Eur J Clin Microbiol Infect Dis (2010) 29:163–170
DOI 10.1007/s10096-009-0834-1

ARTICLE

Characterisation of MRSA from Malta and the description of a Maltese epidemic MRSA strain

E. A. Scicluna · A. C. Shore · A. Thürmer · R. Ehricht · P. Slickers · M. A. Borg · D. C. Coleman · S. Monecke

Received: 24 July 2009 / Accepted: 17 October 2009 / Published online: 13 November 2009

Abstract Malta has one of the highest prevalence rates of methicillin-resistant *Staphylococcus aureus* (MRSA) in Europe. However, only limited typing data are currently available. In order to address this situation, 45 MRSA isolates from the Mater Dei Hospital in Msida, Malta, were characterised using DNA microarrays. The most common strain was ST22-MRSA-IV (UK-EMRSA-15, 30 isolates). Sporadic strains included ST36-MRSA-II (UK-EMRSA-16, two isolates), PVL-positive ST80-MRSA-IV (European Clone, one isolate), ST228-MRSA-I (Italian Clone/South German Epidemic Strain, one isolate) and ST239-MRSA-III (Vienna/Hungarian/Brazilian Epidemic Strain, one isolate). Ten MRSA isolates belonged to a clonal complex (CC) 5/ST149, *spa* type t002 strain. This strain harboured an SCC*mec* IV element (*mecA*, *delta mecR*, *ugpQ*, *dcs*, *ccrA2* and *ccrB2*), as well as novel alleles of *ccrA/B* and the fusidic acid resistance element Q6GD50 (previously described in the sequenced strain MSSA476, BX571857.1:SAS0043). It also carried the gene for enterotoxin A (*sea*) and the *egc* enterotoxin locus, as well as (in nine out of ten isolates) genes encoding the toxic shock syndrome toxin (*tst1*) and enterotoxins C and L (*sec*, *sel*). While the presence of the other MRSA strains suggests foreign importation due to travel between Malta and other European countries, the CC5/t002 strain appears, so far, to be restricted to Malta.

Electronic supplementary material The online version of this article (doi:10.1007/s10096-009-0834-1) contains supplementary material, which is available to authorized users.

E. A. Scicluna · M. A. Borg
Infection Control Unit, Mater Dei Hospital,
Msida, Malta

A. C. Shore · D. C. Coleman
Microbiology Research Unit, Dublin Dental School & Hospital,
University of Dublin, Trinity College Dublin,
Dublin, Ireland

A. Thürmer · S. Monecke (✉)
Institute for Medical Microbiology and Hygiene,
Faculty of Medicine "Carl Gustav Carus",
Technical University of Dresden,
Fetscherstrasse 74,
01307 Dresden, Germany
e-mail: monecke@rocketmail.com

R. Ehricht · P. Slickers
CLONDIAG GmbH,
Loebstedter Strasse 103–105,
07749 Jena, Germany

Introduction

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) differs widely across European countries. In Scandinavia, Denmark, Iceland and the Netherlands, MRSA is very rare and contact tracing as well as typing are possible for essentially all cases. On the other hand, in Southern European countries, the United Kingdom and Ireland, the proportion of MRSA approaches or even exceeds 50% of *S. aureus* infections (http://www.rivm.nl/earss/database/).

There are only limited data currently available concerning epidemic MRSA strains from Southern European countries, since comparatively few articles have been published that provide typing data from, e.g. Spain, Italy, Malta or Greece. The limited number of studies in the literature that are available indicate that some internationally spread MRSA strains also occur in Southern European countries. For example, ST228-MRSA-I is common not only in Germany [1], but also in Italy [2]. The pandemic ST239-MRSA-III strain has also been found in Greece [3] and Spain [4, 5]. ST22-MRSA-IV (UK-EMRSA-15) and

ST36-MRSA-II (UK-EMRSA-16) occur in Spain [4, 5]. The European community-acquired MRSA (caMRSA) clone ST80-MRSA-IV is common in Greece [3, 6, 7]. On the other hand, some data indicate that there are Southern European epidemic strains which are not present in Central and Northern Europe. An example is a recently described Spanish epidemic clone (CC5, *spa* type t067, [8]), which has only been detected sporadically in central and Northern Europe, e.g. in returning travellers [1].

Similar to other Southern European countries, Malta has a very high prevalence of MRSA. This is clearly demonstrated by EARSS data, which indicate an oxacillin/methicillin resistance rate of ca. 55% among invasive *S. aureus* isolates recovered from patients in Maltese hospitals in 2008 and 2009 (http://www.rivm.nl/earss/database/) and by a report of the Maltese Department of Health (http://www.slh.gov.mt/ICUnit/pdf/anti_resist_report.pdf). However, to our knowledge, there is only one published study describing typing data of MRSA isolates from Malta [9], indicating the presence of ST22-MRSA-IV, of ST36-MRSA-II and of another strain which had a novel pulsed field gel electrophoresis (PFGE) pattern, but which was not described in further detail.

In order to gain a better understanding of the Maltese MRSA population, the present study aimed to perform a comprehensive molecular characterisation of Maltese MRSA isolates recovered from patients in the Mater Dei Hospital in Msida, Malta, over a 4-month period, using diagnostic DNA microarrays. These arrays have been described previously in detail [1, 10]. They allow the extensive characterisation of *S. aureus* isolates by detecting the carriage of virulence and resistance genes, as well as of a number of core genome genes. The DNA microarray also assigns isolates to multilocus sequence typing (MLST) clonal complexes based on the analysis of their hybridisation patterns [1, 10].

