View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

ORIGINAL ARTICLE 10.1111/j.1469-0691.2009.02732.x

# High-resolution melting analysis of the *spa* locus reveals significant diversity within sequence type 93 methicillin-resistant *Staphylococcus aureus* from northern Australia

**S. Y. C. Tong, R. A. Lilliebridge, D. C. Holt, M. I. McDonald, B. J. Currie and P. M. Giffard**

*Menzies School of Health Research, Institute of Advanced Studies, Charles Darwin University, Darwin, NT, Australia*

## Abstract

High-resolution melting analysis is an inherently robust, easy and inexpensive approach to the examination of genomic regions containing single-nucleotide polymorphisms and hypervariable loci. *Staphylococcus aureus* sequence type (ST) 93 is a singleton, Panton–Valentine leukocidin-positive clone unique to Australia. A high-resolution melting-based method for the identification of ST93 was developed, and a similar approach was used to reveal diversity within the *spa* locus of this lineage. Statistical and graphical methods that account for instrumental and operator-dependent variation in high-resolution melting curves were developed, to allow greater confidence and reproducibility in deciding whether another curve is truly different from the baseline curve of an amplicon with known sequence. The data support a very early acquisition, or multiple independent acquisitions, of SCC*mec* by ST93 methicillin-susceptible *S. aureus* (MSSA), and the coexistence of MSSA and methicillin-resistant *S. aureus* versions of the same lineage within northern Australia.

**Keywords:** High resolution melting, *Staphylococcus aureus*, *SPA*, ST93
**Original Submission:** 8 October 2008; **Revised Submission:** 15 January 2009; **Accepted:** 15 January 2009
Editor: G. Lina
**Article published online:** 15 April 2009
*Clin Microbiol Infect* 2009; **15:** 1126–1131

**Corresponding author and reprint requests:** P. M. Giffard, Menzies School of Health Research, PO Box 41096, Casuarina, NT 0811, Australia
**E-mail: Phil.Giffard@menzies.edu.au**

## Introduction

In the evolving Australian epidemics of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA), the most common multilocus sequence types (MLSTs) are 1, 93 and 30 [1]. Sequence type (ST) 93 methicillin-resistant *S. aureus* (MRSA), first described in 2000 [2], is an MLST singleton clone, is typically Panton–Valentine leukocidin (PVL)-positive, and is a major cause of severe CA-MRSA disease in Australia [2–4]. The other common PVL-positive clone, ST30-MRSA, probably arrived in Australia via large population migrations in the 1980s [5]. ST93-MRSA is the most rapidly expanding CA-MRSA clone throughout Australia [1], and now constitutes 33% of Australian CA-MRSA isolates (13th International Symposium on Staphylococci and Staphylococcal Infections (ISSI, 2008), Abstract 110), and has also been exported to the UK by travellers (13th ISSI, 2008, Abstract 604). Previous investigators found little genetic diversity within ST93-MRSA, suggesting that it arose from one PVL-positive subtype of ST93 methicillin-susceptible *S. aureus* (MSSA) [6].

The polymorphic X region of the staphylococcal protein A (*spa*) gene rapidly evolves, and *spa* sequence typing can elucidate patterns of diversity within sequence types. High-resolution melting (HRM) analysis, a new approach to analysing genetic variation, is cost-effective, simple, and robust. It has been shown to be highly effective for resolving *spa* types [7]. Although empirical methods to define HRM curves as the same or different have been used, such methods have not been based upon statistical analysis [7].

Our aims were to: (i) develop an HRM-based method for the rapid identification of ST93; and (ii) determine the *spa* locus diversity of ST93-MRSA, ST93-MSSA and ST30-MRSA isolates from northern Australia. Of particular interest was whether ST93-MRSA represents a highly conserved clone with a very recent origin as compared with ST93-MSSA.

