RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2011) 14:33-39
DOI: 10.2436/20.1501.01.133 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

New *Staphylococcus aureus* genetic cluster associated with infectious osteomyelitis

Javier Donate-Correa,[1,2] Julia Alcoba-Flórez,[3] Sebastián Méndez-Álvarez[1,2]*

[1]Research Unit, University Hospital Ntra. Sra. de Candelaria, Santa Cruz de Tenerife, Spain. [2]Department of Microbiology and Cell Biology, University of La Laguna, Santa Cruz de Tenerife, Spain. [3]Microbiology Service, University Hospital Ntra. Sra. de Candelaria, Santa Cruz de Tenerife, Spain

Received 16 February 2011 · Accepted 15 March 2011

Summary. Diverse genotyping methods, including multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA), pulsed field gel electrophoresis (PFGE), and multilocus sequence typing (MLST), were used for genotyping *Staphylococcus aureus* in samples recovered from a clinical case of osteomyelitis. An unexpected genetic diversity of strains was determined, including four new sequence types (ST 1521, 1522, 1628 and 1629) belonging to the same genetic lineage, implying the appearance of a new subgroup derived from clonal complex CC121 isolated from that hospital. A close phylogenetic relationship among the STs was demonstrated, reflecting a possible diversifying evolution process. To our knowledge, there have no been previous reports of staphylococcal genetic variability observed within a single individual with such a high degree of variation. These findings emphasize the need for infection control measures to monitor the high genetic variability continuously occurring in this often dangerous infectious agent. [**Int Microbiol** 2011; 14(1):33-39]

Keywords: *Staphylococcus aureus* · osteomyelitis infections · genetic diversity

Introduction

Osteomyelitis is an acute or chronic inflammatory process of both the bone and its structures that occurs secondary to infection with pyogenic organisms [13]. *Staphylococcus aureus* is the main cause of purulent infections in humans, including bone and joint infections [5], and generates lethal and increasingly common nosocomial infections [19]. Occasionally, *S. aureus* acquires enhanced virulence and antimicrobial resistance through horizontal DNA transfer, which increases its variability. The central genome core, however, is relatively stable and mainly diversifies by the accumulation of single-nucleotide substitutions in the absence of frequent interstrain recombination [8]. It is therefore possible to discern different clones and clonal lineages by molecular typing methodologies.

Molecular typing of the *S. aureus* pathogen has been used to examine both long-term or global epidemiology and short-term or local epidemiology. In another context, the typing of organisms in a defined setting over a short period of time may be used to study the relationships between isolates associated with carriage and infection in a given individual [20]. This last approach was used for this study.

***Corresponding author:** S. Méndez-Álvarez
Unidad de Investigación
Hospital Universitario Nuestra Señora de Candelaria
Carr. del Rosario, 100
38010 Santa Cruz de Tenerife, Spain
Tel +34-922600080 / +34-922600545
Fax: +34-922600562
E-mail: sebastianmendez@funcis.org, smendeza@terra.es

Multilocus sequence typing (MLST) is based on housekeeping genes that represent exclusively core genome genes. These genes can only evolve through mutation and gene replacement. MLST has been used to analyze and compare worldwide genetic variation in collections of bacterial isolates [14]. The number of nucleotide differences between two alleles at a single divergent locus can be used to gauge the likelihood that the allele in the founder genotype has changed by mutation or recombination [9]. Sequence data of *S. aureus* strains analyzed thus far show that genetic differences between a single-locus variant and its ancestral strains appear 15-fold more frequently by point mutations than by recombination [8], indicating a predominantly clonal evolution. The clonality of a delimited population could also be elucidated by this highly discriminatory, precise molecular typing method.

It is known that the genotypes of healthcare-associated methicillin-resistant *S. aureus* (HA-MRSA) clones may differ both within this group and, markedly, from methicillin-sensitive (HA-MSSA) isolates from the same hospitals [7]. However, there is little evidence for differences among HA-MSSA or HA-MRSA isolates causing infections in the same individual.