Materials and methods

Patients and isolates

Mater Dei Hospital is the only tertiary care hospital on the island of Malta, with 850 beds and around 90% bed occupancy, although in the general medical wards, the beds are always full, especially in the winter months. It provides services for an estimated catchment population of about 350,000. Mater Dei Hospital is a new hospital that started functioning in November 2007 following a move from the older hospital (St. Luke's Hospital).

Between October 2008 and January 2009, 45 MRSA isolate were randomly collected from the Bacteriology Laboratory of Mater Dei Hospital. These were submitted for genotyping at the Institute for Medical Microbiology and Hygiene, Dresden, Germany. All isolates were recovered from different patients (15 females and 30 males). These patients included five paediatric patients in a neonatal intensive care unit (n=3) and paediatric ward (n=2). All other isolates were recovered from adults. All but four of the isolates recovered from adults were in-patients; three were from an adult intensive care unit, one from a burns unit, 14 from medical wards, ten from general surgical wards, five from orthopaedic wards, two from urology wards and from one ENT (ear, nose and throat) ward. The three out-patient cases consisted of two renal patients, who frequently attend the hospital for dialysis, and a patient undergoing cardiac catheterisation. One isolate was recovered from a hospital employee. The isolates were from various specimens, including 35 clinical samples (20 wound swabs, five sputa, four blood cultures, three urines, two aspirates and one eye swab) and ten screening samples. One methicillin-susceptible *S. aureus* (MSSA) isolate was included because it was initially tested to be methicillin-resistant and because of similarity to one of the MRSA strains (see below).

S. aureus were identified by first performing a Gram stain on the cultured isolate. Gram-positive cocci were followed up with catalase, coagulase and DNAse tests. Screening specimens for MRSA were plated on MRSA selective medium (Oxoid) and followed-up according to the manufacturer's recommendations. Sensitivity testing was performed using VITEK (bioMérieux) cards. Confirmation of MRSA was done with the PBP2' latex agglutination method (Denka Seiken Co., Ltd.). MRSA strains were stored frozen at −70°C using cryobeads (Mast Diagnostics).

Details on the isolates are summarised in Supplement 1.

Array procedures

Staphylococcal cultures were grown overnight on Columbia blood agar. The culture material was then treated with lysostaphin, lysozyme and ribonuclease A. Following this, all samples were treated with proteinase K and chromosomal DNA was purified using Qiagen's EZI DNA tissue kit according to the manufacturer's instructions.

For the characterisation of isolates, microtitre stripe-mounted DNA microarrays (ASP system by CLONDIAG) were used, which covered 334 target sequences. This included species markers, resistance and exotoxin genes, genes encoding microbial surface components recognising adhesive matrix molecules of the host (MSCRAMMs), as well as SCC*mec*, capsule and *agr* group typing markers. The target genes, the principle of the assay used for this study, as well as related procedures have been described previously in detail [1, 10]. Protocols are provided by the manufacturer (CLONDIAG).

All targets were amplified in a linear primer elongation reaction using a single primer per target only in order to

facilitate a multiplex reaction covering all targets simultaneously. Within this step, biotin-16-dUTP was incorporated into the amplicons, which then were hybridised to the array. This was followed by washing and blocking. Horseradish-peroxidase-streptavidin conjugate and, after incubation and washing, Seramun Green precipitating dye (Seramun, Heidesee, Germany) were added. An image of the array was recorded and analysed using a designated reader and software (Arraymate, Iconoclust, both by CLONDIAG). Raw data were interpreted as 'positive,' 'negative' or 'ambiguous' using a previously described algorithm [10].

Clonal complexes were determined by comparison of the array results to those of a collection of previously characterised and MLST-typed reference strains (for details, see [10]).

Epidemic strains (ST22-MRSA-IV, ST36-MRSA-II, PVL-positive ST80-MRSA-IV, ST228-MRSA-I and ST239-MRSA-III) were identified by comparison to this set of reference strains [10] and to other previously characterised isolates [1, 11–13] with special regard to CC background and SCC*mec*-associated genes. An additional epidemic strain belonging to CC5 has not yet been characterised using that method, and is described below.

DNA isolation for use in PCRs for the characterisation of SCC*mec* elements and *spa* typing

Chromosomal DNA was prepared from each isolate using the Qiagen DNeasy blood and tissue kit system according to the manufacturer's instructions.

PCRs for the characterisation of SCC*mec* elements

Ten CC5 MRSA isolates were identified among the 45 Maltese MRSA isolates investigated during the present study. Using the microarray, all CC5 isolates yielded hybridisation signals indicating the presence of SCC*mec* IV, but also yielded hybridisation signals for an additional set of *ccr* genes (*ccrA3*) and a putative fusidic acid resistance gene (Q6GD50), the latter of which (GenBank BX571857.1, locus tag SAS0043) has previously only been described from a non-*mecA* staphylococcal chromosomal cassette (SCC) element identified in the MSSA strain MSSA476. The SCC*mec* elements of two CC5 Maltese MRSA isolates, representative of those CC5 isolates that yielded similar combinations of hybridisation signals for SCC*mec*, were further characterised using PCR. SCC*mec* typing was performed on these two isolates using four multiplex PCRs to identify: (i) the *mec* complex type (class A, B and C *mec*, [14]), (ii) the *ccr* complex type (*ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4* and *ccrC*, [14]), (iii) the J regions [15] and (iv) the SCC*mec* IV subtype (SCC*mec* IVa–IVh, [16]). The MRSA SCC*mec* typing control isolates described previously [17] were used in the present study as positive controls for multiplex PCRs (i)–(iii) described above. The following *S. aureus* control strains and clinical isolates were used as positive controls for SCC*mec* IV subtyping: CA05 (IVa) [18], 8/63P (IVb) [18], JCSC4788 (IVc) [19], JCSC4469 (IVd) [19], isolate M04/0177 (IVg) and isolate E1749 (IVh).