## Materials and Methods

Isolates were collected in community-based studies of impetigo conducted in remote Aboriginal communities from 2003

©2009 The Authors
Journal Compilation ©2009 European Society of Clinical Microbiology and Infectious Diseases

**TABLE 1. High-resolution melting (HRM) curves and corresponding *spa* types and repeats**

| | HRM curve (no. of isolates) | *spa* genotype (no. sequenced) | *spa* repeats |
|---|---|---|---|
| ST93-MRSA[a] | HRM curve 1 (23) | t202 (8) | 11-17-23-17-17-16-16-25 |
| (Fig. 1c) | HRM curve 3 (2) | t1819 (2) | 11-17-23-17-16-16-25 |
| | HRM curve 4 (1) | t4675 (1) | 11-17-17-17-16-16-25 |
| | HRM curve 5 (4) | t1811 (4) | 11-17-17-16-16-25 |
| ST93-MSSA | HRM curve 1 (26)[a,b] | t202 (5) | 11-17-23-17-17-16-16-25 |
| (Fig. 1d) | HRM curve 2 (1)[c] | t4178 (1) | 11-17-23-17-17-16-16-16-25 |
| | HRM curve 6 (2)[c] | t4699 (2) | 11-17-16-16-25 |
| | HRM curve 10 (1)[b] | t4698 (1) | 04-16-16-25 |
| ST30-MRSA[a] | HRM curve 7 (27) | t019 (7) | 08-16-02-16-02-25-17-24 |
| (Fig. 1e) | HRM curve 8 (2) | t138 (2) | 08-16-02-25-17-24 |
| | HRM curve 9 (1) | t4700 (1) | 08-275-02-16-02-25-17-24 |

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; ST, sequence type.
[a]All ST93-MRSA, ST30-MRSA and 21 ST93-MSSA isolates were from the 2006–2007 Royal Darwin Hospital collection.
[b]Six ST93-MSSA isolates were from the 2003–2005 community-based studies.
[c]Three ST93-MSSA isolates were from the 1991 Royal Darwin Hospital collection.

to 2005 [8], and in a prospective study of CA-MRSA infections at the Royal Darwin Hospital (RDH) from 2006 to 2007 (47th ICAAC, 2007, Abstract L1141); three ST93-MSSA isolates were collected in 1991 at the RDH (Table 1). The community studies had revealed the highly divergent clonal complex (CC) 75 to be the predominant *S. aureus* clone in remote communities in northern Australia [8].

A robust and efficient method for identifying ST93 was developed. An ST93-specific single-nucleotide polymorphism (SNP), *glpF* 165, was identified in the *S. aureus* MLST database using the software Minimum SNPs [9]. This is a three-state SNP, with the 'C' allele completely specific for ST93, the 'A' allele completely specific for CC75, and the 'T' allele possessed by all other known STs. To add redundancy, we also used the MLST database-derived SNP *aroE* 252, which has been determined to be diagnostic for ST93. This SNP also has three allelic states [10], with the 'G' allele being unique to ST93. Primers were designed to generate amplicons containing these SNPs.

The diversity of ST93 was assessed by performing HRM analysis of the *spa* locus of 30 isolates each, identified as ST93-MRSA and ST93-MSSA. Additionally, 30 isolates of PVL-positive ST30-MRSA from northern Australia, previously characterized using a kinetic PCR method to examine eight SNPs [10], were similarly analysed.

We used a Rotor-Gene 6000 instrument (Corbett Life Science) for the HRM analysis. DNA was extracted using the QIAamp DNA minikit protocol for Gram-positive bacteria with lysostaphin. Each reaction contained 5 $\mu$L of Platinum SYBR Green qPCRSuperMix-UDG (2x; Invitrogen Life Technologies), 0.5 $\mu$M each primer and 1 $\mu$L of a 1 : 5 dilution of the DNA template in a final volume of 10 $\mu$L. The real-time PCR thermocycling parameters were: 50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 5 s and 60°C for 30 s; 72°C for 2 min; and 50°C for 20 s; this was followed by HRM ramping with fluorescence data acquisition at 0.05°C increments. The primers and HRM ramping temperatures are shown in Table 2. Reactions were routinely carried out in duplicate. The exported HRM curves are available as Supporting Information. The *spa* locus was sequenced in representative isolates that yielded the different curves, and *spa* types were assigned according to the *spa* database [11].