The aim of this investigation was to describe the HA-MSSA strains recovered from a single patient suffering from osteomyelitis, and to explore the diversification of these strains in hospital settings. Isolates were characterized by genotypic methods, thereby inferring the relationships among them.

Materials and methods

Bacterial isolates, and phenotypic identification. A 48-year-old female patient of University Hospital Nuestra Señora de Candelaria (HUNSC) with very aggressive rheumatoid arthritis, fitted with total prostheses in both knees, presented with destruction of the left femoral head and pubic symphysis. Pathological and microbial evaluation showed active, chronic osteomyelitis from which MSSA were isolated. Ten isolates of *S. aureus*, comprising nine isolates from osseous samples and one from articular liquid, were simultaneously recovered from the patient. Phenotypic identification and antimicrobial resistance were performed with Vitek2 (bioMérieux, Lyon, France). The antimicrobial susceptibility of the patient's isolates was tested in accordance with Clinical and Laboratory Standards Institute guidelines, including clindamycin, ciprofloxacin, erythromycin, fusidic acid, gentamicin, levofloxacin, penicillin G, rifampicin, trimethoprim, mupirocin, tobramycin, teicoplanin, vancomycin, oxacillin, linezolid and tetracycline; methicillin and mupirocin resistance were confirmed by multiplex polymerase chain reaction (PCR) as described below.

PCR. In addition to the phenotypic characterization of the isolates, a multiplex PCR assay, which permits simultaneous identification of *S. aureus* and detection of the methicillin and mupirocin resistance genes, was performed as previously described [22,23]. The isolates were also tested for the presence of Panton-Valentine leucocidin (PVL) genes (*lukF-PV* and *lukS-PV*) by using described primers and methods [9,12,25], and for the presence of the intercellular adhesion gene cluster (*ica*) [15,16].

Pulsed field gel electrophoresis (PFGE). All *S. aureus* isolates were characterized by macrorestriction analysis of *Sma*I-digested genomic DNA by PFGE as described [24]. Restriction fragments were resolved by PFGE with a CHEF-DRIII contour-clamped homogeneous electric field apparatus (Bio-Rad Laboratories, Richmond, CA). Following electrophoresis, the 1% Seakem Gold agarose gels were stained with ethidium bromide (0.5 μg/ml), visualized under UV illumination, and photographed with the Gel Doc 2000 system (Bio-Rad). Digital images were stored electronically as TIFF files.

Multiple locus VNTR analysis (MLVA). MLVA typing was performed as previously described [10,25,26] with minor modifications. Gel images were exported as TIFF files for further analysis.

Computer-monitored fingerprinting analysis. Computer analyses of the banding patterns obtained by PFGE and MLVA were performed with the InfoQuest FP software v4.5 (Bio-Rad) as previously described. A dendrogram of banding patterns using the unweighted pair-group method with arithmetic averages (UPGMA) was constructed by using the Dice coefficient. As in previous studies, a similarity cut-off of 80% was used to define a PFGE type [27,28].

Multilocus sequence typing (MLST). MLST was performed for all the isolates as described by Enright et al. [6]. Sequences were determined with an ABI Prism 310 genetic analyzer with BigDye fluorescent terminator chemistry (Applied Biosystems, Warrington, UK). Allele numbers were assigned by using the MLST website (http://www.mlst.net), and resulting allelic profiles were assigned to particular sequence types (ST) for each isolate. The eBURST algorithm, available at [http://eBURST.mlst.net], was used to classify different STs into clusters or clonal complexes (CCs) of phylogenetic relationships by comparison against the MLST database. Such CCs consisted of two or more isolates with the same ST(s), which differed either at a single locus (single-locus variants) or at two loci (double-locus variants). A minimum spanning tree (MST) constructed with InfoQuest FP software v4.5 (BioRad) using the concatenated seven gene fragments was also used to show the phylogenetic relationships between isolates. The categorical coefficient was used to calculate the MST. The software applies a rule by which, in case of identical calculated distances between types, the type that has the highest number of single-locus variants is linked first [2].