Primer names and sequences are provided in Table 1. Primers were designed to amplify and sequence the *ccrA3*, *ccrB3* and the putative fusidic acid resistance gene Q6GD50 based on previously published sequences. Amplimers were obtained for *ccrB3* and the putative fusidic acid resistance gene only and were sequenced to confirm their identities using the PCR primers. Analysis of chromatograms and sequences was performed using the BioNumerics software package (version 5.1; Applied Maths, Ghent, Belgium) and homology searches were performed using BLAST software (http://ncbi.nih.gov/BLAST). Additional primers (fw_ccrA3 cons and rv_ccrB5_5p, Table 1) were designed to amplify and sequence a specific fragment of the novel *ccrA* gene identified among CC5 isolates. The entire SCC*mec* IV element of both of these isolates was amplified using a combination of previously described and newly designed primers, to determine if the target sequences for the additional amplimers were located within the SCC*mec* element (Table 1).

The left junction region to *ccrA/B-2* and *ccrA/B-2* to *mecA* were amplified using primer pairs IRLFIVa/ccrA2IVaR and ccrA2IVaF/mecAR, respectively (Table 1). Template DNA was amplified from both isolates using these primer pairs and the Expand Long Template PCR System (Roche) according to the manufacturer's instructions with an annealing temperature of 50°C and extension time of 10 min. The *mecA* to *orfX* region was amplified using primer pairs mecAF/ISmecR, ISmecF/dcsR and dcsF/orfX (Table 1). Template DNA was amplified from both isolates using these primer pairs and GoTaq Flexi DNA polymerase (Promega) according to the manufacturer's instructions with an annealing temperature of 50°C and extension time of 2 min for primer pairs ISmecF/dcsR and dcsF/orfX and 4 min for primer pair dcsF/orfX. All PCR products were separated by agarose gel electrophoresis and the sizes of the amplimers were compared to those obtained by amplification using the same primers on template DNA from the SCC*mec* IVa control strain CA05.

Two primers, ccrXF and IRLIVaR (Table 1), based on the novel *ccr* gene identified in the present study and the left extremity of the SCC*mec* IVa element, respectively, were designed to investigate the location of the novel *ccrA/B* genes in the two representative CC5 isolates. Template DNA was amplified from both isolates using these primer pairs and the Expand Long Template PCR System (Roche) according to the manufacturer's instructions with an annealing temperature of 50°C and extension time of 10 min.

Table 1 Primers used for the characterisation of staphylococcal chromosomal cassette (SCC) elements

Target	Primer pair	Nucleotide sequence (5′–3′)	Nucleotide coordinates	Reference
ccrA3	ccrA3F ccrA3R	AGCTCCAAAGCACAATATTC GGTTCACAATGTTCAATGGT	5513–5532[a] 6750–6731[a]	This study
ccrB3	ccrB3F ccrB3R	GTCGGTATCTATGTTCGTGT TCGCAGAACATACTTTTGAG	5513–5532[a] 6750–6731[a]	This study
Putative fusidic acid resistance gene Q6GD50	Far476F Far476R	AGTAAAGCAGTTAGTATATC GATTATTTATATCATCTAGG	33–52[b] 610–591[b]	This study
SCC*mec* IVa left junction region to *ccrAB2*	IRLFIVa ccrAR	CTCTGCTTATCAGTTAATGA GCTTCGATAGCCTGTTTCTG	972–991[c] 9948–9968[c]	This study, [38]
SCC*mec* IVa *ccrAB2* to *mec*A	ccrA2IVaF mecAR	CAAGTTATAGGCTATTACG CCTATCTCATATGCTG	9894–9913[c] 18160–18145[c]	This study, [38]
SCC*mec* IVa *mecA* to IS431*mec*	mecAF ISmecR	GATTGGGATCATAGCGTCAT ACGGTGATCTTGCTCAATGA	18021–18040[c] 22780–22761[c]	[38]
SCC*mec* IVa IS431*mec* to *dcs*	ISmecF dcsR	CCTGACTGTCATTGTAC CGGTCATGGCTATGATTTAG	22724–22740[c] 23943–23924[c]	[38]
SCC*mec* IVa *dcs* to *orfX*	dcsF orfXR	GTCAATGAGATCATCTACAT CCCAAGGGCAAAGCGAC	23679–23698[c] 25395–25379[c]	[38]
Novel *ccr* to SCC*mec* IVa left junction	ccrxF IRLIVaR	GAACCAACCTATTAACAGG TCATTAACTGATAAGCAGAG	991–972[c]	This study
Conserved region of *ccrA-3* and 5′-end of the Maltese *ccrB* sequence	fw_ccrA3 cons rv_ccrB5_5p	TCATGATGGCTATTTTGATATGGA TCGAAATGATGAAAGTTACAATATTCT		This study

