typhimurium, *S.* Typhimurium, or *Salmonella* serovar Typhimurium. For other information regarding serovar designations, see *Antigenic Formulae of the Salmonella Serovars*, 9th ed. (P. A. D. Grimont and F.-X. Weill, WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris, France, 2007; see http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089). For a summary of the current standards for *Salmonella* nomenclature and the Kaufmann-White criteria, see the article by Brenner et al. (J Clin Microbiol **38:**2465–2467, 2000), the opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes (Int J Syst Evol Microbiol **55:**519–520, 2005), and the article by Tindall et al. (Int J Syst Evol Microbiol **55:**521–524, 2005).

The spelling of bacterial names should follow the *Approved Lists of Bacterial Names (Amended) & Index of the Bacterial and Yeast Nomenclatural Changes* (V. B. D. Skerman et al., ed., American Society for Microbiology, Washington, DC, 1989) and the validation lists and notification lists published in the *International Journal of Systematic and Evolutionary Microbiology* (formerly the *International Journal of Systematic Bacteriology*) since January 1989. In addition, two sites on the World Wide Web list current approved bacterial names: Prokaryotic Nomenclature Up-to-Date (http://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html) and List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/). If there is reason to use a name that does not have standing in nomenclature, the name should be enclosed in quotation marks in the title and at its first use in the abstract and the text and an appropriate statement concerning the nomenclatural status of the name should be made in the text. "*Candidatus*" species should always be set in quotation marks.

For guidelines regarding new names and descriptions of new genera and species, see the articles by Tindall (Int J Syst Bacteriol **49:**1309–1312, 1999) and Stackebrandt et al. (Int J Syst Evol Microbiol **52:**1043–1047, 2002). To validate new names and/or combinations, authors must submit three copies of their published article to the *International Journal of Systematic and Evolutionary Microbiology*.

It is recommended that a strain be deposited in at least two recognized culture collections in different countries when that strain is necessary for the description of a new taxon (Int J Syst Evol Microbiol **50:**2239–2244, 2000).

Since the classification of fungi is not complete, it is the responsibility of the author to determine the accepted binomial for a given organism. Sources for these names include *The Yeasts: a Taxonomic Study*, 5th ed. (C. P. Kurtzman, J. W. Fell, and T. Boekhout, ed., Elsevier Science, Amsterdam, Netherlands, 2011), and *Dictionary of the Fungi*, 10th ed. (P. M. Kirk, P. F. Cannon, D. W. Minter, and J. A. Stalpers, ed., CABI International, Wallingford, Oxfordshire, United Kingdom, 2008); see also http://www.speciesfungorum.org/Names/Fundic.asp.

Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) and reported on the ICTV Virus Taxonomy website (http://www.ictvonline.org/index.asp). In addition, the recommendations of the ICTV regarding the use of species names should generally be followed: when the entire species is discussed as a taxonomic entity, the species name, as with other taxa, is italic and has the first letter and any proper nouns capitalized (e.g., *Tobacco mosaic virus, Murray Valley encephalitis virus*). When the behavior or manipulation of individual viruses is discussed, the vernacular (e.g., tobacco mosaic virus, Murray Valley encephalitis virus) should be used. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used.

Microorganisms, viruses, and plasmids should be given designations consisting of letters and serial numbers. It is generally advisable to include a worker's initials or a descriptive symbol of locale or laboratory, etc., in the designation. Each new strain, mutant, isolate, or derivative should be given a new (serial) designation. This designation should be distinct from those of the genotype and phenotype, and italicized genotypic and phenotypic symbols should not be included. Plasmids are named with a lowercase "p" followed by the designation in uppercase letters and numbers. To avoid the use of the same designation as that of a widely used strain or plasmid, check the designation against a publication database such as Medline.

Genetic Nomenclature

To facilitate accurate communication, **it is important that standard genetic nomenclature be used whenever possible and that deviations or proposals for new naming systems be endorsed by an appropriate authoritative body.** Review and/or publication of submitted manuscripts that contain new or nonstandard nomenclature may be delayed by the editor or the Journals Department so that they may be reviewed.

Bacteria. The genetic properties of bacteria are described in terms of phenotypes and genotypes. The phenotype describes the observable properties of an organism. The genotype refers to the genetic constitution of an organism, usually in reference to some standard wild type. Use the recommendations of Demerec et al. (Genetics **54:**61–64, 1966) as a guide to the use of these terms. If your manuscript contains information including genetic nomenclature, please refer to the Instructions to Authors of the *Journal of Bacteriology*.

"Mutant" versus "mutation." Keep in mind the distinction between a mutation (an alteration of the primary sequence of the genetic material) and a mutant (a strain carrying one or more mutations). One may speak about the mapping of a mutation, but one cannot map a mutant. Likewise, a mutant has no genetic locus, only a phenotype.

"Homology" versus "similarity." For use of terms that describe relationships between genes, consult the articles by Theissen (Nature **415:**741, 2002) and Fitch (Trends Genet **16:** 227–231, 2000). "Homology" implies a relationship between genes that have a common evolutionary origin; partial homology is not recognized. When sequence comparisons are discussed, it is more appropriate to use the term "percent sequence similarity" or "percent sequence identity," as appropriate.

Tetracycline resistance determinants. The nomenclature for tetracycline resistance determinants is based on the proposal of Levy et al. (Antimicrob Agents Chemother **43:**1523–1524, 1999). The style for such determinants is, e.g., Tet B; the space helps distinguish the determinant designation from that for phenotypes and proteins (TetB). The above-referenced article also gives the correct format for genes, proteins, and determinants in this family.

Locus tags. Locus tags are systematic, unique identifiers that are assigned to each gene in GenBank. All genes mentioned in a manuscript should be traceable to their sequences by the reader, and locus tags may be used for this purpose in manuscripts to identify uncharacterized genes. In addition, authors should check GenBank to make sure that they are using the correct, up-to-date format for locus tags (e.g., uppercase versus lowercase letters and the presence or absence of an underscore, etc.). Locus tag formats vary between different organisms and also may be updated for a given organism, so it is important to check GenBank at the time of manuscript preparation.

Viruses. The genetic nomenclature for viruses differs from that for bacteria. In most instances, viruses have no phenotype, since they have no metabolism outside host cells. Therefore, distinctions between phenotype and genotype cannot be made. Superscripts are used to indicate hybrid genomes. Genetic symbols may be one, two, or three letters.

Eukaryotes. FlyBase (http://flybase.org/) is the genetic nomenclature authority for *Drosophila melanogaster*. WormBase (http://www.wormbase.org/#01-23-6) is the genetic nomenclature authority for *Caenorhabditis elegans*. When naming genes for *Aspergillus* species, the nomenclature guidelines posted at http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/nomenclature.htm should be followed, and the *Aspergillus* Genome Database (http://www.aspgd.org/) should be searched to ensure that any new name is not already in use. The *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and the *Candida* Genome Database http://www.candidagenome.org/) are authorities for *Saccharomyces cerevisiae* and *Candida albicans* genetic nomenclature, respectively. For more information about the genetic nomenclature of eukaryotes, see the Instructions to Authors for *Eukaryotic Cell* and *Molecular and Cellular Biology*.

ABBREVIATIONS AND CONVENTIONS

Verb Tense

ASM strongly recommends that for clarity you use the **past** tense to narrate particular events in the past, including the procedures, observations, and data of the study that you are reporting. Use the present tense for your own general conclusions, the conclusions of previous researchers, and generally accepted facts. Thus, most of the abstract, Materials and Methods, and Results will be in the past tense, and most of the introduction and some of the Discussion will be in the present tense.

Be aware that it may be necessary to vary the tense in a single sentence. For example, it is correct to say "White (30) demonstrat*ed* that XYZ cells *grow* at pH 6.8," "Figure 2 show*s* that ABC cells fail*ed* to grow at room temperature," and "Air *was* removed from the chamber and the mice *died*, which *proves* that mice *require* air." In reporting statistics and calculations, it is correct to say "The values for the ABC cells *are* statistically significant, indicating that the drug inhibit*ed* . . ."

For an in-depth discussion of tense in scientific writing, see *How To Write and Publish a Scientific Paper*, 7th ed.

Abbreviations

General. Abbreviations should be used as an aid to the reader, rather than as a convenience for the author, and therefore their **use should be limited.** Abbreviations other than those recommended by the IUPAC-IUB (*Biochemical Nomenclature and Related Documents*, 1992) should be used only when a case can be made for necessity, such as in tables and figures.

It is often possible to use pronouns or to paraphrase a long word after its first use (e.g., "the drug" or "the substrate"). Standard chemical symbols and trivial names or their symbols (folate, Ala, and Leu, etc.) may also be used.

Define each abbreviation and introduce it in parentheses the first time it is used; e.g., "Cultures were grown in Eagle minimal essential medium (MEM)." Generally, eliminate abbreviations that are not used at least three times in the text (including tables and figure legends).

Not requiring introduction. In addition to abbreviations for Système International d'Unités (SI) units of measurement, other common units (e.g., bp, kb, and Da), and chemical symbols for the elements, the following should be used without definition in the title, abstract, text, figure legends, and tables:

DNA (deoxyribonucleic acid)
cDNA (complementary DNA)
RNA (ribonucleic acid)
cRNA (complementary RNA)
RNase (ribonuclease)
DNase (deoxyribonuclease)
rRNA (ribosomal RNA)
mRNA (messenger RNA)
tRNA (transfer RNA)
AMP, ADP, ATP, dAMP, ddATP, and GTP, etc. (for the respective 5′ phosphates of adenosine and other nucleosides) (add 2′-, 3′-, or 5′- when needed for contrast)

ATPase and dGTPase, etc. (adenosine triphosphatase and deoxyguanosine triphosphatase, etc.)
NAD (nicotinamide adenine dinucleotide)
NAD^+ (nicotinamide adenine dinucleotide, oxidized)
NADH (nicotinamide adenine dinucleotide, reduced)
NADP (nicotinamide adenine dinucleotide phosphate)
NADPH (nicotinamide adenine dinucleotide phosphate, reduced)
$NADP^+$ (nicotinamide adenine dinucleotide phosphate, oxidized)
poly(A) and poly(dT), etc. (polyadenylic acid and polydeoxythymidylic acid, etc.)
oligo(dT), etc. (oligodeoxythymidylic acid, etc.)
UV (ultraviolet)
PFU (plaque-forming units)
CFU (colony-forming units)
MIC (minimal inhibitory concentration)
Tris (tris[hydroxymethyl] aminomethane)
DEAE (diethylaminoethyl)
EDTA (ethylenediaminetetraacetic acid)
EGTA (ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid)
HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)
PCR (polymerase chain reaction)
AIDS (acquired immunodeficiency syndrome)

Abbreviations for cell lines (e.g., HeLa) also need not be defined.