Results

Isolates characteristics. Ten MSSA isolates were recovered: nine from osseous samples and one from articular liquid. All were confirmed as MSSA by multiplex PCR due to the presence of the *femB* gene (identifying *S. aureus* at the species level) and the absence of *mecA* (encoding high-level methicillin resistance). The presence of the *iles-2* gene (encoding high-level mupirocin resistance) was also discarded with the same triplex PCR experiment. There was no resistance to

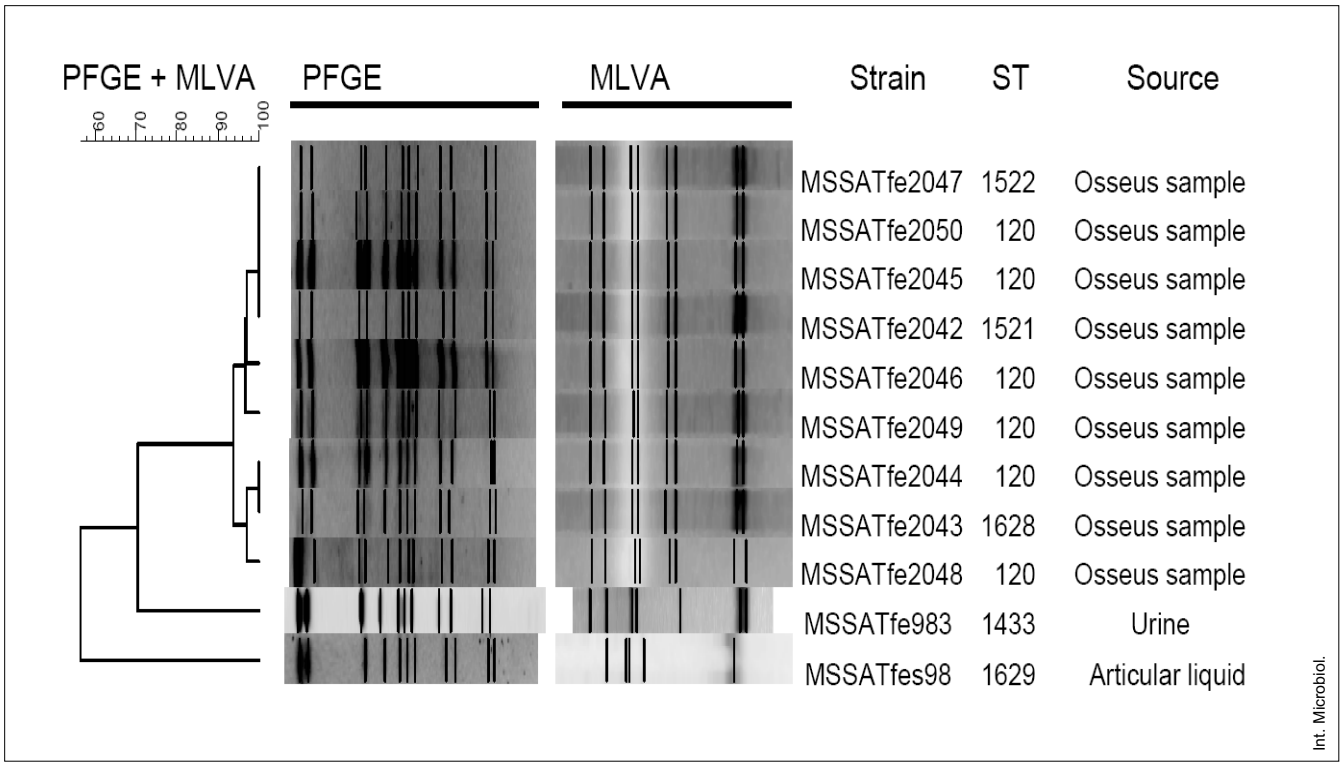

Fig. 1. Dendrogram obtained by the unweighted pair group method using arithmetic averages (UPGMA) and the Dice coefficient, showing the relatedness of the combined PFGE and MLVA banding patterns of *Staphylococcus aureus* isolates. The ST and strain source are indicated to the right of each gel lane. The scale indicates the level of pattern similarity.

methicillin, mupirocin, or a battery of several other antibiotics (vancomycin, clindamycin, ciprofloxacin, linezolid).

