

DNA microarray-based genotyping of methicillin-resistant *Staphylococcus aureus* strains from Eastern Saxony

S. Monecke¹, L. Jatzwauk², S. Weber³, P. Slickers⁴ and R. Ehricht⁴

¹Institute for Medical Microbiology and Hygiene, Faculty of Medicine Carl Gustav Carus, Technical University of Dresden, ²Department of Hospital Hygiene, University Hospital Carl Gustav Carus, Dresden, ³Klinikum Hoyerswerda, Hoyerswerda and ⁴CLONDIAG GmbH, Jena, Germany

ABSTRACT

A diagnostic microarray was used to characterise a collection of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from hospitals in the German region of Eastern Saxony. The most abundant epidemic MRSA (EMRSA) strains were ST5-MRSA II (Rhine-Hesse EMRSA, EMRSA-3), CC5/ST228-MRSA I (South German EMRSA), ST22-MRSA IV (Barnim EMRSA, EMRSA-15) and ST45-MRSA IV (Berlin EMRSA). Other strains were found only as sporadic isolates or in minor outbreaks. These strains included ST1-MRSA IV, ST8-MRSA IV (Hannover EMRSA and others), clonal group 5 strains carrying SCCmec type IV elements (Paediatric clone), ST45-MRSA V, CC8/ST239-MRSA III and ST398-MRSA V. Panton-Valentine leukocidin-positive MRSA isolates were still very rare. The predominant strain was ST80-MRSA IV, although increasing numbers of different strains have recently been detected (ST8-MRSA IV, ST30-MRSA IV and ST59-MRSA V). For more common MRSA strains, it was possible to detect variants that differed mainly in the carriage of additional resistance determinants and certain virulence-associated genes. Detection of such variants can sometimes allow epidemic strains to be resolved beyond *spa* types to a hospital-specific level, which is of significant value for epidemiological purposes.

Keywords DNA microarray, Eastern Saxony, epidemiology, methicillin-resistant *Staphylococcus aureus*, Panton-Valentine leukocidin, SCCmec types

Original Submission: 30 October 2007; **Revised Submission:** 20 December 2007; **Accepted:** 5 January 2008

Clin Microbiol Infect 2008; **14**: 534–545

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) evolved in the late 1960s and has subsequently spread worldwide. MRSA strains carrying SCCmec element types I–III [1] were, for many years, epidemiologically linked to hospital-acquired infections. This link was explained by the adverse effects of high oxacillin resistance on replication [2] and by increased glucose consumption resulting in a decreased growth rate and cell yield [3]. Thus, MRSA had a selective advantage only in a hospital environment with high exposure to antibiotics. Outside of hospitals, where this selective pressure was not maintained,

MRSA could not compete with fast-growing wild-type strains. However, within the last decade, the epidemiological picture has changed profoundly. The novel SCCmec types IV and V [4,5] have appeared, which apparently do not have negative effects on replication [3]. Strains carrying these novel elements spread rapidly in hospitals, and have also appeared in communities outside hospitals, even in remote settings [6]. These strains have been designated 'community-associated' MRSA (caMRSA). Some caMRSA strains produce Panton-Valentine leukocidin (PVL) [7], which is a virulence factor that is associated with chronic or complicated skin and soft-tissue infections, as well as with rapidly fatal pneumonia [8]. Currently, the simultaneous presence of 'hospital-acquired' PVL-negative SCCmecI-IV, 'community-acquired' PVL-negative SCCmecIV or SCCmecV, and 'community-acquired' PVL-positive SCCmecIV or SCCmecV MRSA strains can be observed. The latter strains have also been

Corresponding author and reprint requests: S. Monecke, Institute for Medical Microbiology and Hygiene, Faculty of Medicine Carl Gustav Carus, Technical University of Dresden, Fetscherstrasse 74, D-01307 Dresden, Germany
E-mail: monecke@rocketmail.com

found to cause outbreaks in hospitals [9], which is of particular concern because of their enhanced virulence.

The use of a DNA microarray to genotype *S. aureus* isolates has been reported previously [10–13]. The array was shown to detect a large number of genes related to antibiotic resistance and exotoxin production, and was able to detect the type of *agr* operon. This array has been extended with new probes designed to characterise additional virulence and pathogenicity-related genes. These additional targets include the allelic variants of *ccrA/ccrB*, the more distantly related *ccrAA/ccrC*, *hly*-converting phages, which integrate specifically into the *hly* gene and which carry enterotoxin genes as well as the innate immune evasion gene cluster (IEC) [14], and known variants of the *cap* operon.

S. aureus also possesses several proteins that are excreted and then attached covalently to the cell surface. These proteins have been referred to as microbial surface components recognising adhesive matrix molecules (MSCRAMMs) [15]. The number of these genes and their length are variable. The differences in size can be attributed to a variable number of repeated sequence elements; however, the array was designed to detect more conserved regions, specific for clonal complexes, rather than these repeating units.

The objective of the present study was to apply the new diagnostic microarray to the routine identification and characterisation of MRSA isolates, including the determination of SCC*mec* types, and to obtain an insight into the presence and genetic characteristics of epidemic MRSA (EMRSA) strains in Eastern Saxony.

MATERIALS AND METHODS

Bacterial strains and isolates

The study was performed with isolates sampled at healthcare facilities in Eastern Saxony, including a university hospital, a long-term rehabilitation facility and four county hospitals. Isolates were included from routine diagnostic samples, as well as from outbreak investigations, obtained between 2000 and 2007. Eighty representative MRSA isolates were selected from among a collection of *c.* 300 isolates characterised by a microarray described previously [10–13]. Additional sequenced strains (see below) and representatives of epidemic strains from other sources were also tested in order to obtain hybridisation profiles for comparison by genotype pattern matching (pattern matching algorithm in the partisan array-LIMS software; CLONDIAG GmbH, Jena, Germany).

Preparation of genomic DNA

The protocol for DNA preparation has been described previously [11]. In brief, *S. aureus* isolates were cultured on Columbia blood agar. Two inoculation loops of colony material were suspended in a lysis buffer containing lysostaphin, lysozyme, ribonuclease A, Tris-HCl, EDTA and Triton X-100. After incubation, proteinase K and buffer AL (both from the DNeasy kit; QIAGEN, Hilden, Germany) were added. Finally, the sample was processed using the EZ1 device (QIAGEN).