**TABLE 2. Primers and ramping temperatures for high-resolution melting reactions**

| Primer name | Primer sequence (5′–3′) | Ramping temperatures (°C) |
|---|---|---|
| *glpF* 165F | ACCCAGCGGTGTCTTTAGCTCTTGCAT | 72–84 |
| *glpF* 165R | ATACAATCGTTGCTCCGACA | |
| *aroE* 252F | GTGGATAGGGTATAATACAG | 68–81 |
| *aroE*252R | ACCTGCGCCCAAAATTAAAA | |
| *spa* 1095F | AGACGATCCTTCGGTGAGC | 75–87 |
| *spa*1517R | GCTTTTGCAATGTCATTTACTG | |

We defined 95% CIs for the HRM curves corresponding to isolates of the same sequenced *spa* type by exporting the normalized fluorescence data of the HRM curves to calculate the mean and standard deviation (SD) at each temperature of the melting protocol. This was only performed when there were at least six isolates of the same *spa* type. At each temperature, the 95% CIs were calculated as the mean ± (1.96 × SD). We generated difference graphs by subtracting this mean normalized fluorescence from the normalized fluorescence of each curve at each temperature, and defined the 95% CIs for the difference graph as 0 ± (1.96 × SD).

## Results

We developed HRM-based methods for the examination of both SNP regions, SNP *glpF* 165 and SNP *aroE* 252, using