Typing of isolates. According to the criterion of Tenover [28], the isolates were grouped into two PFGE types. Nine of the ten isolates (all recovered from osseous tissue) were clustered within one type. Similarly, by applying the 80% similarity criterion above mentioned, we were able to group the MLVA patterns into two clusters homologous to those obtained with PFGE. A combined dendrogram deduced from the cluster analysis of macrorestriction and MLVA patterns was constructed (Fig. 1).

PFGE and MLVA clearly distinguished between the osseous sample strains, which formed a defined group underlining their relatedness, and the articular liquid strain. A high preponderance of isolates with the *ica* cluster was obtained in both clusters.

Four new STs not previously recorded (submitted to the MLST database and designated ST1521, ST1522, ST1628 and ST1629) were found, and only one previously described MSSA ST120. All of the new STs were represented by one unique isolate, with the other six isolates belonging to ST120 (Fig. 1), which was the most represented ST. New STs were all single- or double-locus variants of ST120.

Population structure. By using the BURST, we were able to group the STs in only one CC without singletons among the five STs. The predicted ancestor ST120 included most of the isolates (Fig. 1). Accordingly, the clonal complex was called CC120. The complex contained sequence types that differed by not more than two loci. In addition, in most cases, the STs differed by a few mutations. ST1628 was a single-locus variant (SLV), single-nucleotide variant, in the *aroE* locus from ST120; similarly, ST1629 was a SLV in the *gmK* locus from ST1628, differing by two substitutions; and, finally ST1522 and ST1523 were SLV in the *pta* allele from ST1628, differing by three and five substitutions, respectively, which is consistent with a recombination event. This ST1628 turned out to be an intermediate genetic state between ST120 and the remaining STs described here (Figs. 2 and 3A).

The genotypes described seem to have emerged from ST120, and the evolutionary pattern could be explained by