Microarray-based genotyping

Diagnostic DNA microarrays, based on the ArrayTube platform (CLONDIAG), were used as described previously [10,11,16,17]. The arrays carried covalently immobilised probes for the identification of *agr* (accessory gene regulator) groups [18,19], antibiotic resistance determinants and exotoxins of *S. aureus*, as well as probes for *set/ssl* genes (staphylococcal superantigen-like or exotoxin-like genes) [20–22] and species controls. Markers for capsule identification and SCC*mec* typing (see below), and for the detection of allelic variants of MSCRAMM genes, were also included. A complete list of primers and probes is available upon request from the corresponding author. Array evaluation was performed using sequenced *S. aureus* strains Sanger MSSA476 (an ST1 methicillin-susceptible *S. aureus* strain), MW2 (ST1-MRSA IV), N315, Mu50 (both ST5-MRSA II), NCTC 8325 (ST8 MSSA), COL (CC8/ST250-MRSA I), USA300 (ST8-MRSA IV), and Sanger MRSA252 (ST30-MRSA II). Sequenced strain RF122 was not available, but related veterinary isolates were also used. Published sequences were used to predict hybridisation patterns for these strains, which were then compared to the actual hybridisation data. Additional evaluation procedures for a subset of clinically important probes (toxins, resistance genes) have been described previously [17].

For amplification and labelling, a linear and thermally synchronised primer elongation reaction was performed using a standard thermocycler. This allowed all targets to be amplified and labelled simultaneously, using one specific primer for each target. The labelling reaction comprised a mixture of primers (0.135 μ M each), dNTPs, biotin-16-dUTP, Terminator polymerase [17] and 1–2 μ g of target DNA. Amplification was performed as described previously [17].

Compared with earlier protocols, the hybridisation procedures were modified. Before hybridisation, the ArrayTube was washed with 500 μ L of double-distilled water and 500 μ L of Hybridisation Buffer 1 (CLONDIAG), each for 5 min at room temperature while agitating at 550 rpm on a thermomixing device. The labelled sample was diluted in Hybridisation Buffer 1 to a final volume of 100 μ L, denatured (2 min at 95°C), chilled on ice for 1 min, briefly centrifuged, and then transferred into the ArrayTube. The sample was then incubated for 60 min at 50°C (550 rpm), and then removed completely from the tube. The array was washed three times (each for 5 min at room temperature, 550 rpm), first with 500 μ L of 2 \times SSC (2 \times SSC is 0.3 M NaCl, 0.3 M trisodium citrate, pH 7.0) containing Triton X-100 0.01% v/v, then with 500 μ L of 2 \times SSC, and finally with 500 μ L of 0.2 \times SSC. This was followed by the addition of 100 μ L of a blocking reagent (CLONDIAG) 2% w/v solution, freshly dissolved in 6 \times SSPE (1 \times SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA) containing Triton X-100 0.1% v/v, and incubation of

the ArrayTube for 15 min at 30°C, 550 rpm. Horseradish peroxidase–streptavidin conjugate (Pierce, Rockford, IL, USA) was pre-diluted to 1:50 in 1 × phosphate-buffered saline from a stock solution, and then diluted 1:100 in 6 × SSPE containing Triton X-100 0.1% v/v to give a final concentration of 100 pg/μL. The blocking solution was removed and 100 μL of the diluted conjugate was added to the ArrayTube, followed by incubation (15 min, 30°C, 550 rpm) and another three washing steps as described above. The ArrayTube was then placed in an ATR01 reading device (CLONDIAG) and 100 μL of Seramun Green-precipitating horseradish peroxidase substrate (Seramun, Heidesee, Germany) was added. After staining for 5 min, the resulting array image was recorded and analysed.

Data interpretation

The local precipitation of dye results in clearly visible spots that can be read by superimposition of a coordinate grid over an enlarged digital picture of the processed array; the presence of spots is regarded as a positive hybridisation event. In order to facilitate computerised data interpretation, an algorithm was developed, based on measurement of light transmission through the spots with respect to their surroundings. Break-points for the definition of positive or negative signals were defined for each individual experiment, based on the actual measurements for positive control markers. Details of this procedure have been described previously [11].

agr and *spa* typing

agr groups were determined by microarray hybridisation. *spa* typing was performed according to a method described previously [23], using the SPATypemapper program for interpretation (<http://www.clondiag.com/technologies/download.php?file=spa>).

SCCmec typing

SCCmec typing was performed by detection of the recombinase (*ccrA*, *ccrB*, *ccrC*) and accessory genes that form part of the SCCmec cassettes. The structure and gene content of these cassettes have been described previously [1,5,24]. However, in contrast to published protocols, detection was achieved not by PCR, but by hybridisation to a set of probes on a DNA array. In general, probes for *mecA* and *ugpQ* (glycerophosphoryldiesterase) were used to detect SCCmec resistance islands of any type. SCCmecI was identified by hybridisation results positive for *crrA1*, *crrB1*, truncated *mecR* (Δ *mecR*), *pls* (plasmin-sensitive surface protein) and the *dcs* region. Identification of SCCmecII was based on the detection of *crrA2*, *crrB2*, *mecI*, non-truncated *mecR*, the *kdp* operon (*kdpA*–*kdpE*), *xylR* and the *dcs* region. The *aadD* and *ermA* resistance genes were not regarded as prerequisite for SCCmecII, because of their variable presence. For SCCmecIII identification, probes recognising *crrA3*, *crrB3*, *mecI*, *xylR* and non-truncated *mecR* were utilised. The variable presence of the *tetK* resistance gene meant that it was not regarded as evidence for the presence of a type III cassette. Similarly, detection of the mercury resistance operon (*merA*, *merB*, *merR*, *merT*) was not regarded as positive evidence for a type III cassette, as this operon was also found in isolates carrying other SCCmec types. *ccrA* probes designed for

identification of an atypical SCCmec cassette, originally described as type III, from strain 85/2082 (GenBank AB037671) cross-reacted with both SCCmecIII and SCCmecV. An isolate was assigned to SCCmecIV if *crrA2*, *crrB2*, truncated *mecR* and *dcs* were present, but (in contrast to SCCmecII) the *kdp* genes were absent and occurrence of *xylR* was variable. Identification of SCCmecV was based on positive hybridisation reactions with probes for *ccrAA* (GenBank AM292304.1) and *ccrC* (GenBank AB037671), although the latter also cross-reacted with *crrB3*. SCCmecV isolates were not required to display reactivity with the probe for truncated *mecR*, as published sequence data (GenBank AB121219.1, DQ171730.1) indicate more extensive deletions in SCCmecV than in SCCmecI and SCCmecIV, and also a loss of the binding sites of primer and probe for truncated *mecR*. SCCmecVI was identified on the basis of detection of *ccrA4* and *ccrB4*.

