Identification of a distinctive phenotype for endocarditis-associated clonal complex 22 MRSA isolates with reduced vancomycin susceptibility

Helene Marbach,‡ Eve Boakes,§ Steven Lynham, Malcolm Ward, Jonathan A. Otter, and Jonathan D. Edgeworth

Abstract

Purpose. We previously identified an association between CC22 meticillin-resistant Staphylococcus aureus (MRSA) bloodstream infection isolates with an elevated vancomycin MIC (V-MIC) in the susceptible range (1.5–2 mg l\(^{-1}\)) and endocarditis. This study explores whether these isolates have a specific phenotype consistent with the clinical findings.

Methodology. CC22 and CC30 MRSA isolates with high (1.5–2 mg l\(^{-1}\)) and low (≤0.5 mg l\(^{-1}\)) V-MICs were tested for fibrinogen and fibronectin binding, virulence in a Galleria mellonella caterpillar model, phenol soluble modulin production and accessory gene regulator (agr) expression.

Results. CC22 high V-MIC, but not CC30 high V-MIC isolates, showed sustained fibrinogen binding through a stationary growth phase and increased PSM production, specifically PSM\(_\alpha\), compared with respective low V-MIC isolates. Expression was lower in both CC22 and CC30 high V-MIC isolates compared with respective low V-MIC isolates, although there was no associated reduction in virulence in the caterpillar model.

Conclusions. The identification of a distinct phenotype for CC22 high V-MIC isolates supports the hypothesis that bacterial factors contribute to the mechanism underlying their association with endocarditis. Further study of these isolates could shed light on the molecular mechanism of endocarditis in humans.

INTRODUCTION

Staphylococcus aureus infection has a range of consequences from asymptomatic colonization to bloodstream infection (BSI) with haematogenous complications, of which endocarditis is probably the most devastating [1]. There has been interest in understanding whether strains causing severe infections such as endocarditis have distinct genotypic or phenotypic characteristics [2–6]. Clonal complex (CC) 30 meticillin-resistant S. aureus (MRSA) [3, 4] and other MRSA CCs [5] have been associated with endocarditis, whereas other studies have found no association between clones and endocarditis [6].

Vancomycin-intermediate S. aureus (VISA) (4–8 mg l\(^{-1}\)) and heteroresistant (h)VISA isolates have also been associated with endocarditis [1–3, 7]. VISA and hVISA isolates have multiple phenotypic and metabolic differences compared with susceptible isolates, such as reduced autolysis and gradual thickening of the cell wall, changes in global gene regulators including down-regulation of agr, increased expression of surface adhesins that bind fibrinogen and fibronectin, and attenuated virulence [3, 8–13]. There is also clinical evidence that VISA and hVISA isolates are less virulent, with lower acute mortality and less shock reported in some studies [14, 15]. These data support a hypothesis that molecular changes associated with reduced vancomycin susceptibility result in decreased acute virulence but an increased ability to cause distal focal infections, particularly endocarditis. It may be that the fitness cost of reduced susceptibility to vancomycin is reduced acute virulence, but this allows bacteria to survive in the body for longer and increases the chance of distal focus infections. Given that
vancomycin is the mainstay of therapy for MRSA BSIs [8, 9], the association of VISA and hVISA with endocarditis may also result from prolonged bacteraemia on vancomycin therapy, particularly in the setting of low serum vancomycin levels.

In a previous study, we found evidence through analysis of collection of 821 consecutive MRSA BSI isolates that CC22 isolates with an elevated vancomycin (V)-MIC within the susceptible range (1.5–2 mg l\(^{-1}\)) were strongly associated with endocarditis [2]. This suggests that both lineage-associated characteristics of CC22 and a consequence of reduced vancomycin susceptibility, either bacterial or treatment-related, contribute to the risk of developing endocarditis. The relative contribution of bacterial pathogenic factors versus reduced effectiveness of vancomycin therapy in explaining the association between MRSA isolates with reduced vancomycin susceptibility and endocarditis is unclear. The aim of this study was to investigate whether CC22 isolates with a high V-MIC have a distinct phenotype by using a series of laboratory assays to compare various components of staphylococcal disease pathogenesis, including binding, toxin production, gene regulation and in vivo virulence.

METHODS

A total of 16 isolates from BSIs were selected from a collection defined in a previous study [2] (Table 1): four CC22 high V-MIC (defined as V-MIC 1.5 mg l\(^{-1}\); hereafter 'CC22-high'), four CC30 high V-MIC (hereafter 'CC30-high'), four CC22 low V-MIC (defined as V-MIC 0.38–0.5 mg l\(^{-1}\); hereafter 'CC22-low') and four CC30 low V-MIC (hereafter 'CC30-low'); V-MIC was measured by E-test (AB Biodisk). All isolates in the collection had a V-MIC within the susceptible range (<4 mg l\(^{-1}\)) and there were no hVISA isolates [2]. S. aureus (Newman) was used as positive control for fibrinogen binding and S. aureus (8325-4) was used as a positive control for fibronectin binding (both kindly supplied by R. Massey, Bath, UK).