©2009 The Authors

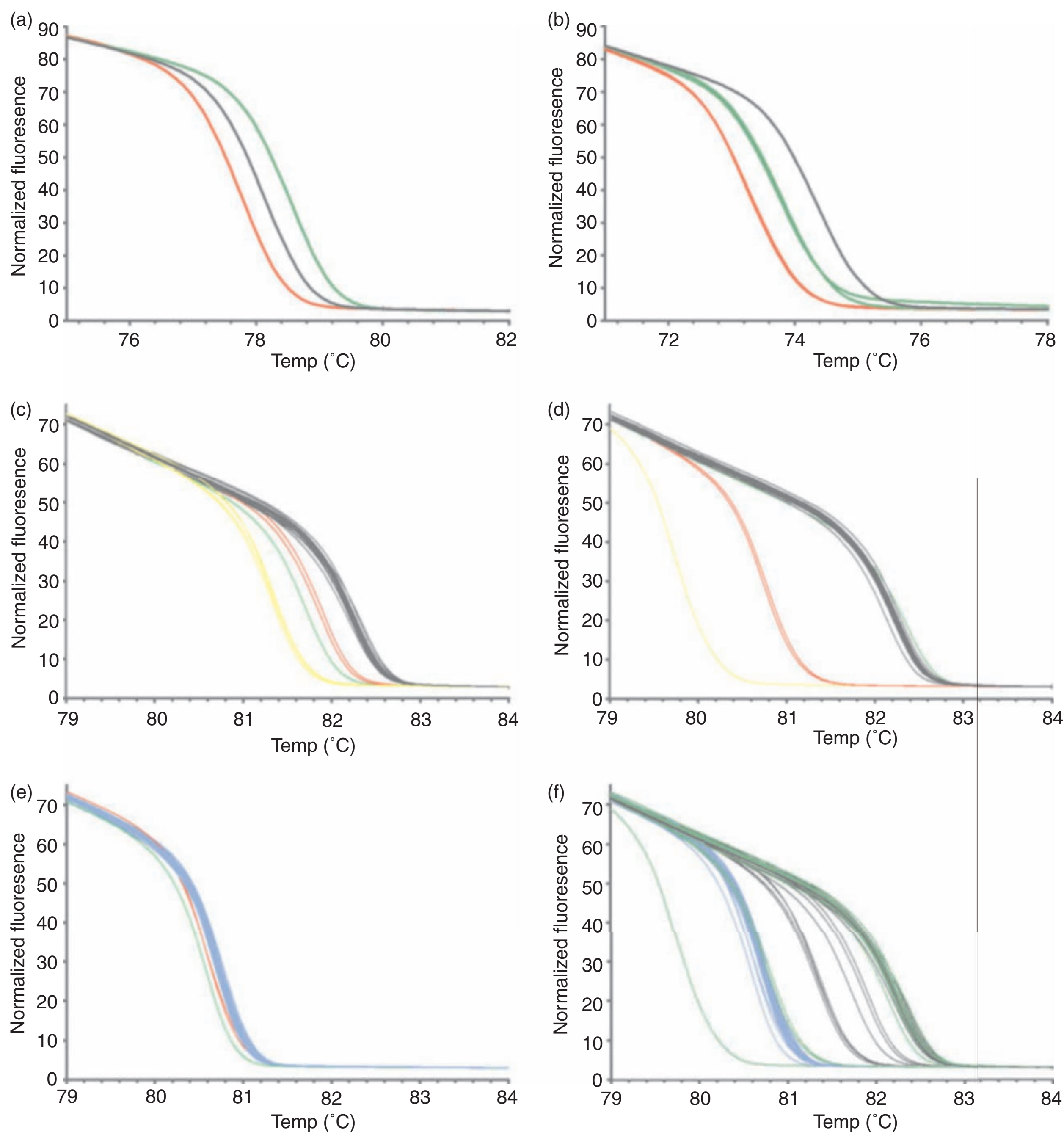


**FIG. 1.** High-resolution melting curves of the *glpF* 165, *aroE* 252 and *spa* loci (c–f). (a) Representative curves corresponding to each allele at *glpF* 165: C (black) is specific for ST93, and A (green) is specific for CC75. (b) Representative curves for the *aroE* 252 region: the black curve is specific for sequence type (ST 93. (c) ST93 methicillin-resistant *Staphylococcus aureus* (ST93-MRSA) showing four curves: curve 1 (black), curve 3 (red), curve 4 (green), and curve 5 (yellow). (d) ST93 methicillin-susceptible *S. aureus* (ST93-MSSA) showing four curves: curve 1 (black), curve 2 (green), curve 6 (red), and curve 10 (yellow). (e) ST30-MRSA showing three curves: curve 7 (blue), curve 8 (red), and curve 9 (green). (f) ST93-MRSA (black), ST93-MSSA (green) and ST30-MRSA (blue) combined.

isolates of known ST. For each SNP region, three easily discriminated curves were obtained (Fig. 1a,b). During the development of the *glpF* 165 assay, some unexpected results were obtained. The initial primer set allowed amplification of a product that generated identical HRM curves for ST93 and CC75, even though CC75 is highly divergent from all other *S. aureus*, and the two PCR products differed at seven positions. Examination of the seven SNPs indicated a neutral

©2009 The Authors

balance of hydrogen bonds, with an identical GC content. Redesign of the primers (*glpF* 165F and *glpF* 165R) allowed clear discrimination of ST93 and CC75, with the amplified regions now having differing GC contents. Of the possible amplified *aroE* 252 regions, ST93, among all the STs, has a unique GC content as well as the highest melting temperature, and therefore generates a unique HRM curve. We were unable to consistently generate an *aroE* amplicon for CC75, and further work to better characterize this locus for CC75 is proceeding. It was concluded that examining the *glpF* 165 and *aroE* 252 SNP regions by HRM analysis is a rapid and robust method for identifying ST93. It has the added benefits of incorporating redundancy and allowing the identification of CC75, which is another major northern Australian clonal complex.