RESULTS

All the isolates tested could be assigned unambiguously to clonal groups by analysing hybridisation patterns once a reference strain that had been MLST/*spa*-typed for each clonal group had been defined. SCCmec typing by hybridisation yielded results in accord with data published previously for the respective strains. A simplified summary of the hybridisation results for discernible epidemic strains and reference strains is provided in Figs 1–4. Full hybridisation results for all isolates tested are available upon request from the corresponding author. Brief summaries of the characteristics of clinical isolates according to their clonal group are given below.

Clonal group 1

USA400, a PVL-positive caMRSA strain from this clonal group, has not yet been found in Eastern Saxony, but closely related PVL-negative ST1-MRSA IV isolates have been detected in three cases (a specimen from paediatric surgery and two specimens from the skin lesions of patients in a dermatological department). In contrast to the sequenced USA400 strain MW2, these isolates lacked *sec*, *sel* and PVL, but their hybridisation patterns were otherwise virtually identical to that of MW2. The isolates belonged to capsule type 8 and carried genes encoding enterotoxin A (*sea*), staphylokinase (*sak*) and staphylococcal complement inhibitor (*scn*), and thus belonged to IEC type D [14]. Carriage of *ssl/set* genes and of genes encoding MSCRAMMs was homogeneous and identical to that of MW2.

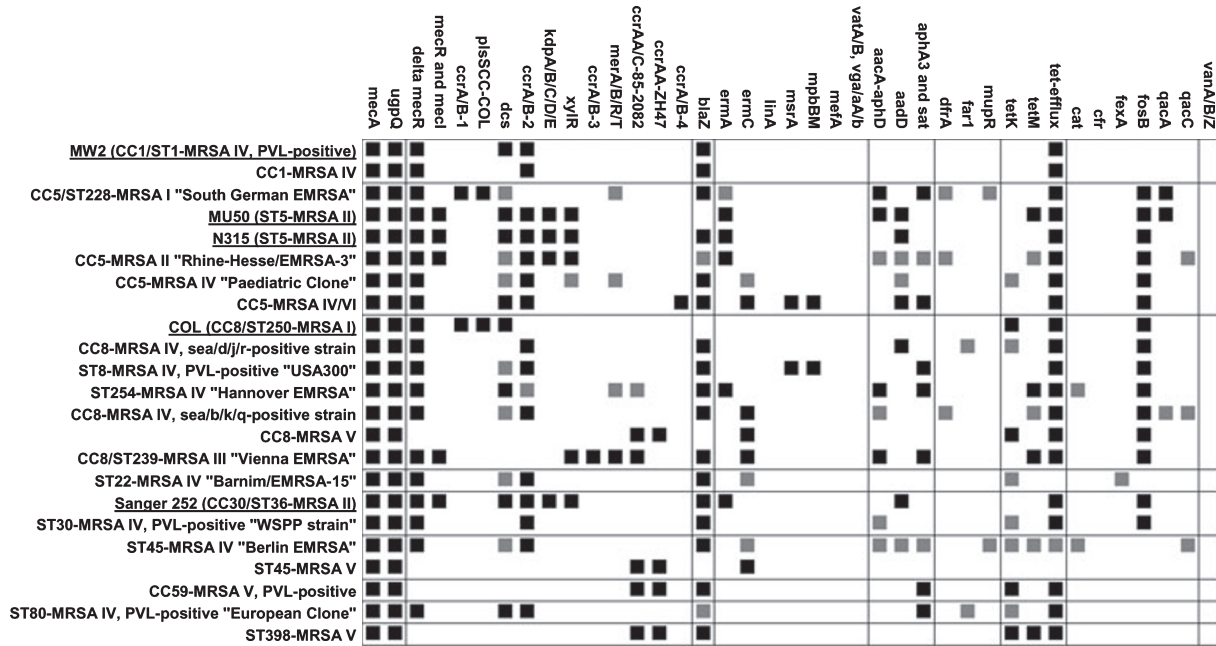


Fig. 1. Hybridisation results for antibiotic resistance genes and genes from SCCmec cassettes. Sequenced reference strains are underlined. Black squares, always positive; grey squares, variable.