The following abbreviations should be used without definition in tables:

amt (amount)
approx (approximately)
avg (average)
concn (concentration)
diam (diameter)
expt (experiment)
exptl (experimental)
ht (height)
mo (month)
mol wt (molecular weight)
no. (number)
prepn (preparation)
SD (standard deviation)
SE (standard error)
SEM (standard error of the mean)
sp act (specific activity)
sp gr (specific gravity)
temp (temperature)
tr (trace)
vol (volume)
vs (versus)
wk (week)
wt (weight)
yr (year)

Drugs. Should an author decide to abbreviate the names of antimicrobial agents in a manuscript, the following standard abbreviations are strongly recommended.

Antibacterial agents. Use the indicated abbreviations for the following antibacterial agents.

amikacin (AMK)
amoxicillin (AMX)
amoxicillin-clavulanic acid (AMC)
ampicillin (AMP)
ampicillin-sulbactam (SAM)
azithromycin (AZM)
azlocillin (AZL)
aztreonam (ATM)
carbenicillin (CAR)
cefaclor (CEC)
cefadroxil (CFR)
cefamandole (FAM)
cefazolin (CFZ)
cefdinir (CDR)
cefditoren (CDN)
cefepime (FEP)
cefetamet (FET)
cefixime (CFM)
cefmetazole (CMZ)
cefonicid (CID)
cefoperazone (CFP)
cefotaxime (CTX)
cefotetan (CTT)
cefoxitin (FOX)
cefpodoxime (CPD)
cefprozil (CPR)
ceftazidime (CAZ)
ceftibuten (CTB)
ceftizoxime (ZOX)
ceftriaxone (CRO)
cefuroxime (axetil) and cefuroxime (sodium) (CXM)
cephalexin (LEX)
cephalothin (CEF)
cephapirin (HAP)
cephradine (RAD)
chloramphenicol (CHL)
cinoxacin (CIN)
ciprofloxacin (CIP)
clarithromycin (CLR)
clinafloxacin (CLX)
clindamycin (CLI)
daptomycin (DAP)
dicloxacillin (DCX)
dirithromycin (DTM)
doxycycline (DOX)
enoxacin (ENX)
erythromycin (ERY)
fleroxacin (FLE)
fosfomycin (FOF)
gatifloxacin (GAT)
gentamicin (GEN)
grepafloxacin (GRX)
imipenem (IPM)
kanamycin (KAN)
levofloxacin (LVX)
linezolid (LZD)
lomefloxacin (LOM)
loracarbef (LOR)
meropenem (MEM)
methicillin (MET)
mezlocillin (MEZ)
minocycline (MIN)
moxalactam (MOX)
moxifloxacin (MXF)
nafcillin (NAF)
nalidixic acid (NAL)
netilmicin (NET)
nitrofurantoin (NIT)
norfloxacin (NOR)
ofloxacin (OFX)
oxacillin (OXA)
penicillin (PEN)
piperacillin (PIP)
piperacillin-tazobactam (TZP)
quinupristin-dalfopristin (Synercid) (Q-D)
rifabutin (RFB)
rifampin (RIF)
rifapentine (RFP)
sparfloxacin (SPX)
spectinomycin (SPT)
streptomycin (STR)
teicoplanin (TEC)
telithromycin (TEL)
tetracycline (TET)
ticarcillin (TIC)
ticarcillin-clavulanic acid (TIM)
tobramycin (TOB)
trimethoprim (TMP)
trimethoprim-sulfamethoxazole (SXT)
trovafloxacin (TVA)
vancomycin (VAN)

β-Lactamase inhibitors. Use the indicated abbreviations for the following β-lactamase inhibitors.

clavulanic acid (CLA)
sulbactam (SUL)
tazobactam (TZB)

Antifungal agents. Use the indicated abbreviations for the following antifungal agents.

amphotericin B (AMB)
clotrimazole (CLT)
flucytosine (5FC)
fluconazole (FLC)
itraconazole (ITC)
ketoconazole (KTC)
nystatin (NYT)
terbinafine (TRB)
voriconazole (VRC)

Antiviral agents. Use the indicated abbreviations for the following antiviral agents.

acyclovir (ACV)
cidofovir (CDV)
famciclovir (FCV)
foscarnet (FOS)
ganciclovir (GCV)
penciclovir (PCV)
valacyclovir (VCV)
zidovudine (AZT)

Reporting Numerical Data

Standard metric units are used for reporting length, weight, and volume. For these units and for molarity, use the prefixes m, μ, n, and p for 10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12}, respectively. Likewise, use the prefix k for 10^3. Avoid compound prefixes such as mμ or μμ. Use μg/ml or μg/g in place of the ambiguous ppm. Units of temperature are presented as follows: 37°C or 324 K.

When fractions are used to express units such as enzymatic activities, it is preferable to use whole units, such as "g" or "min," in the denominator instead of fractional or multiple units, such as μg or 10 min. For example, "pmol/min" is prefera-

ble to "nmol/10 min," and "μmol/g" is preferable to "nmol/μg." It is also preferable that an unambiguous form, such as exponential notation, be used; for example, "$\mu mol\ g^{-1}\ min^{-1}$" is preferable to "μmol/g/min." Always report numerical data in the appropriate SI units.

Representation of data as accurate to more than two significant figures must be justified by presentation of appropriate statistical analyses.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the articles by Olsen (Infect Immun **71:**6689–6692, 2003; Infect Immun **82:**916–920, 2014).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J Virol **79:**669–676, 2005).

Statistics

Statistical analysis of data is a crucial component of scientific publication. Authors who are unsure of proper statistical analysis should have their manuscripts checked by a qualified statistician.

The following is a list of important items that must be considered before manuscript submission. Deficiencies in any of these areas may delay review and/or publication.

(i) Statistical analyses were performed on all quantitative data regardless of how significant the differences look in the tables or figures.

(ii) Data were appropriately analyzed as parametric (normally distributed) or nonparametric data.

(iii) Parametric and nonparametric data are presented appropriately. Means and standard deviations or standard errors are appropriate means of presenting data analyzed by parametric analyses (i.e., *t* test and analysis of variance [ANOVA]), but only medians and surrounding levels (quartiles, quintiles, and 10th and 90th percentiles, etc.) are appropriate for nonparametric statistics (Mann-Whitney test and Kruskal-Wallis test, etc.). Means have no meaning in nonparametric analyses.

(iv) For any data in which there are more than two comparisons (i.e., between one control and more than one experimental group), an analysis must be done for multigroup comparisons. Such an analysis would usually be an ANOVA for parametric data or a Kruskal-Wallis test for nonparametric data. *t* tests cannot be used when more than two groups are being compared (except as indicated below). Failure to use multigroup tests generates type 1 errors: concluding that two data sets within the overall data set being compared are different when in fact they are not. Exception: some statisticians argue that two-group comparisons can be used on multigroup data if the expected outcomes are appropriately anticipated before the experiment. For example, data generated by individually testing two unrelated factors for their effects on a target with only a single, untreated target as a control could be appropriately analyzed by *t* tests instead of ANOVA.

(v) For all appropriate multigroup comparisons, two *P* values must be generated and provided in the manuscript. The main *P* value applies to the overall data set and indicates that within that data set at least two groups differ from each other. The overall *P* value does not indicate which two groups are different. The main *P* value and the overall *P* value should be computed by using a *post hoc* test. For ANOVA, these *post hoc* tests are usually Dunnett's test (used to compare multiple experimental groups to a single control), the Fisher protected least significant difference (PLSD) test, the Tukey-Kramer test, and the Games-Howell test. Others may be used. Note that each *post hoc* test has certain underlying assumptions that may not be applicable to the data under analysis. For a Kruskal-Wallis nonparametric ANOVA, the Dunn procedure is appropriate to generate *P* values for two-group comparisons.

(vi) Data presented as endpoints (i.e., LD_{50} and ID_{50}, etc.) contain both the calculated value and a confidence interval with a statistical significance associated with it (95%, 99%, or similar confidence interval), calculated by logit or probit analysis. Simple LD_{50} values, such as Reed-Muench calculations, may not be used alone.

(vii) When samples are taken multiple times from one experimental entity (i.e., multiple serum samples from one animal, gross pathology scores measured for the same animal over time or growth curves, etc.), one cannot use analyses such as *t* tests, ANOVA, or the Mann-Whitney test, etc., because these tests assume that each measure is independent. An entity with a high score on day 1 is more likely to have a high score on day 2 than is an entity with a low score. It is likely that some expert statistical help will be needed for these situations, usually involving regression analysis or survival analysis, etc.

(viii) Statistical significance and biological significance are not the same. There is nothing magical about a *P* value of 0.05. When results from large sample sizes are compared, a *P* value of <0.05 will often be obtained, as *P* value is a function of both sample size and effect size. If sample sizes are large, then more-rigorous (i.e., smaller) *P* values may be desirable. If sample sizes are small, *P* values of >0.05 may still be important. There should be both statistical and biological significance to the results and conclusions in the manuscript.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the articles by Olsen (Infect Immun **71:**6689–6692, 2003; Infect Immun **82:**916–920, 2014).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J Virol **79:**669–676, 2005).

Isotopically Labeled Compounds

For simple molecules, labeling is indicated in the chemical formula (e.g., $^{14}CO_2$, $^{3}H_2O$, and $H_2{}^{35}SO_4$). Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ^{32}S-ATP) or to a word that is not a specific chemical name (e.g., ^{131}I-labeled protein, ^{14}C-amino acids, and ^{3}H-ligands).