Nucleotide coordinates from: [a] SCC*mec* type III, accession number AB037671; [b] putative fusidic acid resistance gene Q6GD50 from MSSA476, accession number BX571857; [c] SCC*mec* type IVa, accession number AB063172

Additional primers were designed based on *ccrA3* from *S. aureus* (fw_ccrA3cons) and *ccrB5* from *S. pseudintermedius* (strain KM241, AM904731.1; rv_ccrB5_5p), facilitating the amplification and sequencing of a specific fragment of the novel *ccrA* gene (Table 1).

spa typing and MLST

spa typing was performed on the two CC5-MRSA isolates representative of those with unusual SCC*mec* amplimers, using the primers and thermal cycling conditions described by the European Network of Laboratories for Sequence Based Typing of Microbial Pathogens (SeqNet, http://www.seqnet.org). Analysis of *spa* sequences and assignment of *spa* types was performed using the Ridom StaphType software package, version 1.3 (Ridom GmbH, Wurzburg, Germany). MLST was performed as previously described [20] using the MLST website (http://saureus.mlst.net/) for result queries.

Results

Among the 45 Maltese MRSA isolates investigated, six distinct strains were identified using the DNA microarray. These were ST22-MRSA-IV (30/45, 66.7%), ST36-MRSA-II (2/45, 4.4%), ST80-MRSA-IV (1/45, 2.2%), ST228-MRSA-I (1/45, 2.2%), ST239-MRSA-III (1/45, 2.2%) and CC5-MRSA-IV (10/45, 22.2%). Complete hybridisation profiles are provided in Supplement 1.

ST22-MRSA-IV

The most common strain identified among the Maltese isolates was ST22-MRSA-IV, also known as UK-EMRSA-15 or the Barnim Epidemic Strain [21]. Thirty isolates belonged to that strain. The carriage of enterotoxins C and L (*sec*, *sel*), as well as of *ermC* (macrolide/lincosamide resistance), were variable, allowing the distinction of four different variants: [*sec/l*−, *ermC*−], $n=1$; [*sec/l*−, *ermC*+], $n=5$; [*sec/l*+, *ermC*−], $n=5$; and [*sec/l*+, *ermC*+], $n=19$.

The putative fusidic acid resistance gene Q6GD50 was detected in nine ST22-MRSA-IV isolates, seven of which belonged to the [*sec/l*+, *ermC*+] variant. Eight of these nine strains were phenotypically resistant, and one displayed intermediate resistance (Supplement 1).

With regard to other genes covered by the microarray, the Maltese ST22-MRSA-IV isolates were identical to previously described ST22-MRSA-IV isolates from Germany [1].

ST36-MRSA-II

The two isolates belonging to the ST36-MRSA-II strain UK-EMRSA-16 (USA200) carried an SCC*mec* II element. They harboured *sea* (enterotoxin A) and the *egc* enterotoxin

locus (*seg*, *sei*, *sem*, *sen*, *seo*, *seu*). One isolate was also positive for *tst1* (toxic shock toxin). Both isolates yielded hybridisation signals for *sdrD* (serine aspartate repeat protein D). Apart from *tst1* and *sdrD*, the Maltese isolates were identical to the sequenced ST36-MRSA-II strain Sanger MRSA252 (GenBank NC_002952/BX571856, [22]).

ST80-MRSA-IV

The one isolate belonging to the PVL-positive ST80-MRSA-IV European caMRSA strain was isolated from a hospital employee who had no patient contact and no known travel history to continental Europe. The isolate carried beta-lactamase (*blaZ*), neomycin, streptothricin, fusidic acid and tetracycline resistance genes (*aphA3*, *sat*, *far1*, *tetK*). Virulence factors identified included *lukS/F-PV*, *edinB* (epithelial cell differentiation inhibitor) and *etd* (exfoliative toxin D). With regard to the other genes, the isolate was identical to previous descriptions of ST80-MRSA-IV [1, 11, 13].

ST228-MRSA-I

One isolate belonged to ST228-MRSA-I (also known as the South German Epidemic Strain or Italian Clone) matching previously described isolates from Germany [1]. It harboured an SCC*mec* type I element, the beta-lactamase operon, *aphA3*, *sat*, *aacA–aphD* (gentamicin resistance) and *qacA* (an unspecific efflux pump associated with, among others, quaternary ammonium resistance). Contrary to a high proportion of ST228-MRSA-I isolates [1], it lacked a mercury resistance operon. It harboured leukocidin genes *lukD/E* and an un-truncated *egc* enterotoxin gene locus, although both genes in ST228-MRSA-I are commonly affected by deletions [1, 12]. It was also positive for *sea*, although other genes which are usually associated with beta-haemolysin converting phages (staphylokinase, *sak*, staphylococcal complement inhibitor, *scn* and chemotaxis inhibitory protein, *chp*, [23, 24]) were absent.

ST239-MRSA-III

The single isolate belonging to ST239-MRSA-III (Vienna/Hungarian/Brazilian Epidemic Strain) carried an SCC*mec* III element, the mercury resistance operon and *ccrC*. It was also positive for the beta-lactamase operon, *ermA*, *aacA–aphD*, *aphA3*, *sat*, as well as for the tetracycline resistance genes *tetK* and *tetM*. It harboured three enterotoxin genes, *sea*, *sek* and *seq*. Otherwise, it displayed a typical hybridisation pattern as previously described for that strain [1, 13], which was in accordance to the concept that it emerged from some kind of recombination event from CC8 and CC30 parent strains [25].