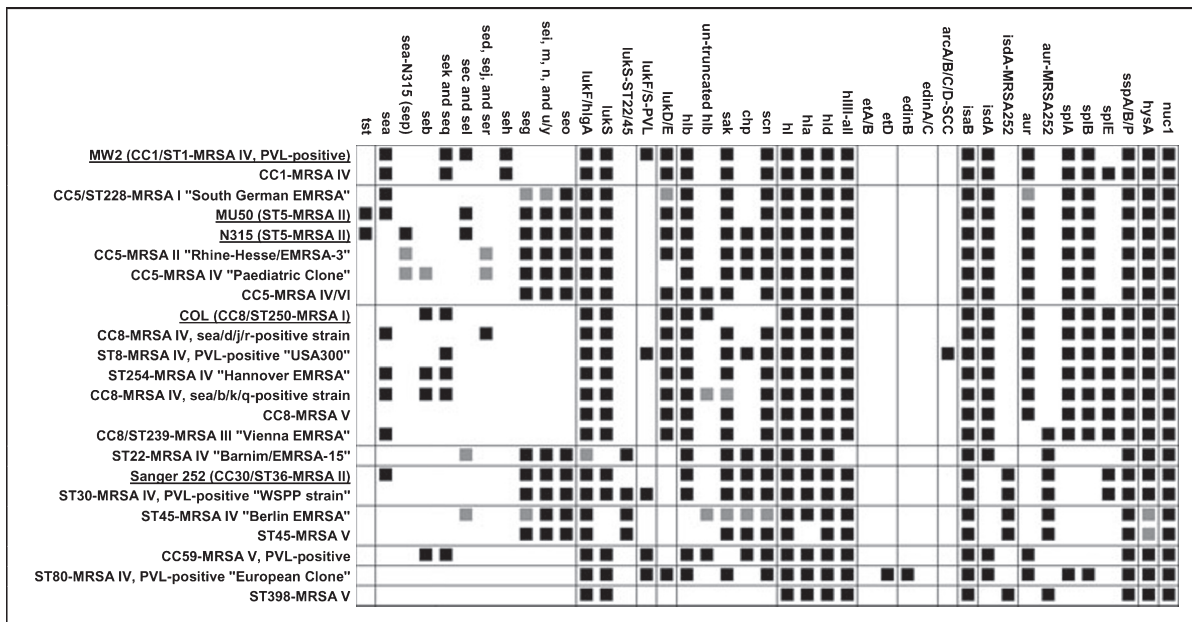


Fig. 2. Hybridisation results for genes encoding virulence-associated factors, including superantigenic toxins, leukocidins, haemolysins, proteases and other exotoxins or enzymes. Sequenced reference strains are underlined. Black squares, always positive; grey squares, variable or ambiguous.

Clonal group 5

Clonal group 5 was represented by two abundant EMRSA strains (South German and Rhine-

Hesse), and by two sporadic strains. All isolates belonged to capsule type 5 and were uniform in terms of carriage of *ssl/set* genes and genes encoding MSCRAMMs. Allelic variants of these

(encoding a metallothiol transferase conferring fosfomycin resistance).

An SCC*mecI* strain from this clonal group is known as the South German Epidemic Strain (ST228, *spa* t001, t041 or t811). This is one of the four most common MRSA strains in Eastern Saxony. It is quite resistant, with isolates carrying the *aacA-aphD*, *aphA*, *sat* and *qacA* (encoding a multidrug efflux protein) genes. In contrast to other clonal group 5 strains, the gene for fibronectin-binding protein B (*fnbB*) was not detectable in this strain. The haemolysin- β (*hlyB*) gene was always disrupted, and all isolates belonged to IEC type D [14]. Altogether, four variants of the South German Epidemic Strain were discernible, based on the variable presence of the mercury resistance operon, *ermA*, *mupR*, *dfrA*, *clfA* (encoding clumping factor A) and *bbp* (bone/sialoprotein binding protein). A distinctive fifth variant of this strain (also *spa* t001) had a truncated *egc* locus, being positive only for the enterotoxin gene *seo*. It also lacked the *lukD/E* leukocidin genes. In addition to the isolate described here, three other isolates were found between 2005 and 2007, all of which were linked to a single ward in a rehabilitation facility.

A related strain is the Rhine–Hesse Epidemic Strain, also known as EMRSA-3 or New-York/Japan Clone (*spa* t003 or t627). Locally, this was one of the four most abundant strains. Isolates carried an SCC*mecII* element, which was in accord with the presence of *ermA* and, in most isolates, of *aadD*. The Rhine–Hesse EMRSA strain is related closely to the sequenced strain N315. Most isolates carried a distinctive allelic variant of *sea* (*sea-N315*, also known as *sep*), described originally in N315 [25]. Enterotoxin carriage (*sea-N315*, *sed* + *sej* + *ser*) and the presence or absence of resistance genes (*blaZ*, *aadD*, *aacA-aphD*, *aphA3*, *sat* and *tetM*) allowed eight variants to be distinguished. Identification of these variants can be helpful for epidemiological purposes. For instance, seven of nine isolates that were *sea-N315*⁻, *sed/j/r*⁺, *blaZ*⁺ were epidemiologically linked, being sampled from a single hospital during 2003–2004. As the *sak*, *scn* and *chp* (encoding chemotaxis inhibitory protein) genes were present, Rhine–Hesse EMRSA isolates belonged either to IEC type B or IEC type F, depending on the carriage of *sea-N315*.

A related strain (*spa* t002, t067) carried an SCC*mecIV* element, corresponding to the interna-

tionally known 'Paediatric clone'. This clone was rare, with only four isolates identified to date. It differed from the Rhine–Hesse EMRSA strain in lacking *ermA*, which is in accord with the presence of an SCC*mecIV* element, but three isolates were positive for *aadD*. Three isolates harboured *sea-N315*, *sed*, *sej* and *ser*, and two were positive for *seb*. The isolates belonged to IEC types B or F [14]. PVL-positive variants of this strain, as reported in other countries, have not been found in Eastern Saxony to date.

A unique isolate belonging to clonal group 5 (t067) was obtained from a patient with severe pneumococcal pneumonia who was repatriated from the Canary Islands. This isolate yielded positive hybridisation signals with the probes for SCC*mecIV*, SCC*mecVI*, *ermA*, *ermC*, *msrA*, *mpbBM* (encoding macrolide 2'-phosphotransferase), *aadD*, *aphA3* and *sat*. It belonged to IEC type E, carrying *sak* and *scn*.

Clonal group 8

MRSA strains from clonal group 8 generally gave hybridisation patterns related to those of the sequenced strains COL, USA300 and NCTC 8325. Carriage of the *ssl/set* genes and the genes encoding MSCRAMMs was rather homogeneous, and resembled that of these sequenced strains. With the exception of ST239 (see below), isolates belonged to capsule type 5 and lacked *cna* (encoding collagen-binding adhesin). Carriage of genes encoding resistance properties and exotoxins, as well as of IEC genes, was variable, thereby allowing several strains to be distinguished.

One strain from this group, known as Ancestral or Ancient MRSA, is a CC8/ST250-MRSA I strain, represented by the sequenced strain COL. No clinical isolates of the Ancient MRSA were found.