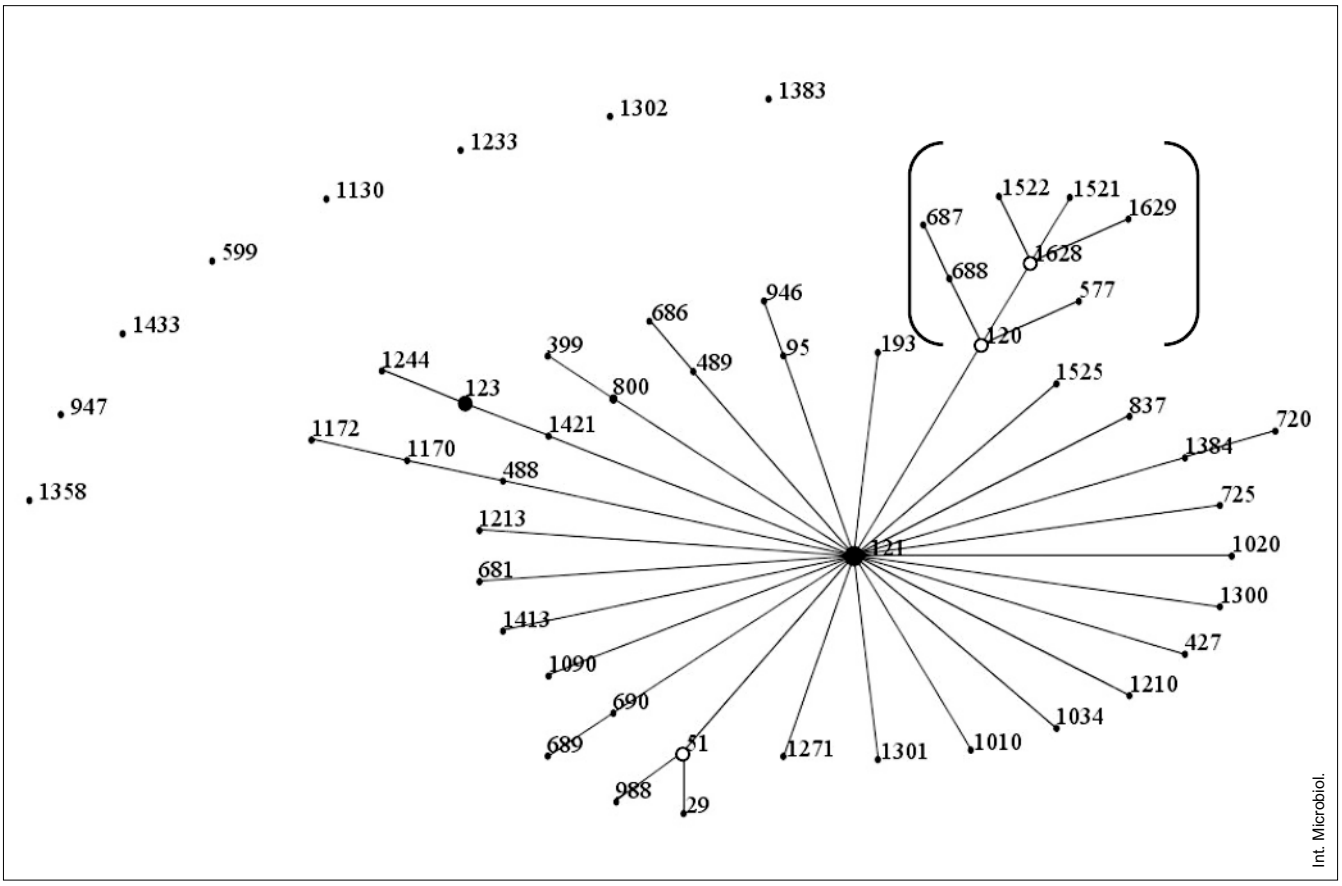

Fig. 2. Application of eBURST algorithm to MLST data for the collection of 52 STs composing the CC121. Each ST is represented by black dots except for subgroup founders identified by white dots. Brackets surround the cluster formed by STs derived from ST120.

means of successive point mutations and recombination processes. Figure 3A shows the probable evolutionary tree resulting from application of the eBURST algorithm. With the objective of validating the clustering and evolutionary model proposed by eBURST, we constructed a cluster analysis using a MST of the concatenated sequences of the seven MLST genes of the five STs and ST121 (Fig. 3B).

Discussion

The new MSSA clonal complex isolated from just one patient afflicted with an osteomyelitic process included five clones, four of which had never been described before. Within the current worldwide epidemic of community-acquired *S. aureus* infections, attention has focused on the role of methicillin-resistant strains. In this study, we characterized new STs of methicillin-susceptible strains that can contribute to this epidemic, either as infectious agents themselves or as reservoirs for the emergence of new MRSA by horizontal acquisition of SCC*mec* element, as described for other STs [21,29].

Most of the MSSA isolates in our study were closely related based on their PFGE and MLVA patterns. Only subtle band changes were observed in the group of strains recovered from osseous tissue. However, there was a high diversity in the MLST. The allelic profiles allowed the isolates to be grouped in a new single clonal complex CC120 with ST120 as founder. None of the genotypes included in the CC120 had been detected in this hospital before and four of them were new STs. These results suggest the appearance of an entirely new group of nosocomial, infectious *S. aureus*. Moreover, the four new strains appeared in a single patient.

Analysis of the sequence data obtained by MLST, based on the slowly evolving genomic "core", provided data that are well suited to studies of global epidemiology and population biology of bacterial pathogens [24]. We attempted to introduce MLST in the analysis of a focused infecting population isolated from one individual. The clones, considered