For specific chemicals, the symbol for the isotope introduced is placed in square brackets directly preceding the part of the name that describes the labeled entity. Note that configuration symbols and modifiers precede the isotopic symbol. The following examples illustrate correct usage:

[^{14}C]urea	UDP-[U-^{14}C]glucose
L-[*methyl*-^{14}C]methionine	*E. coli* [^{32}P]DNA
[2,3-^{3}H]serine	fructose 1,6-[1-^{32}P]bisphosphate
[α-^{14}C]lysine	[γ-^{32}P]ATP

5. ANEXOS

Anexo 1. CBQCA_MRSA online database*

(http://mlva.u-psud.fr/mlvav4/genotyping/view.php?b=CBQCA_MRSA/ [CBQCA_MRSA database])

Strain id[a]	Species	Year of isolation	Country[b]	HVQ Sample id[c]	Host	Isolated from	Skin and soft tissue	Post-surgical wound	Tracheal-bronchial	Blood	Bone marrow	Others	PVL (*LukS/F-PV*)	*ccr* complex	*mec* complex	SCC*mec*	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA0906	SA1132/63_01	SA1194	SA1213	SA1291	SA1425	SA1729	SA1756	SA1866	SA2039	Relatedness_10loc	Distance_10loci	Relatedness_14loci	Distance_14loci
1	MRSA	2009	ECU	60	Human	Wound	1	0	0	0	0	0	0	3	A	III		5	4	2	2	3	3	5	1	2	1	2	3	3	ST8	2	ST241	5
2	MRSA	2009	ECU	438	Human	Axilar abscess	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	2	3	3	1	3	1	2	3	3	ST8	0	ST8	0
3	MRSA	2009	ECU	456	Human	Groing wound	1	0	0	0	0	0	0	2.3.4.5	A	NT		5	3	2	2	3	3	5	1	2	1	2	3	3	ST239	1		
5	MRSA	2009	ECU	629	Human	Blood	0	0	0	1	0	0	1	2	-	NT	10	5	2	6	3	2	3	3	1	3	1	2	3	3	ST8	0	ST8	2
6	MRSA	2009	ECU	673	Human	Foot wound	1	0	0	0	0	0	1	3,5	B	NT	11	5	2	6	2	2	3	3	1	3	1	2	3	3	ST8	1	ST8	1
12	MRSA	2009	ECU	284	Human	Abscess secretion	1	0	0	0	0	0	1	2.5	B	IVh	10	5	3	6	3	2	3	3	1	3	1	2	3	3	ST8	1	ST8	2
13	MRSA	2009	ECU	336	Human	Tracheal secretion	0	0	1	0	0	0	1	2.3.5	B	NT	10	5	3	4		7	5		4		3		3	3	ST228	2		
14	MRSA	2009	ECU	54	Human	Bronchial secretion	0	0	1	0	0	0	1	3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
16	MRSA	2009	ECU	375	Human	Tracheal secretion	0	0	1	0	0	0	0	3.5	A	IIIB	10	5	3	2	2	2	3	5	1	2	1	2	3	3	ST8	2	ST241	3
17	MRSA	2009	ECU	140	Human	Cranium wound	1	0	0	0	0	0	0	3.5	A	IIIB		5	3	2		1	7		1		1		3	3	ST239	3		
18	MRSA	2009	ECU	571	Human	Carotid wound (surgical)	0	1	0	0	0	0	0	3	A	III		5	4	2		3	3		1		1		3	3	ST247	2		
19	MRSA	2009	ECU	568	Human	Gluteus secretion	1	0	0	0	0	0	1	2,3,5	B	NT	11	5	2	6		3	3		1		1		3	3	ST247	0		
20	MRSA	2009	ECU	446	Human	Pleural fluid	0	0	0	0	0	1	0	2.3.4.5	A	NT	10	5	3	2	2	2	3	3	1	3	1	2	3	3	ST8	2	ST239	3
22	MRSA	2009	ECU	503	Human	Thumb abscess	1	0	0	0	0	0	1	2.3	B	NT	10	5	2	6	2	3	3	3	1	3	1	2	3	3	ST8	0	ST8	0
24	MRSA	2009	ECU	325	Human	Thigh secretion	1	0	0	0	0	0	0	2.3.5	A	NT		5	3	2		3	3		1		1		3	3	ST239	1		

Strain id[a]	Species	Year of isolation	Country[b]	HVQ Sample id[c]	Host	Isolated from	Skin and soft tissue	Post-surgical wound	Tracheal-bronchial	Blood	Bone marrow	Others	PVL (*LukS/F-PV*)	*ccr* complex	*mec* complex	SCC*mec*	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA0906	SA1132/63_01	SA1194	SA1213	SA1291	SA1425	SA1729	SA1756	SA1866	SA2039	Relatedness_10loc	Distance_10loci	Relatedness_14loci	Distance_14loci
27	MRSA	2009	ECU	568	Human	Tracheal secretion	0	0	1	0	0	0	0	2.3	A	NT	6	5	3	2		3	3		1		1		3	2	ST239	2		
29	MRSA	2009	ECU	705	Human	Back abscess	1	0	0	0	0	0	1	2.3	B	NT	9	5	2	6	1	2	5	3	1	4	1	2	3	3	ST8	2	ST8	4
31	MRSA	2009	ECU	775	Human	Abscess	1	0	0	0	0	0	1	2.5	B	IVh	10	5	2	6	2	2	3	3	1	1	1	2	3	3	ST8	0	ST8	1
32	MRSA	2009	ECU	780	Human	Abscess	1	0	0	0	0	0	1	2	B	IV	11	5	2	6	2	2	3	3	1	3	1	2	3	3	ST8	1	ST8	1
34	MRSA	2009	ECU	970	Human	Axilar abscess	1	0	0	0	0	0	1	2.5	B	IVh	10	5	2	6	2	2	3	3	1	3	1	2	3	3	ST8	0	ST8	1
37	MRSA	2009	ECU	336	Human	Tracheal secretion	0	0	1	0	0	0	0	2.3.4.5	A	NT		5	3	2		3	3		1		1		3	3	ST239	1		
38	MRSA	2009	ECU	368	Human	Gluteus wound	1	0	0	0	0	0	1	2,5	B	IVh	10	5	2	6	2	2	3	3	1	3	1	2	3	3	ST8	0	ST8	0
39	MRSA	2009	ECU	648	Human	Abscess	1	0	0	0	0	0	1	2,5	B	IVh	10	4	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	2
40	MRSA	2009	ECU	866	Human	Abscess	1	0	0	0	0	0	1	2.3.4.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
41	MRSA	2009	ECU	874	Human	Foot secretion	1	0	0	0	0	0	1	2.3.4.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
43	MRSA	2009	ECU	935	Human	Bone	0	0	0	0	1	0	0	2.3.5	-	NT		5	3	2		2	3		1		1		3	3	ST239	1		
45	MRSA	2009	ECU	10	Human	Wound drainage	0	1	0	0	0	0	0	3.5	-	NT		5	3	2		2	3		1		1		3	3	ST239	1		
46	MRSA	2009	ECU	48	Human	Thigh abscess	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
47	MRSA	2009	ECU	117	Human	Groin abscess	1	0	0	0	0	0	1	2.3	B	NT	9	5	2	6	2	1	7	3	1	3	1	2	3	3	ST8	2	ST8	2
48	MRSA	2009	ECU	271	Human	Abscess	1	0	0	0	0	0	1	2.3	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
49	MRSA	2009	ECU	458	Human	Groin abscess	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	2	3	3	1	3	1	2	3	3	ST8	0	ST8	0
50	MRSA	2009	ECU	582	Human	Finger abscess	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2.5	1	7	3	1	3	1	2	3	3	ST8	2	ST8	3
53	MRSA	2009	ECU	1076	Human	Pharynx secretion	0	0	1	0	0	0	0	2.3.5	B	NT	10	5	2	6	2	2	3	5.5	1	3.5	1	2	3	3	ST8	0	ST8	2

Strain id[a]	Species	Year of isolation	Country[b]	HVQ Sample id[c]	Host	Isolated from	Skin and soft tissue	Post-surgical wound	Tracheal-bronchial	Blood	Bone marrow	Others	PVL (*LukS/F-PV*)	*ccr* complex	*mec* complex	SCC*mec*	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA0906	SA1132/63_01	SA1194	SA1213	SA1291	SA1425	SA1729	SA1756	SA1866	SA2039	Relatedness_10loc	Distance_10loci	Relatedness_14loci	Distance_14loci
54	MRSA	2009	ECU	68	Human	Tracheal secretion	0	0	1	0	0	0	0	2.3.5	B	NT	5	5	3	2		2	3		1		1		3	3	ST239	2		
56	MRSA	2009	ECU	122	Human	Forehead boil	1	0	0	0	0	0	1	2.3.5	A.B	NT	5	5	2	6	2	1	3	3	1	3.5	1	2	3	3	ST8	2	ST8	3
57	MRSA	2009	ECU	260	Human	Groin abscess	1	0	0	0	0	0	1	2.5	B	IVh	10	5	2	6	2	1	3	3	1	3.5	1	2	3	3	ST8	1	ST8	2
58	MRSA	2009	ECU	360	Human	Leg secretion	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
59	MRSA	2009	ECU	308	Human	Abdominal abscess	1	0	0	0	0	0	1	2.5	B	IVh	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	0
62	MRSA	2009	ECU	1066	Human	Groin abscess	1	0	0	0	0	0	1	2.3.5	-	NT	10	3	2	6	2	2	3	3	1	3	1	2	3	3	ST8	1	ST8	1
63	MRSA	2010	ECU	115	Human	Lip abscess	1	0	0	0	0	0	1	2.3.5	-	NT	10	5	2	6	1	1	3	3	1	3	1	2	3	3	ST8	1	ST8	2
65	MRSA	2010	ECU	252	Human	Abscess	1	0	0	0	0	0	1	2.3.5	B	NT	9	4	2	6	2	2	3	3	1	3.5	1	2	3	3	ST8	2	ST8	3
66	MRSA	2010	ECU	286	Human	Wound	1	0	0	0	0	0	1	2.3.5	B	NT	9	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
67	MRSA	2010	ECU	289	Human	Abscess muslo	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	4	2	1	3	3	1	4	1	2	3	2	ST8	2	ST8	3
68	MRSA	2010	ECU	417	Human	Drainage liquid	0	0	0	0	0	1	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	4	1	2	3	3	ST8	1	ST8	2
69	MRSA	2010	ECU	520	Human	Abscess	1	0	0	0	0	0	1	2.3.5	B	NT	11	5	2	6		1	3		1		1		3	3	ST247	1		
70	MRSA	2010	ECU	709	Human	Facial secretion	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	4	1	2	3	3	ST8	1	ST8	2
72	MRSA	2010	ECU	1219	Human	Blood	0	0	0	1	0	0	0	2.3.5	A. B	NT		5	3	2		2	3		1		1		3	3	ST239	1		
73	MRSA	2010	ECU	1275	Human	Thigh abscess	1	0	0	0	0	0	1	3	A	III	10	5	2	6	2	1	3	3	1	5	1	2	3	3	ST8	1	ST8	2
74	MRSA	2010	ECU	800	Human	Skin abscess	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
75	MRSA	2010	ECU	596	Human	Wound	1	0	0	0	0	0	0	3.5	A	IIIB	10	5	3	2	2	2	3	5	1	2	1	2	3	3	ST8	2	ST241	3
77	MRSA	2010	ECU	301	Human	Pericardiac fluid	0	0	0	0	0	1	1	2.3.5	B	NT	9	6.5	3	4		7.5	3		4		5		3	3	ST5	3		