Another strain in this clonal group is PVL-positive ST8-MRSA IV, also known as USA300. Two cases of community-acquired infections with this strain have been identified to date in Eastern Saxony. Two additional cases were referred from a neighbouring county in the Federal State of Brandenburg. All four isolates carried *msrA*, *mpbBM*, *aphA3* and *sat*, but lacked *ermC*, *tetK* and *mupR*, indicating a plasmid content different to that of the sequenced USA300 isolate (NC_007790, NC_007791, NC_007792, NC_007793). The hybridisation results for other

genes were in accord with the published genome, including the presence of PVL genes, the ACME operon, and enterotoxin genes *sek* and *seq*.

Two cases of infection were noted with a related strain that was also ST8, *spa* t008 and SCC*mec*IV. In contrast to USA300, this strain did not carry the PVL, ACME or *sek* or *seq* genes, but harboured *sea*, *sed*, *sej* and *ser*, as well as *aadD*. This strain belonged to IEC type D [14]. A follow-up isolate from one of the two patients was found to have acquired *far1* (fusidic acid resistance) and *tetK*.

Another, locally relevant, strain was the Hannover Epidemic Strain, ST8-MRSA IV, *spa* t009. This strain was apparently introduced to Saxony shortly after German reunification, and was common during the 1990s (L. Jatzwauk, personal communication), but no isolate has been identified since 2001. Isolates of this strain carried *ermA*, *aacA-aphD*, *aphA3*, *sat* and *tetM*, and were resistant to co-trimoxazole, but without harbouring *dfra*. This strain belonged to IEC type D [14]. In addition to *sea*, it harboured the enterotoxin genes *seb*, *sek* and *seq*. In contrast to other ST8-MRSA strains, but in accord with, e.g., NCTC 8325, it lacked *bbp*. Two variants that differed in their carriage of genes associated with SCC*mec* elements were distinguishable. One variant carried the *mecA*, Δ *mecR*, *ugpQ*, *dcs*, *ccrA2* and *ccrB2* genes, and also generated positive signals with the probes for *ccrAA-85/2082* and *ccrC-85/2082*. The other variant was positive for *mecA*, Δ *mecR*, *ugpQ* and *dcs*, and also carried the mercury resistance operon.

Another strain belonged to ST8, *spa* t064 and SCC*mec*IV, and was possibly related to USA500 (<http://spa.ridom.de/spatypes.shtml> [26]). Three imported cases, from Ethiopia (probably community-acquired), Zimbabwe (hospital-acquired) and Mozambique (community-acquired), respectively, were found. This strain harboured *seb*, *sek* and *seq*, and was resistant to erythromycin, clindamycin (*ermA*) and rifampicin. The isolates varied in their carriage of *aacA-aphD*, *tetM*, *sea*, *sak* and *scn*.

One MRSA isolate, belonging to clonal group 8 (t008), carried SCC*mec*V and was positive for *ermC* and *tetK*, but, as with NCTC 8325, did not carry enterotoxin genes. This isolate belonged to IEC type D [14].

ST239-MRSA III (Vienna, Hungarian or Brazilian clones) was found to be involved in a nosocomial outbreak following the repatriation of

a trauma patient from Rhodes, Greece. This strain harboured the *ermC*, *aacA-aphD*, *aphA3*, *sat* and *tetM* genes. It carried only the enterotoxin A gene (*sea*), although ST239-MRSA III may also harbour *sek* and *seq*. IEC gene carriage was in accord with IEC type D [14]. ST239 is unique, as it can be regarded as the result of a large-scale recombination event involving the parental strains of clonal groups 8 and 30 [27]. Indeed, alleles of *ssl/set* and most MSCRAMM genes were typical for ST8 strains, but the *arcC* allele (carbamate kinase, used in multilocus sequence typing (MLST) [28]), capsule type 8 (instead of type 5), *spa* type (t037), the allelic variant of the aureolysin (*aur*) gene and the presence of *cna* indicated the insertion of a chromosomal fragment from clonal group 30.

Clonal group 22

A clonal group 22 strain, ST22-MRSA IV, also known as the Barnim Epidemic Strain [29] or EMRSA-15, was one of the four most abundant MRSA strains in Eastern Saxony. In terms of hybridisation profiles, clonal group 22 was not related to clonal groups 1, 5 and 8, but shared some features with clonal group 45. The *ssl/set* gene patterns were atypical and showed cross-reactivity among probes for different allelic variants of a given gene. Probes for the *hlgA*, *lukF-hlg* and *lukS-hlg* genes regularly gave weak signals. Both of these observations could indicate the presence of, as yet, unsequenced alleles. For *lukS-hlg*, a specific probe was constructed, based on an ST45 sequence (GenBank EF672356). This probe yielded positive results for ST22 and ST45.

All clonal group 22 isolates harboured the *egc* locus, but protease genes *splA*, *splB* and *splE* were not detected. The capsule type was 5, and the vast majority of isolates belonged to IEC type B. Some isolates had non-truncated *hly* and lacked the IEC genes. Depending on the carriage of enterotoxin genes (*sec* and *sel*), haemolysin β -converting phages, *ermC* and the MSCRAMM genes (*fnbB*, *bbp*), a total of eight variants could be distinguished.

ST22-MRSA IV was found among hospital isolates from apparently community-acquired infections. In particular, it was associated with diabetic foot ulcers, although this might, in part, be attributable to an outbreak of one variant

(*sec*/*T*⁻, *ermC*⁻, *IEC*⁺) in a ward and outpatient department specialising in the care of such patients. The PVL-positive ST22-MRSA IV outbreak strain from Bavaria [9] has not yet been detected in Eastern Saxony.

Clonal group 30

Clonal group 30 MRSA strains are ST36-MRSA II, or EMRSA-16 (represented by sequenced strain MRSA 252), and the PVL-positive ST30-MRSA IV strain, also known as WSPP or South-West Pacific clone. The former was not detected in isolates from Eastern Saxony, while the latter was detected in two cases of community-acquired 'insect-bite-like' infections. One of these isolates harboured *aacA-aphD* and *tetK*. Toxin and *set/ssl* gene carriage have been described previously [10]. Carriage of allelic variants of MSCRAMM genes was similar to that of MRSA252; differences from MRSA252 included the presence of *fnbB* and *sdrD*.