CC5-MRSA

Ten MRSA isolates belonged to clonal complex 5 SCC*mec* type IV MRSA. Since they differed from previously known CC5 epidemic strains (see the Discussion section), they are described in detail here. In addition, one isolate was completely identical, in terms of toxin gene carriage, to the ten CC5-MRSA isolates, but lacked all SCC*mec*-associated genes, including *mecA*. As it was originally resistant to oxacillin, it was assumed that this isolate lost its SCC*mec* element during in vitro passage.

All CC5 isolates were PVL-negative. They all carried *sea* and the *egc* enterotoxin locus. Nine out of ten CC5 MRSA isolates harboured *tst1*, *sec* and *sel*. Genes *sak* and *scn* were present, whereas *chp* was absent. No CC5 isolates harboured genes encoding exfoliative toxins (*eta*, *etb*, *etd*) or epithelial cell differentiation inhibitors (*edinA*, *edinB*, *edinC*), and the arginine catabolic mobile element ACME (*arcA/B/C/D*) was always absent. The carriage of leukocidin genes *lukD/E*, staphylococcal exotoxin-like/superantigen-like genes (*ssl/set*), *hsdS* genes (type I site-specific DNAse) and of protease genes (*aur*, *splA*, *splB*, *sspA*, *sspB*, *sspP*) was in accordance to CC5, as represented by sequenced strains Mu50 (NC_002774/AP003367, [26]) and N315 (NC_003140/AP003139, [26]) and by previously hybridised strains [1, 10]. The *agr* group was II and the capsule type was 5. MSCRAMM genes (*bbp*, *clfA*, *clfB*, *ebh*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *map*, *sasG*, *sdrC*, *sdrD* and *vwb*) were all present and allelic variants were identical to other CC5 strains [1, 10], but the *tst1/sec/sel*-negative isolate also lacked the gene encoding the bone-binding protein *bbp*.

The ten CC5-MRSA isolates carried *mecA*, *delta mecR*, *ugpQ*, *dcs-Q9XB68*, *ccrA2* and *ccrB2*, indicating the presence of SCC*mec* IV, as well as the beta-lactamase operon (*blaZ*, *blaR*, *blaI*). Two isolates were positive for *ermC* and in one isolate, *tetK* was detected. In addition to the SCC*mec* IV element, all ten CC5-MRSA isolates yielded hybridisation signals with probes for *ccrA3* (but not with *ccrB3*), as well as with the putative fusidic acid resistance element Q6GD50. This corresponded to phenotypic resistance in seven and intermediate resistance in two isolates (Supplement 1). This CC5-MRSA strain also yielded irregular/ambiguous signals, with one out of two probes specific for *ccrB1*. The CC5-MSSA isolate was negative for the SCC*mec* IV element and did not yield signals with *ccrA3*, *ccrB1* or Q6GD50 probes.

Two of the CC5-MRSA isolates with unusual combinations of SCC-associated hybridisation signals were further investigated. The two isolates were identified as *spa* type t002. One of them was subjected to MLST and yielded ST149. SCC*mec* typing confirmed the presence of SCC*mec* IVa (*ccrAB2*, class A *mec*, *dcs* and J1 region IVa). The *ccrAB2* amplimer obtained for both isolates using SCC*mec*

typing PCR was sequenced to confirm its identity and was found to have 100% homology to the same region of *ccrAB2* from previously described SCC*mec* II and IV elements. No significant size variation was observed in the mobility of amplimers obtained following amplification of the entire SCC*mec* IVa element compared to the sizes expected with typical SCC*mec* IVa using overlapping primer pairs. All amplimers obtained were of the expected size, which rules out major insertions within the SCC*mec* IVa element.

While CC5-MRSA isolates yielded hybridisation signals with *ccrA3* (but not with *ccrB3*), PCR yielded amplimers for *ccrB3* but not for *ccrA3*. The *ccrB3* amplimers from the two isolates were sequenced and they were found to be 100% identical to each other and similar to *ccrB5* from *S. pseudintermedius*. Thus, additional primers were designed based on *ccrA3* and *ccrB5* (fw_ccrA3cons, rv_ccrB5_5p), facilitating the amplification and sequencing of a fragment of the *ccrA3* gene. Altogether, a 2,030-bp sequence was obtained (GenBank accession number GU066221 and Supplement 2). BLAST analysis revealed that the sequence of this amplimer had 99% identity to the cassette chromosome recombinase genes *ccrASHP/ccrBSHP* recently described from an ACME-positive *S. haemolyticus* strain H9 from China (GenBank accession number EU934095.1, [27]). Other similar sequences included *ccrA/B5* genes from an *S. pseudintermedius* SCC*mec* cassette (strain KM241, AM904731.1 and KM1381, AM904732.1, with 85%, or, respectively 82%, identity) and *S. aureus* SCC*mec* III cassettes (DQ196432.1, AB037671.1, AB047088.2, AB014437.1, AB014436.1, with 82% identity). Binding sites of primer/probe used in array hybridisations were localised in non-homologous parts of the *ccrB* sequence, explaining the absence of hybridisation on the array. Sequences and alignments are provided in Supplement 2.