Strain id[a]	Species	Year of isolation	Country[b]	HVQ Sample id[c]	Host	Isolated from	Skin and soft tissue	Post-surgical wound	Tracheal-bronchial	Blood	Bone marrow	Others	PVL (*LukS/F-PV*)	*ccr* complex	*mec* complex	SCC*mec*	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA0906	SA1132/63_01	SA1194	SA1213	SA1291	SA1425	SA1729	SA1756	SA1866	SA2039	Relatedness_10loc	Distance_10loci	Relatedness_14loci	Distance_14loci
78	MRSA	2010	ECU	596	Human	Thigh abscess	1	0	0	0	0	0	0	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
79	MRSA	2010	ECU	627	Human	Groin abscess	1	0	0	0	0	0	1	2.3.5	B	NT	10	4	2	6	1	1	3	3	1	3	1	2	3	3	ST8	1	ST8	3
81	MRSA	2010	ECU	1123	Human	Nail infection	1	0	0	0	0	0	1	2.3.5	B	NT	10	4	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	2
82	MRSA	2010	ECU	1148	Human	Wound	1	0	0	0	0	0	1	2.5	B	IVh	10	4	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	2
85	MRSA	2010	ECU	244	Human	Soft tissue secretion	1	0	0	0	0	0	1	2.3.5	B	NT	10	4	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	2
86	MRSA	2010	ECU	449	Human	Foot wound	1	0	0	0	0	0	1	5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	2	ST8	2	ST8	2
89	MRSA	2010	ECU	1004	Human	Foot wound	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	3.5	1	2	3	2	ST8	2	ST8	2
90	MRSA	2010	ECU	842	Human	Izquiorectal abscess fluid	0	0	0	0	0	1	1	2.5	B	IVh	10	5	2	4	2		3	3	1	3	1	2	3	2	ST8	1	ST8	2
91	MRSA	2010	ECU	1048	Human	Knee wound	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
92	MRSA	2010	ECU	1054	Human	Leg abscess	1	0	0	0	0	0	1	2	B	IV	9	5	2	6	1	1	3	3	1	1	1	2	3	3	ST8	1	ST8	3
94	MRSA	2010	ECU	294	Human	Bone marrow	0	0	0	0	1	0	1	2.3.5	B	NT	10	5	2	6	3	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
95	MRSA	2010	ECU	319	Human	Pleural fluid	0	0	0	0	0	1	1	2.3.5	A	NT		5	3	2		2	3		1		1		3	3	ST239	1		
96	MRSA	2010	ECU	734	Human	Facial abscess	1	0	0	0	0	0	0	2	B	IV	11	5	2	6		1	3		1		1		3	3	ST247	1		
97	MRSA	2010	ECU	928	Human	Surgical wound	0	1	0	0	0	0	0	2.3	A	NT	6	5	3	2		2	3		1		1		3	3	ST239	2		
98	MRSA	2010	ECU	932	Human	Abscess	1	0	0	0	0	0	0	2.3.5	B	NT	10	5	2	3	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
99	MRSA	2010	ECU	933	Human	Boil	1	0	0	0	0	0	1	2.3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
103	MRSA	2010	ECU	636	Human	Knee boil secretion	1	0	0	0	0	0	0	3.5	A.B	NT	6	5	3	2		2	3		1		1		3	3	ST239	2		
104	MRSA	2010	ECU	793	Human	Finger abscess	1	0	0	0	0	0	0	3	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1

Strain id[a]	Species	Year of isolation	Country[b]	HVQ Sample id[c]	Host	Isolated from	Skin and soft tissue	Post-surgical wound	Tracheal-bronchial	Blood	Bone marrow	Others	PVL (*LukS/F-PV*)	*ccr* complex	*mec* complex	SCC*mec*	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA0906	SA1132/63_01	SA1194	SA1213	SA1291	SA1425	SA1729	SA1756	SA1866	SA2039	Relatedness_10loc	Distance_10loci	Relatedness_14loci	Distance_14loci
105	MRSA	2010	ECU	1023	Human	Knee wound	1	0	0	0	0	0	1	3.5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
107	MRSA	2010	ECU	819	Human	Thigh abscess	1	0	0	0	0	0	1	3.5	B	NT	7	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
108	MRSA	2010	ECU	821	Human	Tracheal secretion	0	0	1	0	0	0	1	5	B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	2
109	MRSA	2010	ECU	34	Human	Shoulder secretion	1	0	0	0	0	0	0	2.3.5	A.B	NT		5	3	2		2	3		1		1		3	3	ST239	2		
110	MRSA	2010	ECU	210	Human	Ankle secretion	1	0	0	0	0	0	0	2.3.5	B	NT	10	4	1	4	3	2	7	5	4	3	3	2	3	3	X	X	ST5	3
111	MRSA	2010	ECU	320	Human	Leg secretion	1	0	0	0	0	0	0	2.3.5	B	NT		5	3	2	2	3	3	3	1	3	1	2	3	3	ST241	2		
116	MRSA	2010	ECU	423	Human	Gluteus abscess	1	0	0	0	0	0	0	2.3	A.B	NT	11	5	2	6.5		1	3		1		1		3	3	ST247	2		
117	MRSA	2010	ECU	456	Human	Leg abscess	1	0	0	0	0	0	1	2.3	A.B	NT	11	5	2	6.5		1	3		1		1		3	3	ST247	2		
119	MRSA	2010	ECU	793	Human	Skin	1	0	0	0	0	0	0	3.5	B	NT	9	5	2	6.5	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
123	MRSA	2010	ECU	795	Human	Vertebral body	0	0	0	0	1	0	0	2.3.5	A.B	NT	9	5	2	6.5	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
124	MRSA	2010	ECU	796	Human	Lumbar puncture	0	0	0	0	1	0	1	2.5	A.B	NT	9	5	2	6.5	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
125	MRSA	2010	ECU	729	Human	Blood	0	0	0	1	0	0	0	2.3.5	A.B	NT	9	5	2	6.5	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
126	MRSA	2010	ECU	730	Human	Blood	0	0	0	1	0	0	1	2.3.5	A.B	NT	9	5	2	6.5	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
127	MRSA	2010	ECU	732	Human	Blood	0	0	0	1	0	0	0	2.3.5	A.B	NT	9	5	2	6.5	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
128	MRSA	2010	ECU	763	Human	Bone marrow culture	0	0	0	0	1	0	1	2.3.5	A.B	NT	9	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
130	MRSA	2010	ECU	8501	Human	Blood	0	0	0	1	0	0	0	2.3.5	B	NT	10	5	2	6.5		2	3	3	1				3	3	ST8	2	ST8	5
133	MRSA	2010	ECU	513	Human	Synovial fluid	0	0	0	0	0	1	1	2.3.5	B	NT	9	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
134	MRSA	2010	ECU	873	Human	Sputum	0	0	1	0	0	0	0	1.2.5	B	NT	11	6	1	2		1	7				2		3	2	ST72	1		

Strain id[a]	Species	Year of isolation	Country[b]	HVQ Sample id[c]	Host	Isolated from	Skin and soft tissue	Post-surgical wound	Tracheal-bronchial	Blood	Bone marrow	Others	PVL (*LukS/F-PV*)	*ccr* complex	*mec* complex	SCC*mec*	SA0122/24_01	SA0266/81_01	SA0311	SA0704/67_01	SA0906	SA1132/63_01	SA1194	SA1213	SA1291	SA1425	SA1729	SA1756	SA1866	SA2039	Relatedness_10loc	Distance_10loci	Relatedness_14loci	Distance_14loci
135	MRSA	2010	ECU	896	Human	Right ankle	1	0	0	0	0	0	0	2.3.5	B	NT	10	4	1	4	3	3	7	5.5	4	2	3	2	3	3	X	X	ST5	5
138	MRSA	2010	ECU	1046	Human	Elbow abscess	1	0	0	0	0	0	0	2.3	A.B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
141	MRSA	2010	ECU	513	Human	Blood	0	0	0	1	0	0	1	2	A.B	NT	10	5	2	6	2	1	3	3	1	3	1	2	3	3	ST8	1	ST8	1
142	MRSA	2010	ECU	542	Human	Vulvar pustules	1	0	0	0	0	0	1	2	B	IV	10	4	2	6	2	2	3	3	1	3	1	2	3	3	ST8	1	ST8	1
143	MRSA	2010	ECU	511	Human	Blood	0	0	0	1	0	0	1	2	B	IV	10	6	2	6	2	1	3	3	1	3	1	2	3	3	ST8	2	ST8	2
147	MRSA	2010	ECU	725	Human	Gluteus abscess	1	0	0	0	0	0	0	2.5	B	IVh	10	4	2	6	2	2	3	3	1	3	1	2	3	3	ST8	1	ST8	1
TrSa214		**2007**	**FR**														**9**	**6**	**5.5**	**5**	**0.5**	**2**	**8**	**0**	**1**	**0.5**	**5**	**2**	**3**	**1**	**ST45**	**0**	**ST45**	**0**
TrSa152		**2007**	**FR**														**10**	**6**	**3**	**4**	**3**	**7**	**7**	**5**	**4**	**4**	**5**	**2**	**3**	**3**	**ST5**	**0**	**ST5**	**0**
TrSa150		**2007**	**FR**														**7**	**4**	**3**	**3**	**0.5**	**3**	**5**	**0**	**2**	**0**	**4**	**3**	**2**	**4**	**ST30**	**1**	**ST30**	**1**
TrSa134		**2007**	**FR**														**10**	**5**	**2**	**6**	**2**	**3**	**3**	**3**	**1**	**3**	**1**	**2**	**3**	**2**	**ST8**	**1**	**ST8**	**1**

[a], TrSa214, TrSa152, TrSa150, and TrSa134 are control strains.