Clonal group 45

Clonal group 45 strains resembled those of clonal group 22 in terms of atypical *ssl/set* and *luk/hlgA* gene patterns (see above). ST45 isolates also carried the *egc* cluster, but in contrast to ST22, belonged to capsule type 8. These isolates had a unique allele of the gene for the bone-binding protein (*bbp*). The *sasG* (biofilm production/protein G) gene was conspicuously absent from this clonal group, as in clonal groups 30 and 398.

ST45-MRSA IV, also known as the Berlin Epidemic Strain (*spa* t004 or t040), was one of the most abundant local MRSA strains. Ten variants of this strain could be distinguished using the microarray. The variations observed affected resistance genes *aacA-aphD*, *aadD*, *ermC*, *dfrA*, *aphA3*, *sat* and *tetK*, as well as a common deletion of an enterotoxin gene (*seg*) from the *egc* cluster. All except one of the isolates (which lacked IEC genes) belonged to IEC type B. A related strain, or an eleventh variant, was an ST45-MRSA IV strain, which differed in the presence of additional enterotoxin genes (*sec*, *sel*) and the absence of *aphA3* plus *sat*. This strain was detected only once, but has also been detected in Sweden and The Netherlands (A. Matussek and F. H. J. Schuren, personal communication).

In 2007, a single isolate of an ST45-MRSA V strain was detected which was otherwise very similar to the Berlin Epidemic Strain.

Clonal group 59

A single isolate belonging to clonal group 59 was obtained from a community-acquired infection. The isolate was a PVL-positive SCC*mecV* strain, similar to isolates characterised previously from Australia and the UK [10], and was resistant to erythromycin, clindamycin and quinupristin-dalfopristin, but was susceptible to linezolid. However, the isolate yielded negative hybridisation results for all macrolide and streptogramin resistance genes tested, as well as for *cfr* (23S RNA methylase). The isolate carried *chp* and *scn*, and belonged to IEC type C [14].

Clonal group 80

ST80-MRSA IV, or European Clone (*spa* t044 or t131), was the most prevalent PVL-positive, community-acquired MRSA strain in Eastern Saxony, with eight isolates being obtained from Dresden or nearby counties (Freiberg, Pirna and Hoyerswerda) in the period until October 2007. Three cases were considered to be hospital-associated; two of these have been described previously [10], while the third was a case of septicaemia in a patient suffering from diabetic gangrene. Details of the carriage of toxin and resistance genes have been described previously [10]. In brief, this strain carries exfoliative toxin D (*etD*) and an epidermal cell differentiation inhibitor (*edinB*). Most isolates also harboured a plasmid encoding a β -lactamase, fusidic acid (*far1*) and tetracycline (*tetK*) resistance. The *cna* gene was absent among the MSCRAMM genes, but the IEC genes *chp* and *sak* were present, corresponding to type E [14].

Clonal group 398

ST398-MRSA V is a recently described strain, occurring in humans and a variety of domestic animals [30], that has been involved in outbreaks of infection affecting pig farmers [31,32]. The present study identified a single isolate of a *spa* t011 MRSA strain from a urinary tract infection in a child. This isolate carried an SCC*mecV* element and the *tetK* and *tetM* genes. It differed

from previously described [12,30] veterinary ST398-MRSA V isolates by the absence of *aacA-aphD*, *ermA* and *ermC*. The capsule type was 5, and the *cna* gene was present. Enterotoxin genes (except for a ubiquitous homologue), IEC and protease genes *splA*, *splB* and *splE*, as well as *sasG*, were not detected.

DISCUSSION

Diagnostic DNA microarrays allow the rapid identification of isolates and a comprehensive assessment of virulence properties and antimicrobial resistance genes under routine conditions. Complete hybridisation patterns allow isolates to be assigned to clonal groups [11]. Combined with a novel scheme for hybridisation-based *SCCmec* typing, this facilitates identification and characterisation of MRSA isolates and their assignment to epidemic strains. By analysing isolates from routine diagnostic laboratories and outbreak investigations, it was possible to obtain insights into the local occurrence of MRSA strains and *SCCmec* elements, as well as clinically relevant virulence factors, e.g., superantigenic toxins or PVL.

The vast majority of MRSA infections or colonisations in Eastern Saxony could be attributed to four strains i.e., South German EMRSA (CC5/ST228-MRSA I), Rhine-Hesse EMRSA (ST5-MRSA II), Barnim EMRSA (ST22-MRSA IV) and Berlin EMRSA (ST45-MRSA IV). Other MRSA strains were rare in comparison, and were only detected in isolated cases. The predominant *SCCmec* types were I, II and IV. *SCCmecI* elements are still common, being present in the South German EMRSA strain. The Ancestral MRSA (CC8/ST250-MRSA I) strain was not found. *SCCmecII* was restricted to one of the abundant strains (Rhine-Hesse EMRSA). Variation in this element was noted, as some isolates lacked the tobramycin resistance gene *aadD*. As this gene is carried by the plasmid pUB110, these strains can be classified as either pUB110-deficient variant IID (2A.3.4) or pUB110-deficient variant IIb (2A.2) [33]. A second internationally important *SCCmecII* strain, ST36-MRSA II (EMRSA-16), was not detected, and *SCCmecIII* (ST239-MRSA III) was detected only in a single nosocomial outbreak following its importation from southern Europe. This latter strain is remarkable, as it appears to be some kind of hybrid derived from clonal group 8

and clonal group 30 parental strains. The array data confirmed the data obtained by MLST [27], and allowed the parentage of additional genes to be traced. Approximately half of the MRSA strains detected carried *SCCmecIV* elements, and belonged mostly to the Barnim and Berlin EMRSA strains. Another hospital-acquired MRSA strain carrying *SCCmecIV* was the Hannover EMRSA (ST8-MRSA IV), which appears to have become less common since the 1990s [34]. *SCCmecV* and *SCCmecVI* elements are still rare.