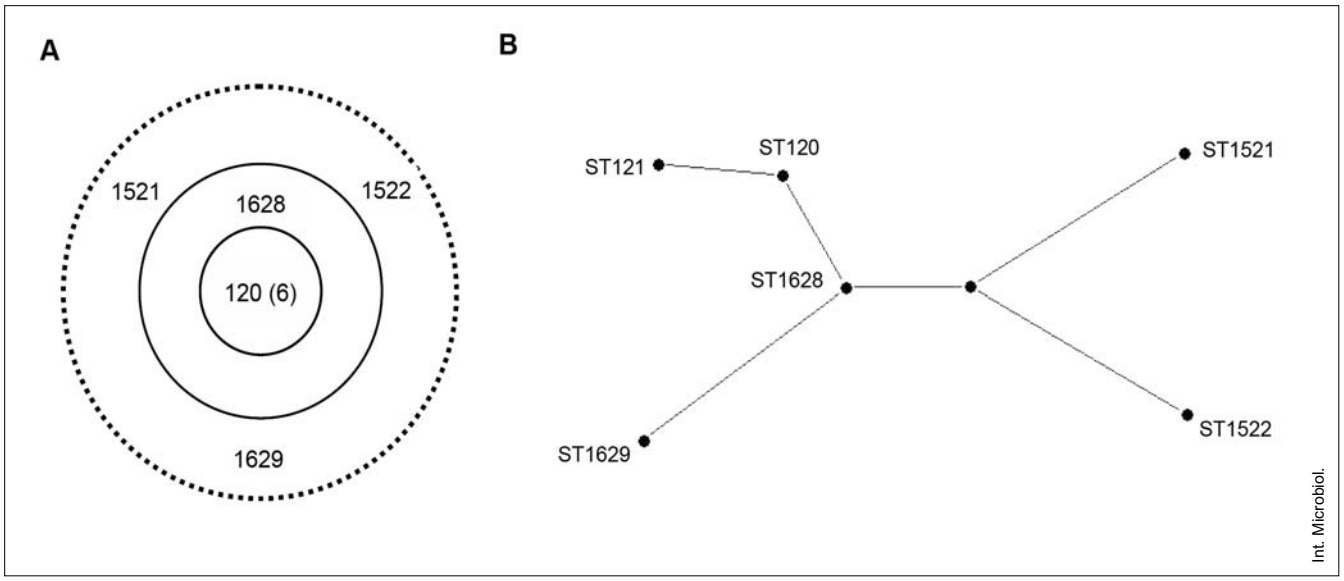

Fig. 3. Clonal complex 120. (**A**) Analysis of *S. aureus* isolates with eBURST. The predicted clonal ancestor is shown in the central ring, the single-locus variant in the middle (solid) ring, and double-locus variants in the outer (dashed) ring. The number of isolates in ST120 is shown in parentheses. No singletons were detected. (**B**) Phylogenetic diversity of the sequenced *Staphylococcus aureus* strains. The split decomposition tree was constructed using concatenated sequences of the seven loci used in MLST for all the STs isolated and ST121. The graph was constructed by using SplitsTree Version 4. Distances were estimated based on Hamming (uncorrected) distances.

similar through the use of PFGE, were distinguished by MLST, which indicates a high genotypic diversity [17].

Staphylococcus aureus is characterized by low levels of recombination, with clones diversifying predominantly by point mutation rather than by recombination [8]. STs belonging to CC120 might have evolved by point mutations in two cases (ST1628 and ST1629) and by recombination in the remaining two (ST1522 and ST1521), as shown by the predicted evolutionary tree (Fig. 3B). A highly related PFGE pattern in ST1433 was previously isolated from this hospital several years before and described by our group. The group of isolates defined in this study might have had a phylogenetic relationship with ST1433. However, this was not supported by the PFGE results. As PVL has been associated with life-threatening infections [22], we tested the presence of PVL genes, which we did not detect in any of the isolates. Although PVL has been associated with the founder clone ST120 [4], this gene was not detected by PCR. Furthermore, even though this ST is commonly described as a community-acquired (CA)-MSSA, in our study, this strain was recovered as an HA-MSSA. This result supports the suggestion to eliminate the artificially established barrier between CA-MRSA and HA-MRSA [F.C. Tenover, personal communication].

The ability of *S. aureus* strains to develop a biofilm has been often considered as a virulence factor in osteomyelitic processes [11]. Therefore, we tested for the presence of the *ica*-gene cluster, whose component icaR (regulatory) and icaADBC (biosynthetic) genes are known to be necessary for biofilm formation and virulence [3,18]. All the isolates but two carried this cluster, which is in accordance with previous work reporting that it is commonly found in *S. aureus* strains involved in osteomyelitis [1].