[b], ECU stands for Ecuador, FR stands for France.

[c], HVQ stands for Hospital Vozandes Quito.

*In all the table, except in loci data, 1 stands for presence and 0 for absence. In loci data numbers represent VNTR repeats.

Anexo 2. Ejemplo de una publicación en la revista seleccionada

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Improved Multiple-Locus Variable-Number Tandem-Repeat Assay for *Staphylococcus aureus* Genotyping, Providing a Highly Informative Technique Together with Strong Phylogenetic Value▽†

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We describe an improved *m*ultiple-*l*ocus *v*ariable-*n*umber *t*andem-*r*epeat (VNTR) *a*nalysis (MLVA) scheme for genotyping *Staphylococcus aureus*. We compare its performance to those of *m*ulti*l*ocus *s*equence *t*yping (MLST) and *spa* typing in a survey of 309 strains. This collection includes 87 epidemic methicillin-resistant *S. aureus* (MRSA) strains of the Harmony collection, 75 clinical strains representing the major MLST clonal complexes (CCs) (50 methicillin-sensitive *S. aureus* [MSSA] and 25 MRSA), 135 nasal carriage strains (133 MSSA and 2 MRSA), and 13 published *S. aureus* genome sequences. The results show excellent concordance between the techniques' results and demonstrate that the discriminatory power of MLVA is higher than those of both MLST and *spa* typing. Two hundred forty-two genotypes are discriminated with 14 VNTR loci (diversity index, 0.9965; 95% confidence interval, 0.9947 to 0.9984). Using a cutoff value of 45%, 21 clusters are observed, corresponding to the CCs previously defined by MLST. The variability of the different tandem repeats allows epidemiological studies, as well as follow-up of the evolution of CCs and the identification of potential ancestors. The 14 loci can conveniently be analyzed in two steps, based upon a first-line simplified assay comprising a subset of 10 loci (panel 1) and a second subset of 4 loci (panel 2) that provides higher resolution when needed. In conclusion, the MLVA scheme proposed here, in combination with available on-line genotyping databases (including http://mlva.u-psud.fr/), multiplexing, and automatic sizing, can provide a basis for almost-real-time large-scale population monitoring of *S. aureus*.

Staphylococcus aureus is a pathogen of worldwide clinical significance. For this reason, it is the subject of intensive investigations in terms of virulence and drug resistance phenotypes and, also, population genetics. Although the latter is not of significant use for short-term patient care, it is essential for understanding the emergence and spread of new phenotypes. For instance, it was initially considered most likely that methicillin (meticillin)-resistant variants were appearing only rarely through the acquisition of a mobile DNA region designated staphylococcal cassette chromosome *mec* (SCC*mec*) and that these variants were spreading efficiently worldwide (34). However, the most recent population genetics investigations suggested instead that SCC*mec* was acquired hundreds of times independently worldwide and that, as a rule, the geographic spread of these resistant strains was limited (28, 36). This knowledge could be produced in the past 10 years due to sequence-based approaches, mainly *m*ulti*l*ocus *s*equence *t*yping (MLST) analysis, in which approximately 3 kb of coding genome sequence (or 1/1,000 of the whole genome) are scanned for polymorphism. MLST has allowed the creation of shared and high-quality databases which can be easily queried over the Internet, and this has proved to be highly valuable (7). However, as the sequences used in MLST schemes evolve slowly and are highly conserved, the resolution provided by MLST is too low for the investigation of recent evolution and, above all, for short-term epidemiological studies. The sequencing of much larger portions of the genome to increase resolution can only be used in dedicated research projects analyzing a limited number of strains (28). Presently, pulsed-field gel electrophoresis (PFGE) remains the most discriminatory technique for *S. aureus* typing, but it allows the constitution of shared databases only at the national level and is not appropriate for population studies (1, 35). There is, consequently, still a need for a technology as discriminatory as PFGE and as portable as MLST at a low cost.

Tandemly repeated sequences provide a very valuable source of polymorphism. *M*ultilocus *v*ariable-*n*umber *t*andem-*r*epeat (VNTR) analysis (MLVA) is now used in genotyping several bacterial species (26, 48). MLVA typing relies upon a basic and widespread methodology, the measurement of the length of DNA fragments. It is not a "pattern"-producing method, even when run on agarose gels. The genotype, in the form of a string of numbers corresponding to the number of repeats at each locus, is highly portable and can be readily incorporated in large databases (13).

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VNTRs were proposed years ago to genotype *S. aureus* isolates, first with a tandem repeat (TR) adjacent to the coagulase gene *coa* (12) and later with a single TR present in the *S. aureus* protein A (*spa*) gene (9). Subsequently, new TRs present in individual genes were analyzed simultaneously to produce patterns, or "fingerprints" (8, 37). A second level of TR-associated polymorphisms due to repeat unit variations was exploited by sequencing TR alleles. The *spa* gene, providing a high level of information, is most frequently used. It allows the relatively correct assignment of isolates to MLST-defined clonal complexes (CCs) (17, 22, 41), with some occasional exceptions. The resulting data have a limited phylogenetic value (28). In order to increase the discriminatory power and phylogenetic content, an assay called *d*ouble-*l*ocus *s*equence *t*yping (DLST) in which a second TR locus (*clfB*) is included has recently been developed (23).

MLVA stricto sensu, in which a repeat copy number is deduced for each locus, was first applied to *S. aureus* genotyping by Hardy et al., using seven members of a class of repeated elements called *s*taphylococcal *i*nterspersed *r*epeat *u*nits (SIRUs) (15, 16). Eleven additional TRs were later identified, and different combinations were used to improve the assay (11). Ikawaty et al. recently described an MLVA scheme with six SIRUs (20) that showed a higher discriminatory power than MLST and *spa* typing. However, the clustering only identified three large clusters of MLVA types and the correlation with MLST CCs was partial. In a recent work, Schouls et al. confirmed that MLVA with as few as eight VNTRs provided clustering similar to that of *spa* typing and PFGE, but they did not demonstrate agreement between MLST complexes and MLVA complexes with their assay (40).

Although MLVA appears to have the potential to provide a technique for short-term epidemiological studies that is fast and reliable in comparison to other techniques, there is still no consensus on the set of VNTR markers to be used for an efficient genotyping protocol and, more generally, for the potential use of MLVA for *S. aureus* typing. Different TRs vary at different rates, and homoplasy levels at individual VNTRs may be high. Due to intraspecies genetic variability in *S. aureus*, some primer combinations fail to amplify a significant fraction of the strains. The loci and primers to be used need to be carefully selected. In the present study, we have investigated new VNTRs, as well as previously described ones, and we propose an MLVA genotyping scheme, the MLVA-14 assay, made up of two complementary panels totaling 14 markers, which provides an easy and highly informative genotyping assay with a strong phylogenetic content. For this purpose, we analyzed three diverse *S. aureus* strain collections which were previously characterized in detail with both MLST and *spa* typing.

MATERIALS AND METHODS

Strains. Eighty-seven strains from the Harmony collection (1) were provided by Alex van Belkum. Twenty-five methicillin-resistant *S. aureus* (MRSA) carriage strains were isolated in the Maastricht University Medical Center (MUMC) in The Netherlands between 2002 and 2006 and comprise five strains each from MLST CC5, CC8, CC22, CC30, and CC45 (29). Fifty methicillin-sensitive *S. aureus* (MSSA) nasal carriage strains were isolated from patients attending their general practitioner in The Netherlands during 2005 and comprise five strains each from MLST CC5, CC7, CC8, CC12, CC15, CC22, CC25, CC30, CC45, and CC51 (6). These 75 strains were previously *spa* typed (30).

One hundred thirty-five nasal carriage strains, two of which were MRSA, were isolated from newly employed hospital personnel during their first medical checkup at a tertiary care hospital in Lausanne, Switzerland, and have been genotyped using MLST, *spa* typing, amplified fragment-length polymorphism, and DLST (39). Reference strain Mu50 was purchased from the Centre de Ressources Biologiques de l'Institut Pasteur (CRBIP). The Ridom nomenclature was used to describe the organization of the *spa* repeats (17).

MLVA. Oligonucleotide primers targeting the 5′ and 3′ flanking regions of the selected loci and matching the sequenced genomes of strains COL, MRSA476, MW2, N315, NCTC8325, JH1, JH9, Newman, USA300 (FPR3757), and USA300 (TCH1516) were used for amplification. Some mismatches existed with the genomes of strains MRSA252 and RF122. DNA was extracted by using a DNeasy tissue kit (Qiagen) after treatment of bacteria with lysostaphin (Sigma) at a concentration of 1 mg/ml. PCRs were performed in 15-μl volumes containing 2 ng DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Qiagen, Courtaboeuf, France), 200 μM of each deoxynucleoside triphosphate, and 0.3 μM of each flanking primer (Eurogentec, Angers, France). Amplification was performed with a PTC 200 thermocycler (Bio-Rad, Marnes-la-Coquette, France) using the following conditions: initial denaturation cycle for 5 min at 94°C, 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at various temperatures (indicated in Table 1), and elongation for 45 s at 72°C plus a final elongation step for 10 min at 72°C. Three microliters of PCR products was separated in a 2% agarose gel (Eurogentec, Angers, France). Electrophoresis was performed in 20-cm-long gels made in 0.5× Tris-borate-EDTA buffer (Sigma), run at 8 V/cm. In each run, the PCR product from reference strain Mu50 was included at least once. The 100-bp DNA size marker was from MBI Fermentas (Euromedex, Souffelweyersheim, France). The gels were stained after the run in 0.5 μg/ml ethidium bromide for 15 to 30 min and then rinsed with water and photographed under UV illumination (Vilber-Lourmat, Marne la Vallée, France). To prevent carryover contamination, the different steps of the procedure were performed in separate rooms with dedicated materials.