While some of the strains detected are also known to be abundant in other European countries (e.g., Rhine-Hesse EMRSA or EMRSA-3, and the Barnim Epidemic strain or EMRSA-15), some important strains found in other countries were completely absent (e.g., EMRSA-16). Data from the German national reference centre show an overall increase in MRSA prevalence, and also a shift in the relative frequencies of different strains during the 1990s, with the North German and Hannover EMRSA strains disappearing, and the Barnim, as well as the Rhine-Hesse EMRSA strains, showing a massive spread [29,34]. A possible explanation is that the dissemination of EMRSA strains into a low-prevalence region (e.g., Saxony during the 1990s) was achieved easily, but that it was mainly a matter of chance which strains actually spread. Thus, the absence of EMRSA-16 was not the result of reduced fitness in comparison with the regionally abundant strains, but rather a chance failure to import this strain to Saxony during the 1990s. When the founder strains reached a certain prevalence, competition among them might have resulted in the elimination of some strains, perhaps because they replicated more slowly, were less infectious, or were more susceptible to environmental conditions. It would be interesting to determine the reasons that led to the decline in importance of the North German and Hannover EMRSA strains. The use of antibiotics was probably not responsible, as these strains harbour more resistance genes than the strains that have supplanted them. Between 2000 and 2007, no new strains were observed to gain ground, and although several importations of strains (including an ST239-MRSA III outbreak) were observed, none became permanently established. It can be assumed that newly imported strains might face difficulties in becoming established in a setting with a high prevalence of previously well-established strains.

Successful establishment in such an environment would require either a major selective advantage resulting from novel pathobiochemical properties (e.g., those associated with USA300 in the USA) or importation on a massive scale. Similarly, a significant influx of community-acquired PVL-positive MRSA strains into the hospital environment was not observed, although sporadic infections with PVL-positive ST80-MRSA IV were recorded in diabetic or long-term rehabilitation patients, but close surveillance of this strain, as well as of other PVL-positive strains, is warranted because of their virulence and their known epidemic potential [9,35,36]. PVL-positive MRSA strains are still rare, although diversification can already be observed, with the recognition of an increasing number of different strains (ST80-MRSA IV in 2004, USA300 in 2005, ST30-MRSA IV in 2006, and ST59-MRSA V in 2007). Other possibly emerging strains might include ST398-MRSA V, which has been involved in outbreaks affecting domestic animals and pig farmers in The Netherlands [31,32], clonal group 5 strains carrying *SCCmecIV* elements (Paediatric clone) and ST45-MRSA V.

The DNA microarray allowed detection of sub-strains or variants, especially within the more common strains, which often differ in the carriage of genes encoding leukocidins, enterotoxins and accessory resistance determinants (e.g., *ermC* in the Barnim EMRSA strain). Such variations in gene content were observed in several major strains revealed in the present study (Hannover, Barnim, Berlin, South German, Paediatric and Rhine-Hesse EMRSA strains, as well as ST80-MRSA IV). The fact that no variations have been observed to date in some other strains (e.g., ST1-MRSA IV) might be attributed to the small sample size and/or the recent proliferation of these strains, rather than their genuine absence. The clinical significance of the loss, or acquisition, of exotoxin genes is not yet clear, but these genes can be useful for subtyping. In the same way, the presence and identity of haemolysin β -converting phages can be used to distinguish variants within a strain. Some variations were also noted with respect to the presence of MSCRAMM genes. However, the variability of these genes affected mainly the number and sequence of repeating units, which are not targeted by the probes on the array. The probes were designed to recognise conserved, non-repeat regions in order to detect

the presence or absence, rather than variation, of these genes. Occasional deletions of the MSCRAMM genes could affect the ability to adhere to implants or intravenous lines, which is of particular relevance for hospital-acquired strains. All these variations in gene content can be used for subtyping, and thus for tracking the epidemiology of strains involved in outbreaks. For instance, all isolates of a characteristic deletion mutant of the South German EMRSA strain (see above) were linked to a single ward. Such discrimination at a level below *spa* types can be very helpful for epidemiological purposes, especially in settings in which most MRSA cases can be attributed to a small number of abundant epidemic strains.

The DNA microarray used for the present study facilitated a comprehensive genomic profiling of *S. aureus* isolates. The laboratory effort is comparable in time and cost to real-time PCR assays for single genomic loci. Thus, the array provides a valuable alternative approach to other typing techniques, being less time-consuming and adding discriminatory power to outbreak investigations. A large number of loci can be analysed within a single experiment, combining the detection of clinically relevant virulence- or resistance-associated genes and the typing of a given isolate. Additional probes used in the present study allowed identification of the *SCCmec* type, the content of *hly*-converting phages and the capsule type. The overall hybridisation patterns generated were found to correlate with the clonal complexes defined by MLST. In contrast, probes for exotoxin and antibiotic resistance determinants target genes located on highly mobile genetic elements. Their variability, even within a given strain, might allow the subtyping of epidemic strains to the level of hospital- or ward-specific variants. An expansion of the database covering these variations might be of great value in epidemiological studies of transmission events and links among individual cases.

ACKNOWLEDGEMENTS

The authors acknowledge A. Ruppelt, H. Kanig, I. Engelmann, E. Müller and J. Sachtschal for excellent technical assistance. We thank the staff at the Bacteriological Laboratory of the Institute for Medical Microbiology and Hygiene (Dresden, Germany), C. Gäbler and S. Vogel for identifying and collecting clinical isolates, and K. Becker, B. Berger-Bächi, G. Coombs, M. Ellington, T. Juratzek, A. Kearns, H.-J. Linde, A. Matussek,

F. O'Brien, F. H. J. Schuren, W. Witte, the Institut Pasteur, Paris and the Network on Antimicrobial Resistance in *Staphylococcus aureus* for providing reference strains. We acknowledge E. Jacobs (Institute for Medical Microbiology and Hygiene, Dresden, Germany) and E. Ermantraut (CLONDIAG) for their support of this work. P. Slickers and R. Ehricht are employees of CLONDIAG. The authors declare that they have no other conflicts of interest in relation to this work.