Sequencing of the amplimers obtained following the amplification of an internal region of the putative fusidic acid resistance genes of these two isolates revealed that they were identical to each other and showed 100% identity to HDE288 (SCC*mec* VI strain, formerly SCC*mec* IV, GenBank AF411935, bases 765-203). This region of SCC*mec* of HDE288 has not been annotated and there is no description of a fusidic acid resistance element in this strain. In addition, the amplimers also showed 99% identity to a putative fusidic acid resistance gene in MSSA476 (BX571857.1:SAS0043, bases 52860–53422). Two additional primers were designed based on the sequenced *ccrB3* amplimer (ccrxF) and the left SCC*mec* IVa junction region (IRLIVaR) in order to determine if the additional *ccr* genes were located adjacent to SCC*mec*. They did not yield any amplimers. PCRs with primers based on *ccrA3/ccrB5* and the putative fusidic acid resistance gene Q6GD50 also did not yield amplimers.

Discussion

DNA microarray analysis of 45 Maltese MRSA isolates revealed the presence of six distinct strains, with ST22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA) predominating, accounting for 66.7% of MRSA isolates investigated. This appears to be an extremely successful, pandemic strain which has spread worldwide among humans and domestic animals [28–30]. This strain has been identified previously in Malta, and it was found to be the most common strain there [9]. Frequent travel between the United Kingdom [9] or other European countries such as Germany or Ireland and Malta could be a reason for the importation of this strain into Malta. The presence of ST36-MRSA-II (UK-EMRSA-16, [31, 32]) may also indicate an epidemiological connection between the UK and Malta [9].

The importation of ST239-MRSA-III could essentially have occurred from any place in Europe, the Mediterranean region or Asia, as this strain is widespread. ST228-MRSA-I have been described from Germany and Italy, and many tourists travel from both of these countries to Malta.

An interesting observation was the detection of a PVL-positive ST80-MRSA-IV in a Maltese hospital employee. This is among the first published cases of PVL-MRSA in Malta after Larsen et al. [33] mentioned the importation of this strain from Malta to Denmark. It fits into a series of observations of infections with that strain in Mediterranean countries to which either Europeans travel for vacation or from which people immigrate to Europe [33, 34].

The most interesting observation of this study was the description of a distinct strain belonging to CC5. This strain was most similar to the Paediatric Clone (ST5-MRSA-IV), but differed in toxin gene carriage and by the presence of an additional set of recombinase genes and a putative fusidic acid resistance gene [1, 13]. It also differed from a Spanish epidemic strain [8] with regard to sequence type, *spa* type and carriage of resistance genes. A closely related strain (a PVL- and *tst1*-positive ST149-MRSA-IV) has been isolated from a Libyan patient in Switzerland [35]. Another similar strain (the Geraldine Clone) has been described from France and Switzerland [36, 37]. This was also a *tst1*-positive, CC5-MRSA which was phenotypically resistant to fusidic acid. However, it differed in enterotoxin carriage (*sea*-negative but *sed*-positive) and SCC*mec* type [36].

The most distinguishing feature of the Maltese CC5-MRSA-IV isolates is the presence of an additional set of recombinase genes. Sequencing revealed that it harboured a second set of *ccrA/B* genes in addition to *ccrA/B*2 from its SCC*mec* IV element. Their sequences are virtually identical to *ccrASHP/ccrBSHP* genes from an ACME-positive *S. haemolyticus* strain from China (GenBank accession number EU934095.1, [27]). This might indicate gene transfer from *S. haemolyticus* to *S. aureus* (or vice versa). However,

ACME genes were not detectable in the Malta strain. The second set of *ccrA/B* genes were found not to be located within the SCC*mec* IVa element. Also, a direct link between the putative fusidic acid resistance gene and the additional *ccrA/B* genes was not proven, as PCRs using primers to amplify from *ccrA/B* to Q6GD50 yielded no amplimers. Thus, a possible presence of a second SCC element harbouring the fusidic acid genes cannot be proven yet. However, the detection of a putative deletion mutant which lacks SCC*mec* IV, the second set of *ccrA/B* and Q6GD50 suggests a link between these genes.

The fusidic acid resistance gene Q6GD50 was not only found in the Maltese CC5-MRSA-IV strain, but also in nine out of 30 ST22-MRSA-IV isolates from Malta. Its presence, indeed, correlated with phenotypic resistance to fusidic acid (Supplement 1). A higher rate of carriage of a particular resistance gene might be related to higher selective pressure. Gene movement/acquisition is a random process having neither direction nor purpose. Selective pressure, such as the presence of an antibiotic drug, can provide a direct selective advantage to sub-populations of bacterial cells that have acquired a resistance gene by chance. Thus, a clone that has acquired a fusidic acid resistance gene might spread in an environment characterised by a widespread therapeutic use of this drug. Fusidic acid is licensed in Malta for intravenous application and anecdotal evidence suggests high use and abuse of fusidic acid topical formulations (M. Borg, unpublished observation). In other places where this drug is not used extensively, such a gene transfer might also occur. Since the resulting variant strain would have no survival advantage over other strains, it might either remain rare or disappear altogether. Indeed, Q6GD50 has not yet been observed in German [1] or Irish (A. Shore, unpublished observation) isolates that have been processed through the same DNA microarray.