In the first phase of the study, the size of the amplicons was measured with the assistance of BioNumerics 5.1 (Applied-Maths, Sint-Martens-Latem, Belgium), and the number of repeats was deduced using the Mu50 genome sequence as a reference. Thereafter, the size of the amplicons was directly estimated by eye before import and conversion into a character data set in BioNumerics.

Nomenclature and description of MLVA profiles. The repeat lengths and numbers of repeat units in the different sequenced genomes were determined by using the Microorganisms Tandem Repeats Database (http://minisatellites.u-psud.fr) (4, 13, 25). For each locus, the size of the PCR product, *S*, in the first sequenced genome (as predicted by in silico analysis of reference strain Mu50 with the primer pair used here), the size of the repeat unit, *U*, and the corresponding number of repeat units, *N*, are indicated in Table 1. Amplification of DNA from reference strain Mu50 produced amplicons of the expected size. The exact copy number for each allele was calculated as follows: *S* was subtracted from the estimated allele size, and the result was divided by *U*, added to *N*, and rounded up to the nearest integer that was distant by less than 0.2. Alleles which could not be rounded up following this rule were double-checked and eventually sequenced to confirm the existence of intermediate alleles (0.5) and to establish the reason for this intermediate size (which may result from small deletions in the flanking sequence).

The polymorphism index of individual or combined VNTR loci was calculated using the Hunter-Gaston diversity index (19), an application of Simpson's index of diversity (43). Confidence intervals (CI) were calculated as described by Grundmann et al. (14). The results of using the MLVA-14 assay, with 14 VNTRs in two panels, to genotype a strain are expressed as its allelic profile, corresponding to the number of repeats at each VNTR in the order Sa0122 (*spa*), Sa0266 (*coa*), Sa0311, Sa0704, Sa1132, Sa1194, Sa1291 (SIRU13), Sa1729, Sa1866, and Sa2039 (panel 1) and Sa0906, Sa1213, Sa1425, and Sa1756 (SIRU15) (panel 2) (Table 1). The genotype of Mu50, deduced from its genomic sequence, is 10 6 3 4 6 7 4 5 3 3 (panel 1) 3 5 4 2 (panel 2). The categorical coefficient (also called Hamming's distance) and the unweighted pair group method with arithmetic mean clustering method were run within BioNumerics. A cutoff value of 45% similarity was applied to define clusters. It corresponds to differences at a maximum of 3 VNTRs out of 14. This is still empirical since there is no precise knowledge about the VNTR evolutionary mechanism and speed, but it seems to correctly define clusters when compared to those defined by other genotyping methods. The minimum spanning tree was produced in BioNumerics, allowing the creation of missing links. The circle size is proportional to the number of isolates. A logarithmic scale was used when drawing branches.

The MLVA profiles and allele size ranges are available for comparison in the MLVAbank for Bacterial Genotyping (http://mlva.u-psud.fr/ [*Staphylococcus aureus* database]).

TABLE 1. VNTRs used in this study

VNTR locus and MLVA-14 panel[a]	Gene or SIRU where located	Size (bp) of repeat unit (U)[f]	No. of repeats in Mu50 genome (N)[f]	Size (bp) of PCR product in Mu50 (S)[f]	Oligonucleotide[g]	Annealing temp (°C)	Gene or region amplified
Panel 1							
Sa0122[b]	*spa* SIRU21	24	10	392	L, AGCAGTAGTGCCGTTTGCTT R, AAGACGATCCTTCAGTGAGCA	60	*spa*
Sa0266[c]	*coa*	81	6	630	L, TTGGATATGAAGCGAGACCA R, CTTCCGATTGTTCGATGCTT	60	*coa*
Sa0311		55	3	272	L, AGGGTTAGAGCCCGAGACAT R, CACGGGATTGGAACAGAAAT	60	STAR
Sa0704		67	4	380	L, CGCGCGTGAATCTCTTTTAT R, AGTCCCATATCGTGCGTTAAA	60	Intergenic
Sa1132		63	6	532	L, CGTGCATAATGGCTTACGAA R, AAGCAGCAGAAAAAGCTAAAGAA	60	SAV1078
Sa1194		67	7	524	L, AGTGCAAGCGGAAATTGAAG R, ATCGTGAAAAAGCCCAAAAA	60	Intergenic
Sa1291	SIRU13	64	4	369	L, GGGGGAAATTCTAAGCAACC R, CGAAATTTTCCACGTCGATT	60	Intergenic
Sa1729		56	5	499	L, TACTTAAAAATARGAATACATAATTAG R, CAACAATAAATTACTTATTTGAAGTT	53	STAR
Sa1866		159	3	607	L, CTGTTTTGCAGCGTTTGCTA R, GCAACTTGAAGAAACGGTTG	60	SAV1738
Sa2039		56	3	282	L, TTCGTTCTACCCCAACTTGC R, GAGCCTGGGTCATAAATTCAA	60	STAR
Panel 2							
Sa0906		56	3	864	L, CATGTATTCATGGGATTGCAGC R, CAGATTTTCCTTCAACAATTATCAC	55	STAR
Sa1213		56	5	868	L, GGCTGATGCTAAAGTTGCATTAGA R, GTGGCATGTTCTACAAACGTAAAC	55	STAR
Sa1425		58	4	630	L, TCGTTATTAAACTACGAATTCTCGATT R, ATTTCGRGAATGATTCAATTCAATTTT	55	STAR
Sa1756[d]	SIRU15	131	2	365	L, AATTATAGCATATTAGAGCCCCTTA R, ACGTAAAGGTCGCGACAAAA	60	Intergenic
Other VNTRs							
Sa2547		15	5	257	L, AAAGATGCTGAAAAGAAAGTGG R, TGATCAATCGCACCTTTGTA	58	*sbi*
Sa387[d]	SIRU1	55	2	299	L, CATGAGCAGTGCCTCCTTTA R, CGCCTTGTATCTTAAATTTGTTG	55	Intergenic
Sa2821	SAV2654[e] SAV2655	54	7	396	L, CTGAGTTTGAGTCTACTCCGC R, CGTTTAAGAGCGAGAGTGTT	58	SAV2654
Sa964	SAV0920[e] SAV0921	43	6	468	L, CAACACCATCATGTCCAATA R, CAACCTGTTAATCCGATGTT	58	Intergenic

[a] Locus tags indicate the genomic localization in strain Mu50 (GenBank accession number AP009324) in kilobases.
[b] Primers are different from those in references 42 and 15.
[c] Primers are different from those in reference 37.
[d] SIRU1 and SIRU15 primers are those described in reference 15.
[e] Locus tag is from reference 10.
[f] *U*, *N*, and *S* were used to calculate the exact copy number of each allele as described in Materials and Methods.
[g] L, left; R, right.

RESULTS

Selection of a VNTR panel. In a preliminary study performed in 2003, 14 VNTRs were selected by comparing the available sequenced genomes of strains Mu50, N315, MRSA252, NCTC8325, MW2, and MSSA476 using the strain comparison tool developed by Denoeud and Vergnaud (4) and available at http://minisatellites.u-psud.fr/ (Microorganism Tandem Repeats database and Strain Comparison pages) (Table 1). They were tested on a collection from the Pasteur Institute given to us by Nevine El Sohl and previously typed by PFGE. These initial results showed that MLVA could efficiently cluster strains with similar pulsotypes (31). We then investigated the informativeness and potential use of additional VNTRs described in other studies. Most of the published primers did not perfectly match the genome of the strains analyzed or they were predicted by in silico analysis of thirteen sequenced genomes to amplify more than one locus. This is the case, for instance, for the *sdr* locus in which the three highly informative TRs present in genes *sdrC*, *sdrD*, and *sdrE* could not be amplified independently. In addition to the *spa* locus (SIRU21), only SIRU01, SIRU13, and SIRU15 (15) and SAV920 and SAV1078 (10) were retained and tested on a larger collection of strains (data not shown). For SAV920 and SIRU01, lack of amplification was observed in about 10% of strains, and therefore, these markers were not kept for a first-line MLVA scheme. The selected set of loci comprises 14 VNTRs that are present in all of the sequenced genomes, 6 of which correspond to *S. aureus* repeat (STAR) elements (Sa0311, Sa0906, Sa1213,

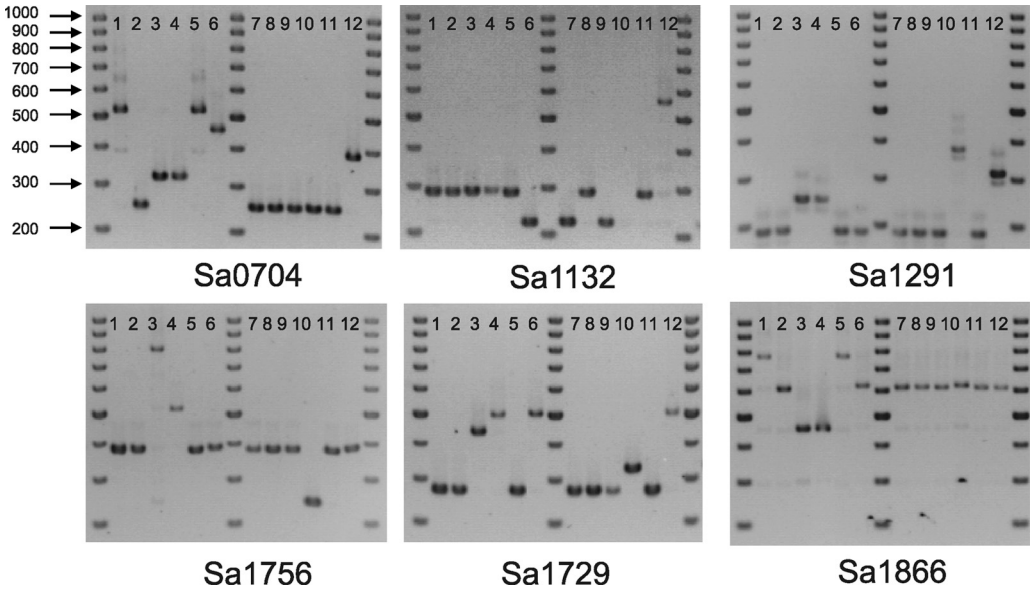

FIG. 1. Polymorphism of six VNTRs in 12 isolates (lanes numbered 1 to 12) as shown by agarose gel electrophoresis of PCR products. PCR was performed on two groups of 6 unrelated isolates, and the products are migrated next to the DNA size marker (the sizes in base pairs are shown on the left side of the first panel).