REFERENCES

- Ito T, Katayama Y, Asada K *et al.* Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2001; **45**: 1323–1336.
- Ender M, McCallum N, Adhikari R *et al.* Fitness cost of SCC*mec* and methicillin resistance levels in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2004; **48**: 2295–2297.
- Lee SM, Ender M, Adhikari R *et al.* Fitness cost of staphylococcal cassette chromosome *mec* in methicillin-resistant *Staphylococcus aureus* by way of continuous culture. *Antimicrob Agents Chemother* 2007; **51**: 1497–1499.
- Daum RS, Ito T, Hiramatsu K *et al.* A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. *J Infect Dis* 2002; **186**: 1344–1347.
- Ito T, Ma XX, Takeuchi F *et al.* Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. *Antimicrob Agents Chemother* 2004; **48**: 2637–2651.
- O'Brien FG, Lim TT, Chong FN *et al.* Diversity among community isolates of methicillin-resistant *Staphylococcus aureus* in Australia. *J Clin Microbiol* 2004; **42**: 3185–3190.
- Panton P, Valentine F. Staphylococcal toxin. *Lancet* 1932; **i**: 506–508.
- Vandenesch F, Naimi T, Enright MC *et al.* Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Pantone–Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis* 2003; **9**: 978–984.
- Linde H, Wagenlehner F, Strommenger B *et al.* Healthcare-associated outbreaks and community-acquired infections due to MRSA carrying the Pantone–Valentine leukocidin gene in southeastern Germany. *Eur J Clin Microbiol Infect Dis* 2005; **24**: 419–422.
- Monecke S, Slickers P, Hotzel H *et al.* Microarray-based characterisation of a Pantone–Valentine leukocidin-positive community-acquired strain of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 2006; **12**: 718–728.
- Monecke S, Berger-Bächi B, Coombs C *et al.* Comparative genomics and DNA-array-based genotyping of pandemic *Staphylococcus aureus* strains carrying Pantone–Valentine leukocidin. *Clin Microbiol Infect* 2007; **13**: 236–249.
- Monecke S, Kuhnert P, Hotzel H *et al.* Microarray-based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. *Vet Microbiol* 2007; **125**: 128–140.
- Monecke S, Slickers P, Ellington M *et al.* High diversity of Pantone–Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated MRSA. *Clin Microbiol Infect* 2007; **13**: 1157–1164.
- van Wamel WJB, Rooijackers SHM, Ruyken M *et al.* The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. *J Bacteriol* 2006; **188**: 1310–1315.
- Clarke SR, Foster SJ. Surface adhesins of *Staphylococcus aureus*. *Adv Microb Physiol* 2006; **51**: 187–224.
- Monecke S, Leube I, Ehricht R. Simple and robust array-based methods for the parallel detection of resistance genes of *Staphylococcus aureus*. *Genome Lett* 2003; **2**: 106–118.
- Monecke S, Ehricht R. Rapid genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using miniaturised oligonucleotide arrays. *Clin Microbiol Infect* 2005; **11**: 825–833.
- Jarraud S, Mougel C, Thioulouse J *et al.* Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. *Infect Immun* 2002; **70**: 631–641.
- Robinson DA, Monk AB, Cooper JE *et al.* Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. *J Bacteriol* 2005; **187**: 8312–8321.
- Fitzgerald JR, Reid SD, Ruotsalainen E *et al.* Genome diversification in *Staphylococcus aureus*: molecular evolution of a highly variable chromosomal region encoding the staphylococcal exotoxin-like family of proteins. *Infect Immun* 2003; **71**: 2827–2838.
- Lina G, Bohach GA, Nair SP *et al.* Standard nomenclature for the superantigens expressed by *Staphylococcus*. *J Infect Dis* 2004; **189**: 2334–2336.
- Williams RJ, Ward JM, Henderson B *et al.* Identification of a novel gene cluster encoding staphylococcal exotoxin-like proteins: characterization of the prototypic gene and its protein product, SET1. *Infect Immun* 2000; **68**: 4407–4415.
- 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.
- Oliveira DC, Wu SW, de Lencastre H. Genetic organization of the downstream region of the *mecA* element in methicillin-resistant *Staphylococcus aureus* isolates carrying different polymorphisms of this region. *Antimicrob Agents Chemother* 2000; **44**: 1906–1910.
- Kuroda M, Ohta T, Uchiyama I *et al.* Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 2001; **357**: 1225–1240.
- Diep BA, Carleton HA, Chang RF *et al.* Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 2006; **193**: 1495–1503.
- Robinson DA, Enright MC. Evolution of *Staphylococcus aureus* by large chromosomal replacements. *J Bacteriol* 2004; **186**: 1060–1064.
- Enright MC, Day NPJ, Davies CE *et al.* Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 2000; **38**: 1008–1015.
- Witte W, Enright M, Schmitz FJ *et al.* Characteristics of a new epidemic MRSA in Germany ancestral to United Kingdom EMRSA 15. *Int J Med Microbiol* 2001; **290**: 677–682.

30. Cuny C, Strommenger B, Witte W. Emergence of MRSA of multilocus sequence type ST398 in animals and in humans (RFP06). *Int J Med Microbiol* 2006; **296** (suppl 3): 153.
31. de Neeling AJ, van den Broek MJ, Spalburg EC *et al.* High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. *Vet Microbiol* 2007; **122**: 366–372.
32. Huijsdens XW, van Dijke BJ, Spalburg E *et al.* Community-acquired MRSA and pig-farming. *Ann Clin Microbiol Antimicrob* 2006; **5**: 26.
33. Deurenberg RH, Vink C, Kalenic S *et al.* The molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 2007; **13**: 222–235.
34. Witte W, Bralke C, Heuck D *et al.* Methicillin resistant *Staphylococcus aureus* in German hospitals develop narrower patterns of antimicrobial resistance. *Euro Surveill* 2000; **5**: 31–34.
35. Baggett HC, Hennessy TW, Rudolph K *et al.* Community-onset methicillin-resistant *Staphylococcus aureus* associated with antibiotic use and the cytotoxin Panton–Valentine leukocidin during a furunculosis outbreak in rural Alaska. *J Infect Dis* 2004; **189**: 1565–1573.
36. Moran GJ, Krishnadasan A, Gorwitz RJ *et al.* Methicillin-resistant *S. aureus* infections among patients in the emergency department. *N Engl J Med* 2006; **355**: 666–674.