Together, these findings reflect that the spread of infections caused by genetically closely related methicillin-susceptible strains sharing phenotypic characteristics other than susceptibility to methicillin involves a driving force probably different from that of methicillin resistance and related to other undefined fitness characteristics. Our results also indicate the need to reinforce microbiological controls in infection processes involving osteomyelitis, persistent bacteraemia, and other deep-seated infections, all of which are associated with high mortality rates and are often difficult to treat. In addition, the data can be applied to examine the potential role of combination therapy in resolving these infections.

Previous works have compared strains in a given individual over a short period of time [5]. These studies and many

others have found higher indexes of diversity by PFGE than by MLST, in which only one ST corresponds to a group of related PFGE patterns [30]. Conversely, here we argue that focusing only on PFGE patterns may lead to a subdiscrimination with respect to variability as applied to microepidemiological purposes. Nonetheless, for many strictly epidemiological applications, PFGE is the system of choice, with MLST is reserved for more profound population genetic studies.

The history of staphylococcal infections has been marked by the rapid emergence of new strains with distinctive epidemiological and clinical characteristics. We are currently in such a period. We conclude that such an emergence and clonality of *S. aureus* can occur within a restricted location, such as a single individual, in which a highly localized bacterial strain diversity is possible. Thus, continued epidemiological surveillance is of great importance to monitor the course of this development, to provide further evidence concerning the evolution of this human pathogen, and to predict additional probable changes.

Acknowledgements. This work was partially supported by projects FUNCIS 28/07 and FUNCIS 20/10 from the Canarian Autonomous Government (Spain) and FIS10/00125 from the Spanish Health Ministry to S.M.A. J.D.C. was partially supported by a FUNCIS contract.

Competing interests. None declared.