In conclusion, this study has provided a detailed analysis of the molecular characteristics of MRSA in Malta. Similar to some other studies, our study suggests that, in addition to the presence of pandemic strains such as ST239-MRSA-III or ST22-MRSA-IV, there are also locally important MRSA clones restricted to certain regions. Other examples of such endemic clones include the Spanish t067 clone [8] or the Geraldine Clone in France [36]. In addition to strain properties (such as growth rate, presence of virulence factors etc.), the geographically restricted spread of certain clones might be determined by mere accident or by the size and habits of a host population in which the strain first appeared. However, the factors which determine whether a clone remains restricted to a region like Malta or achieves pandemic spread are largely unknown.

Acknowledgements The authors want to thank Antje Ruppelt, Hanna Kanig (TU Dresden), Elke Müller, Ines Engelmann and Jana Sachtschal (CLONDIAG) for their excellent technical assistance, as well as Dr. Wolfram Rudolph for the sequencing. We acknowledge the staff of the Bacteriology Laboratory of Mater Dei Hospital and the Infection Control Unit of Mater Dei Hospital for providing the isolates. *Staphylococcus aureus* control isolates for SCC*mec* typing were kindly provided by Prof. Teruyo Ito, Juntendo University, Japan, Prof. Herminia de Lencastre, Rockefeller University, New York, USA, and Prof. Robert Daum, University of Chicago, USA. We acknowledge Prof. E. Jacobs for his support.

Ralf Ehricht and Peter Slickers are employees of CLONDIAG. The authors declare no other conflicting interests.

References

1. Monecke S, Jatzwauk L, Weber S, Slickers P, Ehricht R (2008) DNA microarray-based genotyping of methicillin-resistant *Staphylococcus aureus* strains from Eastern Saxony. Clin Microbiol Infect 14:534–545
2. Mato R, Campanile F, Stefani S, Crisóstomo MI, Santagati M, Sanches SI, de Lencastre H (2004) Clonal types and multidrug resistance patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) recovered in Italy during the 1990s. Microb Drug Resist 10:106–113
3. Aires de Sousa M, Bartzavali C, Spiliopoulou I, Sanches IS, Crisóstomo MI, de Lencastre H (2003) Two international methicillin-resistant *Staphylococcus aureus* clones endemic in a university hospital in Patras, Greece. J Clin Microbiol 41:2027–2032
4. Cuevas O, Cercenado E, Bouza E, Castellares C, Trincado P, Cabrera R, Vindel A (2007) Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Spain: a multicentre prevalence study (2002). Clin Microbiol Infect 13:250–256
5. Alcoceba E, Mena A, Cruz Pérez M, Ruiz de Gopegui E, Padilla E, Gil J, Ramírez A, Gallegos C, Serra A, Pérez JL, Oliver A (2007) Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Majorcan hospitals: high prevalence of the epidemic clone EMRSA-15. Clin Microbiol Infect 13:599–605
6. Vourli S, Perimeni D, Makri A, Polemis M, Voyiatzi A, Vatopoulos A (2005) Community acquired MRSA infections in a paediatric population in Greece. Euro Surveill 10:78–79
7. Chini V, Petinaki E, Foka A, Paratiras S, Dimitracopoulos G, Spiliopoulou I (2006) Spread of *Staphylococcus aureus* clinical isolates carrying Panton-Valentine leukocidin genes during a 3-year period in Greece. Clin Microbiol Infect 12:29–34
8. Pérez-Vázquez M, Vindel A, Marcos C, Oteo J, Cuevas O, Trincado P, Bautista V, Grundmann H, Campos J (2009) Spread of invasive Spanish *Staphylococcus aureus spa*-type t067 associated with a high prevalence of the aminoglycoside-modifying enzyme gene *ant(4')-Ia* and the efflux pump genes *msrA/msrB*. J Antimicrob Chemother 63:21–31
9. Gould SW, Rollason J, Hilton AC, Cuschieri P, McAuliffe L, Easmon SL, Fielder MD (2008) UK epidemic strains of meticillin-resistant *Staphylococcus aureus* in clinical samples from Malta. J Med Microbiol 57:1394–1398
10. Monecke S, Slickers P, Ehricht R (2008) Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. FEMS Immunol Med Microbiol 53:237–251
11. Monecke S, Slickers P, Hotzel H, Richter-Huhn G, Pohle M, Weber S, Witte W, Ehricht R (2006) Microarray-based characterisation of a Panton-Valentine leukocidin-positive community-acquired strain of methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect 12:718–728