HRM analysis of the *spa* locus revealed four and three clearly distinct curves for ST93-MRSA and ST93-MSSA isolates, respectively (Fig. 1c,d and Table 1). Of 14 sequenced ST93 isolates associated with HRM curve 1, 13 had the same *spa* type of t202, and one contained an additional 24-bp repeat, t4178. Closer examination of the t4178 HRM difference curve, with the mean t202 curve as the baseline, revealed part of the curve sitting outside the 95% CI curve, suggesting that the t4178 and t202 HRM curves are indeed different (Fig. 2a). The five other curves correlated with different *spa* types. Comparison of the raw data of HRM curves of two ST93-t202 isolates produced in another laboratory [7] showed that they were consistent with our t202 curves, although a part of the curve at 72°C, well away from the melting temperature, was outside the 95% CI (Fig. 2a). Within ST30-MRSA, three slightly different curves could be distinguished on both the normalized fluorescence and the difference curves (Figs 1e and 2b). The *spa* loci of seven isolates with HRM curve 8 were sequenced; all had the same *spa* type of t019. The two other curves correlated with different *spa* types (Table 1). Fig. 1f shows curves from all three groups of isolates.

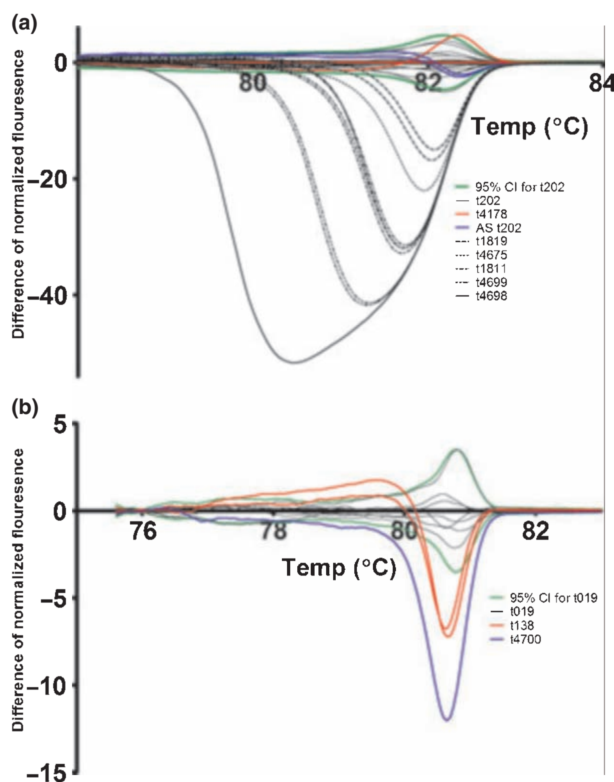


FIG. 2. Difference graph of high-resolution melting curves of *spa* loci. (a) Curves for sequence type (ST) 93 isolates. The mean of the known t202 curves was used as the baseline. The green lines indicate the 95% CIs for the 13 t202 curves, and the thin black lines indicate each t202 curve. The blue lines (AS t202) refer to t202 curves derived from the raw data of the study by Stephens *et al.* [7], and sit within the 95% CI, whereas part of the t4178 (red) curve sits outside the 95% CI. (b) Curves for ST30 isolates. The mean of the known t019 curves was used as the baseline. The green lines indicate the 95% CIs for the seven t019 curves, and the thin black lines indicate each t019 curve.

## Discussion

All of the ST93-MRSA isolates were from the 2006–2007 hospital-based study, and the finding of four *spa* types within these 30 isolates revealed surprising *spa* type diversity for a group of chronologically and geographically localized isolates from a hospital serving a population of only 176 000. Similarly localized [12–14], and some even larger [15,16], studies of CA-MRSA revealed no *spa* type diversity among ST30, ST80 and USA300 isolates. The diversity in ST93-MRSA more closely mirrors that seen in studies across broader geographical regions and time periods, where single STs contained up to five *spa* types [17–20]. Larsen *et al.* [19,20] linked this diversity within ST80 and USA300 to multiple international importations of these strains into Denmark. Such importation could explain the diversity that we demonstrated in the widely distributed ST30-MRSA, but not that in ST93-MRSA.