References

1. Brady RA, Leid JG, Calhoun JH, Costerton JW, Shirtliff ME (2008) Osteomyelitis and the role of biofilms in chronic infection. FEMS Immunol Med Microbiol 52:13-22
2. Chen KW, Chen YC, Lo HJ, Odds FC, Wang TH, Lin CY, Li SY (2006) Multilocus sequence typing for analyses of clonality of *Candida albicans* strains in Taiwan. J Clin Microbiol 44:2172-2178
3. Cramton SE, Ulrich M, Götz F, Döring G (2001) Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Infect Immun 69:4079-4085
4. Daskalaki M, Rojo P, Marin-Ferrer M, Barrios M, Otero JR, Chaves F (2010) Panton-Valentine leukocidin-positive *Staphylococcus aureus* skin and soft tissue infections among children in an emergency department in Madrid, Spain. Clin Microbiol Infect 16:74-77
5. Dohin B, Gillet Y, Kohler R, et al. (2007) Pediatric bone and joint infections caused by Panton-Valentine leukocidin-positive *Staphylococcus aureus.* Pediatr Infect Dis J 26:1042-1048
6. Enright MC, Day NPJ, Davies CE, Peacock SJ, Spratt BG (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol 38:1008-1015
7. Fan J, Shu M, Zhang G, et al. (2009) Biogeography and virulence of *Staphylococcus aureus*. PLoS One 4:e6216 doi:10.1371/journal.pone.0006216
8. Feil EJ, Cooper JE, Grundmann H, et al. (2003) How clonal is *Staphylococcus aureus*? J Bacteriol 185:3307-3316
9. Holmes A, Ganner M, McGuane S, Pitt TL, Cookson BD, Kearns AM (2005) *Staphylococcus aureus* isolates carrying Panton-Valentine leucocidin genes in England and Wales: frequency, characterization, and association with clinical disease. J Clin Microbiol 43:2384-2390
10. Ikawaty R, Willems RJL, Box ATA, Verhoef J, Fluit AC (2008) Novel multiple-locus variable-number tandem-repeat analysis method for rapid molecular typing of human *Staphylococcus aureus*. J Clin Microbiol 46:3147-3151
11. Klein JL, Petrovic Z, Treacher D, Edgeworth J (2003) Severe community-acquired pneumonia caused by Panton-Valentine leukocidin-positive *Staphylococcus aureus*: first reported case in the United Kingdom. Intensive Care Med 29:1399
12. Lina G, Piémont Y, Godail-Gamot F, et al. (1999) Involvement of Panton-Valentine leukocidin–producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis 29:1128-1132
13. Mader JT, Mohan D, Calhoun J (1997) A practical guide to the diagnosis and management of bone and joint infections. Drugs 54:253-264
14. Maiden MC (2006) Multilocus sequence typing of bacteria. Annu Rev Microbiol 60:561-588
15. Martín-López JV, Pérez-Roth E, Claverie-Martín F, et al. (2002) Detection of *Staphylococcus aureus* clinical isolates harboring the *ica* gene cluster needed for biofilm establishment. J Clin Microbiol 40:1569-1570
16. Martín-López JV, Díez-Gil O, Morales M, Batista N, Villar J, Claverie-Martín F, Méndez-Alvarez S (2004) Simultaneous PCR detection of *ica* cluster and methicillin and mupirocin resistance genes in catheter-isolated *Staphylococcus*. Int Microbiol 7:63-66
17. Mira A, Martín-Cuadrado AB, D'Auria G, Rodríguez-Valera F (2010) The bacterial pan-genome: a new paradigm in microbiology. Int Microbiol 13:45-57
18. Moscoso M, García E, López R (2009) Pneumococcal biofilms. Int Microbiol 12:77-85
19. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, et al. (2003) Comparison of community- and health care–associated methicillin-resistant *Staphylococcus aureus* infection. JAMA 290:2976-2984
20. Peacock SJ, de Silva GD, Justice A, Cowland A, Moore CE, Winearls CG, Day NPJ (2002) Comparison of multilocus sequence typing and pulsed-field gel electrophoresis as tools for typing *Staphylococcus aureus* isolates in a microepidemiological setting. J Clin Microbiol 40:3764-3770
21. Pérez-Roth E, Alcoba-Flórez J, López-Aguilar C, Gutiérrez-González I, Rivero-Pérez B, Méndez-Álvarez S (2010) Familial furunculosis associated with community-acquired leukocidin-positive methicillin-susceptible *Staphylococcus aureus* ST152. J Clin Microbiol 48:329-332
22. Pérez-Roth E, Claverie-Martín F, Batista N, Moreno A, Méndez-Álvarez S (2002) Mupirocin resistance in methicillin-resistant *Staphylococcus aureus* clinical isolates in a Spanish hospital. Co-application of multiplex PCR assay and conventional microbiology methods. Diagn Microbiol Infect Dis 43:123-128
23. Pérez-Roth E, Claverie-Martín F, Villar J, Méndez-Álvarez S (2001) Multiplex PCR for simultaneous identification of *Staphylococcus aureus* and detection of methicillin and mupirocin resistance. J Clin Microbiol 39:4037-4041
24. Pérez-Roth E, Lorenzo-Díaz F, Batista N, Moreno A, Méndez-Álvarez S (2004) Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998 to 2002) in a Spanish hospital. J Clin Microbiol 42:4649-4656

25. Ribeiro A, Dias C, Silva-Carvalho MC, et al. (2005) First report of infection with community-acquired methicillin-resistant *Staphylococcus aureus* in South America. J Clin Microbiol 43:1985-1988
26. Sabat A, Krzyszton-Russjan J, Strzalka W, et al. (2003) New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. J Clin Microbiol 41:1801-1804
27. Struelens MJ, Deplano A, Godard C, Maes N, Serruys E (1992) Epidemiologic typing and delineation of genetic relatedness of methicillin-resistant *Staphylococcus aureus* by macrorestriction analysis of genomic DNA by using pulsed-field gel electrophoresis. J Clin Microbiol 30:2599-2605
28. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33:2233-2239
29. Tristan A, Bes M, Meugnier H, et al. (2007) Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus*, 2006. Emerg Infect Dis 13:594-600
30. Vandenesch F, Naimi T, Enright MC, et al. (2003) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis 9:978-984