Sa1425, Sa1729, and Sa1866), a family of intergenic elements whose copy numbers vary from 13 to 21 in individual strains (3). The VNTR set contains four additional intergenic TRs (Sa1756 [SIRU15], Sa0704, Sa1194, and Sa1291 [SIRU13]) and four TRs located inside the coding regions of the *spa*, *coa*, SAV1078, and SAV1738 genes (corresponding to Sa0122, Sa0266, Sa1132, and Sa1866, respectively). The MLVA scheme was run with individual PCRs and agarose gel electrophoresis of amplicons in this study, as shown in Fig. 1 for a subset of VNTRs. The size of the amplicons can be easily estimated by eye on agarose gels. For markers Sa0906 and Sa1213, the absence of amplification was frequently observed when using primers localized about 20 bp on each side of the TRs. Therefore, primers inside the two genes flanking the STAR element were selected, allowing correct amplification in all the strains. A large number of alleles could be obtained for Sa0906, reflecting the complexity of the locus as confirmed by examination of the sequence (see "The structure of Sa0906" below).

This MLVA assay is perfectly reproducible, as attested by the repeated use of Mu50 DNA as control, which always gave the same result. In addition, the stability of the VNTRs in cultured bacteria is demonstrated by the use of different batches of Mu50 DNA and by the fact that the observed amplicon sizes are identical to those predicted by the genome sequence.

Comparison of MLVA, MLST, and *spa* typing in 300 isolates. To assess the informativeness of MLVA compared to that of MLST and *spa* typing, we genotyped three complementary and well-referenced strain collections previously analyzed by these two techniques and belonging to the major MLST CCs. Overall, the efficiency of PCR amplification was excellent. Only in three instances could no amplification be obtained, which may be due to the absence or the mutation of the target of one of the primers: Sa0311 failed to amplify two MLST sequence type 398 (ST398) samples from nasal carriage, and Sa1291 failed to amplify the ST8 sample NL33. The data were used to perform a clustering analysis based upon the categorical distance coefficient and unweighted pair group method with arithmetic mean clustering method. With a cutoff value of 45%, 21 clusters were defined, two of which corresponded to a single isolate (2 strains of nasal carriage, Laus167 of ST50 and Laus325 of ST78) (see dendrogram in Fig. S1 in the supplemental material). These clusters correspond to CCs defined by MLST and by *spa* typing together with the algorithm Based Upon Repeat Pattern. Figure 2 shows a minimum spanning tree which produces a more condensed representation of the clustering and suggests relationships between the clusters. An almost perfect correlation between the results of the different techniques was observed, with the exception of a few isolates which did not cluster by MLVA with strains of the same ST or *spa* type. Strain NL33, *spa* typed as t701 that is associated with CC8, is grouped with CC7, and Laus356, ST45 and *spa* type t1081, is grouped with CC12, both with long branches. In general, a much higher diversity was found with MLVA than with MLST, particularly inside the major CCs, due to polymorphism at one or several VNTRs (Fig. 3). For example, the groups of 19 ST8 and t008 isolates, 16 ST5 and t002 isolates, and 18 ST30 and t012 isolates were each resolved into 12 MLVA genotypes. Thirteen ST45 t015 isolates were resolved into seven MLVA genotypes (44). The diversity index of the MLVA-14 assay is 0.9965 (95% CI, 0.9947 to 0.9984) (243 genotypes). In comparison, the diversity index of the MLST assay with the same isolates (52 different STs) is 0.9314 (95% CI, 0.9198 to 0.9431) and the diversity of the *spa* assay (127 *spa* types) is 0.9802 (95% CI, 0.9755 to 0.9849).

In order to test whether the complete set of 14 VNTRs was necessary to get a good resolution, clustering was performed with different combinations of markers, and we found that very satisfying clustering was already obtained with 10 VNTRs (panel 1), excluding those occasionally showing very small al-

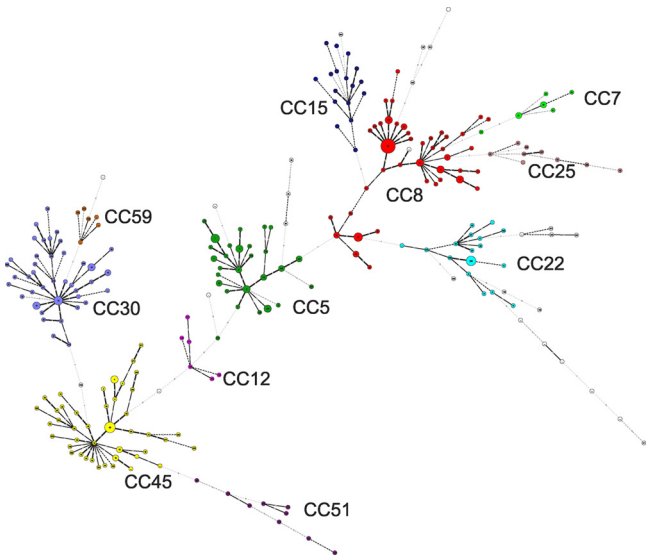

FIG. 2. Minimum spanning tree representation of the MLVA clustering. The MLVA data for 311 isolates, including 10 reference strains, was analyzed in BioNumerics. Each circle represents a genotype, and the size is proportional to the number of isolates. Isolates in the main MLST CCs are indicated by the different colors.

leles (Sa0906, Sa1123, Sa1425, and Sa1756). The only inconsistency observed with panel 1 is the clustering of an ST121 (CC51) isolate with CC45 strains. Therefore, we propose to use panel 1 to assign the isolates to a cluster and four additional markers (panel 2) for more informativeness. Panel 1 discriminates 215 genotypes with a diversity index of 0.9946 (95% CI, 0.9925 to 0.9967).

The structure of Sa0906. As explained above, VNTR Sa0906 is a TR present in a STAR element, which shows a complex organization requiring the use of primers in the flanking coding regions for efficient PCR amplification (SAV0834 and SAV0835) (Fig. 4). A large number of alleles was observed, and in order to better assess their nature, these alleles were sequenced. The basic organization of a STAR element is a group of three sequences, or "boxes," in the order B, C, A that

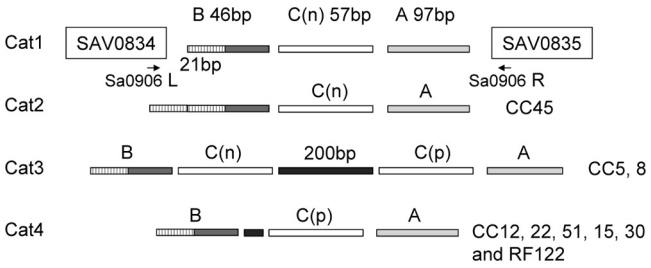

FIG. 4. Organization of the STAR element containing the Sa0906 VNTR. The diagram is drawn from the sequencing of PCR products from isolates of different CCs. Arrows represent PCR primers; the light gray bars represent sequence ("box") A, the dark gray bars box B, and the open bars box C. The black bars represent inserted sequences. Cat, category.

are 46 bp, 57 bp, and 97 bp long, respectively (24). Box C can be tandemly repeated (Fig. 4, cat1). The shortest allele, corresponding to a basic structure, was observed in only four isolates from the present collection of strains (NL33 and a group of nasal carriage isolates, Laus253, Laus369, and Laus292), all localized in out-group positions. In isolates of CC45, the presence of a 21-bp duplication (Fig. 4, cat2) was observed and different alleles were seen to possess one to four C sequences. CC5 and CC8 isolates had an identical structure, with an insertion within the box C repeats of the STAR element and variations in copy number on both sides (Fig. 4, cat3). In several other CCs (and in strain RF122), it seemed that a deletion had removed part of this insertion (Fig. 4, cat4). An artificial convention is proposed so that all the different alleles and deletion combinations can be coded as in the case of an ordinary TR variation (described in the support website at http://minisatellites.u-psud.fr/MLVAnet/index.php?&largeur-1680).

DISCUSSION

MLVA. We demonstrate in this study that the results of MLVA genotyping of *S. aureus* are highly informative and congruent with those of two other widely used techniques,

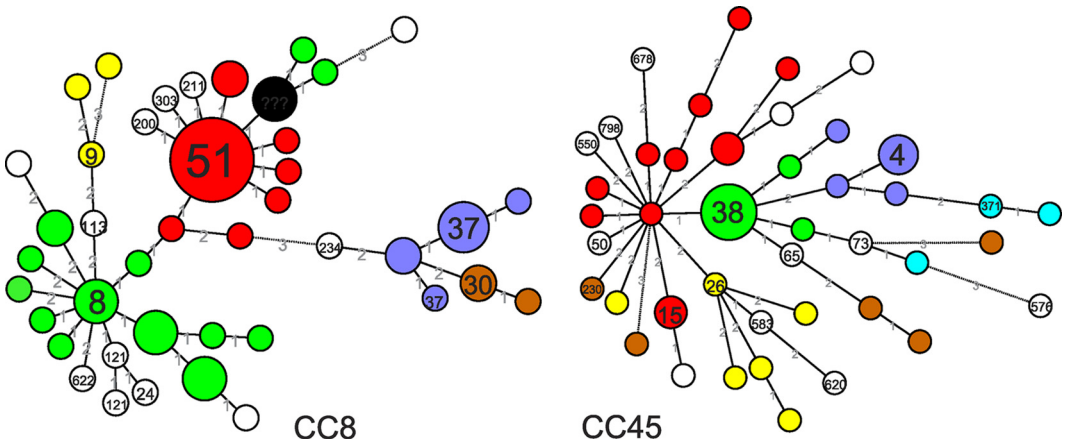

FIG. 3. Minimum spanning tree representation of the MLVA clustering of isolates belonging to CC8 and CC45. The size of each circle is proportional to the number of isolates. *Spa* types are indicated inside circles and by using different colors. The dark circle means that more than one *spa* type is present. The color codes for CC8 and CC45 are independent.