The diversity in both ST93-MRSA and ST93-MSSA does not support the hypothesis that ST93-MRSA is undergoing an explosive clonal expansion derived from a recent single instance of SCC*mec* acquisition. Rather, the data support an early acquisition of SCC*mec*, with subsequent rearrangements of the *spa* sequence or multiple independent acquisitions of SCC*mec*, and coexistence of MSSA and MRSA versions of the same lineage. A recent study of ST5 revealed

©2009 The Authors

that SCC*mec* has probably been acquired repeatedly in geographical regions within ST5, and predicted that MRSA haplotypes within a geographical region should also be present in MSSA from that region [21]. Our findings support this prediction; for instance, the commonest *spa* type, t202, is present in ST93-MRSA from RDH and in ST93-MSSA from both RDH and community study collections. Interestingly, all 21 ST93-MSSA isolates from the 2006–2007 hospital collection were *spa* type t202, raising the possibility of a current epidemic of ST93-MSSA-t202, as compared with endemic ST93-MRSA. However, these 21 isolates were not linked epidemiologically in terms of time or patient residence, and only one was nosocomially acquired.

HRM analysis is a single-step closed-tube reaction. It is inherently robust, easy, and inexpensive to optimize and perform. It can also be used to examine hypervariable loci [7]. The derivation of SNP sets from sequence alignments using the software Minimum SNPs, and the development of HRM-based assays for the examination of these SNPs, is a straightforward and efficient approach for assembling genotyping procedures for specific purposes. We have been able to accurately discriminate two major clones in northern Australia from all other clones and also from each other.

HRM-mediated *spa* examination can differentiate closely related *spa* types, and provides further resolving power for genotyping. It is less expensive than full *spa* sequencing; each sequencing reaction in our institution costs US $20, as compared with US $0.50 for reagents for each HRM run. The Rotor-Gene 6000 software and previous publications have used a single HRM curve of an amplicon of known sequence as the baseline to generate difference curves. However, despite the robust methods, there is inevitably instrumental and operator error, resulting in slight variations in HRM curves generated from identical amplicons. We have developed a simple technique that incorporates this variation and allows greater confidence and reproducibility in deciding whether another curve is truly different from the baseline curve of known sequence. With appropriate calibration of instruments, it should also allow portability of the HRM curves and interlaboratory comparison, as demonstrated in this article.

## Acknowledgements

These findings were presented in part at the 13th International Symposium on Staphylococci and Staphylococcal Infections, 7–10 September 2008, Cairns, Australia, Abstract 709. We thank the RDH microbiology laboratory for its assistance, and F. O'Brien for providing the 1991 RDH isolates. This publication made use of the *spa* typing website (http://www.spaserver.ridom.de/), which is developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/).

## Transparency Declaration

S. Y. C. Tong is an Australian National Health and Medical Research Council PhD scholar (NHMRC grant 436033), and financial support was also provided by the Co-operative Research Centre for Aboriginal Health. P. M. Giffard is an inventor on a patent application that describes the software "Minimum SNPs" and is eligible for royalty payments if this is commercialized. The other authors have no competing interests.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Exported numerical data defining the HRM curves generated in this study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## References

1. Nimmo GR, Coombs GW, Pearson JC *et al.* Methicillin-resistant *Staphylococcus aureus* in the Australian community: an evolving epidemic. *Med J Aust* 2006; 184: 384–388.
2. Munckhof WJ, Schooneveldt J, Coombs GW, Hoare J, Nimmo GR. Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infection in Queensland, Australia. *Int J Infect Dis* 2003; 7: 259–267.
3. Peleg AY, Munckhof WJ, Kleinschmidt SL, Stephens AJ, Huygens F. Life-threatening community-acquired methicillin-resistant *Staphylococcus aureus* infection in Australia. *Eur J Clin Microbiol Infect Dis* 2005; 24: 384–387.
4. Tong SY, Anstey NM, Lum GD, Lilliebridge RA, Stephens DP, Currie BJ. Fatal community-associated methicillin-resistant *Staphylococcus aureus* pneumonia after influenza. *Med J Aust* 2008; 188: 61.
5. Nimmo GR, Schooneveldt J, O'Kane G, McCall B, Vickery A. Community acquisition of gentamicin-sensitive methicillin-resistant *Staphylococcus aureus* in southeast Queensland, Australia. *J Clin Microbiol* 2000; 38: 3926–3931.
6. Munckhof WJ, Nimmo GR, Carney J *et al.* Methicillin-susceptible, non-multiresistant methicillin-resistant and multiresistant methicillin-resistant *Staphylococcus aureus* infections: a clinical, epidemiological and microbiological comparative study. *Eur J Clin Microbiol Infect Dis* 2008; 27: 355–364.