12. Monecke S, Ehricht R, Slickers P, Wiese N, Jonas D (2009) Intra-strain variability of methicillin-resistant *Staphylococcus aureus* strains ST228-MRSA-I and ST5-MRSA-II. Eur J Clin Microbiol Infect Dis (in press)
13. Monecke S, Berger-Bächi B, Coombs G, Holmes A, Kay I, Kearns A, Linde HJ, O'Brien F, Slickers P, Ehricht R (2007) Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin. Clin Microbiol Infect 13:236–249
14. Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, Etienne J, Hiramatsu K (2007) Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. Antimicrob Agents Chemother 51:264–274
15. Oliveira DC, de Lencastre H (2002) Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 46:2155–2161
16. Milheiriço C, Oliveira DC, de Lencastre H (2007) Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* type IV in methicillin-resistant *Staphylococcus aureus*: 'SCC*mec* IV multiplex'. J Antimicrob Chemother 60:42–48
17. Shore AC, Rossney AS, O'Connell B, Herra CM, Sullivan DJ, Humphreys H, Coleman DC (2008) Detection of staphylococcal cassette chromosome *mec*-associated DNA segments in multi-resistant methicillin-susceptible *Staphylococcus aureus* (MSSA) and identification of *Staphylococcus epidermidis ccrAB4* in both methicillin-resistant *S. aureus* and MSSA. Antimicrob Agents Chemother 52:4407–4419
18. Ma XX, Ito T, Tiensasitorn C, Jamklang M, Chongtrakool P, Boyle-Vavra S, Daum RS, Hiramatsu K (2002) Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrob Agents Chemother 46:1147–1152
19. Ma XX, Ito T, Chongtrakool P, Hiramatsu K (2006) Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant *Staphylococcus aureus* strains isolated in Japanese hospitals from 1979 to 1985. J Clin Microbiol 44:4515–4527
20. 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
21. Witte W, Enright M, Schmitz FJ, Cuny C, Braulke C, Heuck D (2001) Characteristics of a new epidemic MRSA in Germany ancestral to United Kingdom EMRSA 15. Int J Med Microbiol 290:677–682
22. Holden MTG, Feil EJ, Lindsay JA, Peacock SJ, Day NPJ, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabbinowitsch E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whitehead S, Barrell BG, Spratt BG, Parkhill J (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci USA 101:9786–9791
23. Coleman DC, Sullivan DJ, Russell RJ, Arbuthnott JP, Carey BF, Pomeroy HM (1989) *Staphylococcus aureus* bacteriophages mediating the simultaneous lysogenic conversion of beta-lysin, staphylokinase and enterotoxin A: molecular mechanism of triple conversion. J Gen Microbiol 135:1679–1697
24. van Wamel WJB, Rooijakkers SHM, Ruyken M, van Kessel KPM, van Strijp JAG (2006) The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. J Bacteriol 188:1310–1315
25. Robinson DA, Enright MC (2004) Evolution of *Staphylococcus aureus* by large chromosomal replacements. J Bacteriol 186:1060–1064
26. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, Lian J, Ito T, Kanamori M, Matsumaru H, Maruyama A, Murakami H, Hosoyama A, Mizutani-Ui Y, Takahashi NK, Sawano T, Inoue R, Kaito C, Sekimizu K, Hirakawa H, Kuhara S, Goto S, Yabuzaki J, Kanehisa M, Yamashita A, Oshima K, Furuya K, Yoshino C, Shiba T, Hattori M, Ogasawara N, Hayashi H, Hiramatsu K (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet 357:1225–1240
27. Pi B, Yu M, Chen Y, Yu Y, Li L (2009) Distribution of the ACME-*arcA* gene among meticillin-resistant *Staphylococcus haemolyticus* and identification of a novel *ccr* allotype in ACME-*arcA*-positive isolates. J Med Microbiol 58:731–736
28. Grinberg A, Kingsbury DD, Gibson IR, Kirby BM, Mack HJ, Morrison D (2008) Clinically overt infections with methicillin-resistant *Staphylococcus aureus* in animals in New Zealand: a pilot study. N Z Vet J 56:237–242
29. Walther B, Monecke S, Ruscher C, Friedrich AW, Ehricht R, Slickers P, Soba A, Wleklinski CG, Wieler LH, Lübke-Becker A (2009) Comparative molecular analysis substantiates zoonotic potential of equine methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 47:704–710
30. Walther B, Wieler LH, Friedrich AW, Hanssen AM, Kohn B, Brunnberg L, Lübke-Becker A (2008) Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from small and exotic animals at a university hospital during routine microbiological examinations. Vet Microbiol 127:171–178
31. Johnson AP, Aucken HM, Cavendish S, Ganner M, Wale MC, Warner M, Livermore DM, Cookson BD (2001) Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). J Antimicrob Chemother 48:143–144
32. [No authors listed] (1993) EMRSA-16: a new epidemic strain of *Staphylococcus aureus*. Commun Dis Rep CDR Wkly 3:25
33. Larsen AR, Böcher S, Stegger M, Goering R, Pallesen LV, Skov R (2008) 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 46:62–68
34. Maier J, Melzl H, Reischl U, Drubel I, Witte W, Lehn N, Linde H (2005) Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* in Germany associated with travel or foreign family origin. Eur J Clin Microbiol Infect Dis 24:637–639
35. Francois P, Harbarth S, Huyghe A, Renzi G, Bento M, Gervaix A, Pittet D, Schrenzel J (2008) Methicillin-resistant *Staphylococcus aureus*, Geneva, Switzerland, 1993–2005. Emerg Infect Dis 14:304–307
36. Dauwalder O, Lina G, Durand G, Bes M, Meugnier H, Jarlier V, Coignard B, Vandenesch F, Etienne J, Laurent F (2008) Epidemiology of invasive methicillin-resistant *Staphylococcus aureus* clones collected in France in 2006 and 2007. J Clin Microbiol 46:3454–3458
37. Durand G, Bes M, Meugnier H, Enright MC, Forey F, Liassine N, Wenger A, Kikuchi K, Lina G, Vandenesch F, Etienne J (2006) Detection of new methicillin-resistant *Staphylococcus aureus* clones containing the toxic shock syndrome toxin 1 gene responsible for hospital- and community-acquired infections in France. J Clin Microbiol 44:847–853
38. Shore A, Rossney AS, Keane CT, Enright MC, Coleman DC (2005) Seven novel variants of the staphylococcal chromosomal cassette *mec* in methicillin-resistant *Staphylococcus aureus isolates* from Ireland. Antimicrob Agents Chemother 49:2070–2083