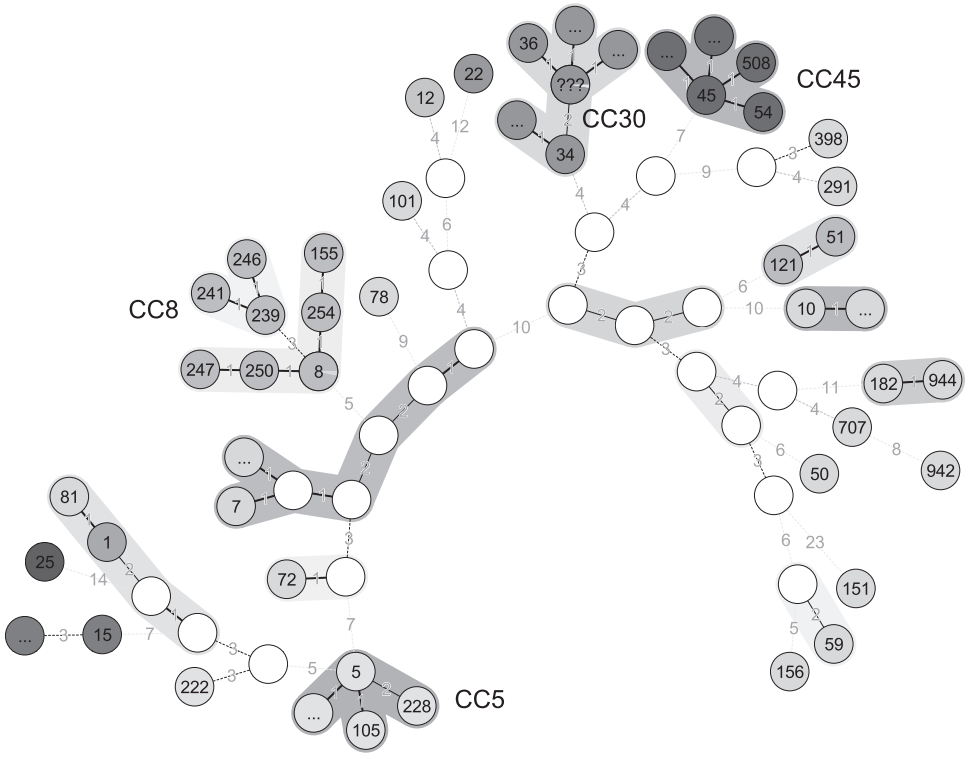

FIG. 5. Minimum spanning tree representation of the MLST clustering, performed using the MLST sequence data. Missing links (empty circles) and logarithmic branch length parameters were used.

MLST and *spa* typing. MLVA provided better resolution and was more congruent with the results of MLST than with those of *spa* typing. VNTRs were previously used to genotype *S. aureus* in a method called *m*ulti*l*ocus *v*ariable-number tandem-repeat *f*ingerprinting (MLVF) to reflect the fact that the data are not interpreted in terms of repeat copy number at each locus but simply used to compare multiple banding patterns as is done with PFGE (21, 27, 38, 45). The discriminatory power of MLVF is similar to that of PFGE, but the profiles are not easily comparable between laboratories and this precludes the constitution of international databases. The reproducibility of MLVF might be also limited by the performance of 10-plex PCRs. Other PCR-based genotyping techniques, such as repetitive sequence-based PCR, are satisfactory for determining strain relatedness, but their informativeness is low and the data are not suitable for population studies (47). Although MLVA is a gel-based method (using either traditional gels or sophisticated capillary electrophoresis equipment), it relies on coding the results as a string of numbers. This, in contrast to all fingerprinting methods, including PFGE and MLVF, makes the resulting profiles highly reproducible and portable, like MLST and *spa* typing.

In the present MLVA scheme, markers which could be easily analyzed using agarose gel electrophoresis were favored (i.e., with a size difference between two alleles that is greater than 5% of the amplicon size across the whole allele size range). As such, the assay can be performed in a laboratory equipped with simple molecular biology equipment. In addition, preliminary experiments showed that the assay can be easily adapted to capillary electrophoresis. For instance, six markers could be analyzed simultaneously on a Beckman CEQ8000 apparatus in a multiplex PCR, using three fluorophores and selecting VNTRs according to the allele size range (see Fig. S2 in the supplemental material). Further development of this procedure should allow the analysis of the complete set of markers in two to three PCRs.

In addition to informativeness and portability, the cost issue is also very important when choosing a genotyping technique, particularly when thousands of isolates need to be investigated. Based on the cost of consumables only, the cost of in-house MLST typing for one sample (seven PCR amplifications and seven sequencing reactions) was estimated to be $30, with an initial start-up cost of $100,000 to $300,000 for equipment. When sequencing is performed outside the laboratory, it amounts at the very least to $50 per strain for sequencing costs alone. The cost for consumables alone for MLVA with 14 VNTRs amounts to $10 per strain whether the analysis is done with agarose gels or capillary electrophoresis, although the later requires a considerably higher start-up investment and expensive maintenance, whereas the former requires more hands-on time.

Population structure. In recent years, MLST has provided a clear view of the *S. aureus* population and its evolution, with the definition of a number of well-defined CCs that are evolving, largely independently from each other, to such an extent that some authors suggest giving species status to each CC (46).

The collection of strains analyzed here covers the two groups or clades identified by MLST analysis (2, 18). This is illustrated in Fig. 5, using a minimum spanning tree representation and

allowing the prediction of missing links. The phylogenetic relationships observed by using MLVA data also separate the isolates into two main groups (Fig. 2). The two clades are not as sharply defined as those found by using MLST data, but the main relationships between CCs are similar using either MLVA or MLST. MLVA clearly allows the identification of CCs and the emergence of new families, as shown here with ST239 emerging from ST8, for example (33).

In the nasal carriage isolates, the clustering produced by MLVA is very similar to that identified by eBURST analysis of MLST data (39). In this previous work, ST942 and ST707 were grouped by DLST, although, when grouped by MLST, they differed by more than two mutations. This was also the case for ST291 and ST398. The MLVA-14 assay, which investigates 14 loci throughout the genome, also groups these isolates, suggesting a closely related origin of at least some part of their genome. Their *spa* types are compatible with a recent common origin. In the previous study mentioned above, amplified fragment-length polymorphism analysis placed Laus356, an ST45 isolate with a *spa* type characteristic for CC45, with a long branch next to the main CC45 cluster. By MLVA, this isolate is positioned with a long branch near the CC12 cluster.

Among the 14 selected VNTRs, 6 are present in STAR elements. The polymorphism of box C in STAR elements is used in a typing protocol based on RFLP and PCR (32) and contributes significantly to the present MLVA-14 assay. Additional polymorphism is provided by different rearrangements, including the insertion or deletion of sequences which can be further described upon sequencing. In particular, marker Sa0906 shows at least four structures which appear to be of phylogenetic value. Indeed, MLVA clustering places certain strains in an ancestral position to specific clusters, for example, NL33, a CC8 isolate from The Netherlands.

Antibiotic resistance. In the present collection, strains belonging to CC7, CC15, and CC25 were all MSSA, whereas both MSSA and MRSA isolates were found intermixed in all the other clusters. CC8 and CC5 isolates are mainly MRSA, as previously observed. This is in agreement with the CCs observed in typical MRSA and MSSA lineages worldwide (reviewed in reference 5).

CC45. We describe in CC45 an unusual polymorphism at locus Sa0906, as well as in loci Sa1213 and Sa1425, with no or only a fraction of one repeat (data not shown). In addition, this CC also possesses only one copy of the repeat for loci Sa1132, Sa1291, Sa2039, and Sa1756 (SIRU15). Among these VNTRs, only locus Sa1132 is within a hypothetical protein-coding gene. In addition, CC45 displays the highest level of *spa* polymorphism, apparently through loss of motifs, whereas the other VNTRs show relatively less diversity (44). Therefore, we believe that in this CC, there is for some reason an important level of recombination leading to deletion. We are presently exploring the basis for this phenomenon. At the moment, there is no complete sequenced genome available for a CC45 strain.

Conclusion. There is a need to expand investigations of pathogenic bacterial populations on a worldwide scale. The currently available data come from very few countries (36), and the main typing method (MLST) has an insufficient discriminatory power for epidemiological and shorter-term evolutionary studies, which is well exemplified by the study of Nubel et al. (28). Single-nucleotide polymorphism typing might provide an interesting alternative to MLST. However, in a highly clonal species like *S. aureus*, the use of single-nucleotide polymorphisms is an inherently biased approach which will fail to explore CCs not previously identified by other means. For epidemiological studies, MLVA entails costs and time investment similar to those of *spa* typing while providing considerably higher resolution. MLVA appears to be adapted to large-scale investigations and, in addition, might give some insight into the effects of DNA plasticity and recombination through the analysis of the TR mechanism of instability. In contrast to MLVF, in which VNTRs are used to produce a pattern, MLVA investigates each locus independently. The MLVA-14 assay described here, comprising 10 loci in panel 1 and 4 loci in panel 2, is highly discriminatory, cost efficient, reproducible, and portable. The assay can be used in automated or more manual protocols, as is best adapted to local conditions. If necessary, additional VNTRs described in the literature (Table 1) could be added by taking advantage of the numerous available genome sequences to design new primer sets able to amplify most if not all strains. In addition to the present collection of strains, the MLVA-14 assay has been applied to about 300 isolates from French patients with cystic fibrosis, allowing a follow-up during chronic infection (H. Vu Thien, K. Hormigos, G. Corbineau, B. Fauroux, H. Carvol, D. Moissenet, G. Vergnaud, and C. Pourcel, unpublished data).

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