©2009 The Authors

7. Stephens AJ, Inman-Bamber J, Giffard PM, Huygens F. High-resolution melting analysis of the *spa* repeat region of *Staphylococcus aureus*. *Clin Chem* 2008; 54: 432–436.
8. McDonald M, Dougall A, Holt D *et al.* Use of a single-nucleotide polymorphism genotyping system to demonstrate the unique epidemiology of methicillin-resistant *Staphylococcus aureus* in remote Aboriginal communities. *J Clin Microbiol* 2006; 44: 3720–3727.
9. Price EP, Inman-Bamber J, Thirvenkataswamy V, Huygens F, Giffard PM. Computer-aided identification of polymorphism sets diagnostic for groups of bacterial and viral genetic variants. *BMC Bioinformatics* 2007; 8: 278.
10. Huygens F, Inman-Bamber J, Nimmo GR *et al. Staphylococcus aureus* genotyping using novel real-time PCR formats. *J Clin Microbiol* 2006; 44: 3712–3719.
11. Harmsen D, Claus H, Witte W *et al.* Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *J Clin Microbiol* 2003; 41: 5442–5448.
12. Ben Nejma M, Mastouri M, Bel Hadj Jrad B, Nour M. Characterization of ST80 Panton–Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* clone in Tunisia. *Diagn Microbiol Infect Dis* 2008; [Epub ahead of print] doi: 10.1016/j.diagmicrobio.2008.02.010.
13. Ho PL, Cheung C, Mak GC *et al.* Molecular epidemiology and household transmission of community-associated methicillin-resistant *Staphylococcus aureus* in Hong Kong. *Diagn Microbiol Infect Dis* 2007; 57: 145–151.
14. Ofner-Agostini M, Simor AE, Mulvey M *et al.* Methicillin-resistant *Staphylococcus aureus* in Canadian aboriginal people. *Infect Control Hosp Epidemiol* 2006; 27: 204–207.
15. Tenover FC, McDougal LK, Goering RV *et al.* Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. *J Clin Microbiol* 2006; 44: 108–118.
16. Faria NA, Oliveira DC, Westh H *et al.* Epidemiology of emerging methicillin-resistant *Staphylococcus aureus* (MRSA) in Denmark: a nationwide study in a country with low prevalence of MRSA infection. *J Clin Microbiol* 2005; 43: 1836–1842.
17. Damkjaer Bartels M, Boye K, Rhod Larsen A, Skov R, Westh H. Rapid increase of genetically diverse methicillin-resistant *Staphylococcus aureus*, Copenhagen, Denmark. *Emerg Infect Dis* 2007; 13: 1533–1540.
18. Cookson BD, Robinson DA, Monk AB *et al.* Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* strains: the HARMONY collection. *J Clin Microbiol* 2007; 45: 1830–1837.
19. Larsen A, Stegger M, Goering R, Sorum M, Skov R. Emergence and dissemination of the methicillin resistant *Staphylococcus aureus* USA300 clone in Denmark (2000–2005). *Euro Surveill* 2007; 12.
20. Larsen AR, Bocher S, Stegger M, Goering R, Pallesen LV, Skov R. Epidemiology of European community-associated methicillin-resistant *Staphylococcus aureus* clonal complex 80 type IV strains isolated in Denmark from 1993 to 2004. *J Clin Microbiol* 2008; 46: 62–68.
21. Nübel U, Roumagnac P, Feldkamp M *et al.* Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. *Proc Natl Acad Sci USA* 2008; 105: 14130–14135.

©2009 The Authors