Fibrinogen and fibronectin binding assay

The assay was adapted from Christensen et al. [16]. Fibrinogen (Sigma-Aldrich) and fibronectin (Merck Chemicals) from human plasma were dissolved in PBS (Sigma-Aldrich) to a concentration of 10 mg l\(^{-1}\). Ninety-six-well flat-bottomed polystyrene microtitre plates (Nunc; Fisher Scientific) were coated with 100 µl of each of the protein solutions at 4 °C overnight. Wells were rinsed twice with PBS, blocked with 2 % BSA (Sigma-Aldrich) at 37 °C for 1 h then washed twice with PBS. Bacteria were cultured in 10 ml tryptic soy broth (TSB; Oxoid) with 1 % glucose at 225 g at 37 °C to stationary phase overnight. For stationary phase binding, an overnight cell suspension was adjusted with TSB/10 % glucose to an OD\(_{570}\) nm of 1.0, corresponding to approximately \(10^8\) c.f.u. ml\(^{-1}\), and 100 μl inoculated in each well. For exponential phase binding, the overnight cell suspension was diluted 1:100, grown at 37 °C for 2 h, adjusted to an OD\(_{570}\) nm of 1.0, and 100 μl was inoculated in each well. The inoculated microtitre plates were incubated for 3 h at 37 °C and then washed twice with PBS to remove non-adhered bacteria. Adhered bacteria were fixed with 25 % formaldehyde for 30 min, then stained with 0.1 % crystal violet (Pro Lab Diagnostics) for 15 min and rinsed under running tap water. Absorbance was measured with a microplate reader (SpectraMax 190; Molecular Devices) at OD\(_{570}\) nm. Sterile TSB was used as a negative control to provide background absorbance. The OD\(_{570}\) nm for each well was the reading after subtraction of the background absorbance. Three wells were inoculated with each isolate on each plate, and each assay was performed in triplicate on separate days. Mean binding for each group was compared between exponential and stationary phases using t-tests [12].

In vivo virulence assay

A Galleria mellonella (wax moth) larvae in vivo pathogenicity model was performed [13]. Larvae (Livefoods) were used within 7 days of purchase and checked to ensure that they had normal colour, size and mobility. TSB was inoculated with five pure colonies of each isolate from nutrient agar plates (Oxoid) and incubated overnight at 37 °C at 225 r.p.m. Overnight cultures were adjusted to an OD\(_{600}\) nm of 0.150. Adjusted cultures (1.5 ml) were spun at 14,000 g and resuspended in 1 ml saline three times. A 25 μl glass syringe (Hamilton) was disinfected by filling it completely with 100 % ethanol, washed with sterile saline and used to inject 10 μl of the washed culture into each caterpillar, as described by Peleg et al. [13]. Twelve caterpillars were inoculated for each strain, placed in filter-paper-lined Petri dishes and incubated at 37 °C for 96 h along with a saline-injected and non-injected control group for each batch. Each caterpillar was scored as dead/live at 16, 40, 64 and 96 h. Caterpillars were considered dead when they displayed no movement in response to touch. Experiments that had more than two dead caterpillars in either control group were discarded and repeated. Three replicate experiments were performed for each strain. Log-rank tests were used to compare Kaplan–Meier survival curves for CC22-high versus CC22-low, CC30-high versus CC30-low, and all CC22 versus all CC30 isolates.

Mass spectrometry for phenol soluble modulin (PSM) production

Nano-electrospray liquid chromatography and a multiple reaction monitoring (MRM) method of mass spectrometry was used to measure relative production of PSM α1–4, β1–2 and γ-haemolysin [17–19]. Heat-treated MRSA strains were measured for total protein content using the Bradford method [20] at OD\(_{595}\) nm. A C\(_{18}\) Zip-Tip (manufacturer’s protocol; Millipore) was used to clean up the peptides, which were dried to completion in a Speed-Vac (Thermo Scientific) prior to liquid chromatography–tandem mass spectrometry (LC-MS/MS). Peptides were resolved by reversed phase chromatography using an Ultimate LC system (Dionex). PSM peptide sequence masses were determined by Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.htm). Each peptide sequence was...
Table 1. Description of the isolates included in the study

<table>
<thead>
<tr>
<th>spa type</th>
<th>SCCmec</th>
<th>Clone</th>
<th>Teicoplanin MIC</th>
<th>Vancomycin MIC</th>
<th>Vancomycin classification</th>
<th>Antibiotic resistance*</th>
<th>Gender</th>
<th>Specialty</th>
<th>Age</th>
<th>Year</th>
<th>BSI focus</th>
<th>Distal focus</th>
<th>In-hospital death</th>
</tr>
</thead>
<tbody>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>0.25</td>
<td>1.5</td>
<td>High</td>
<td>CIP</td>
<td>Female</td>
<td>Other</td>
<td>55</td>
<td>2008</td>
<td>Endocarditis</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>0.38</td>
<td>1.5</td>
<td>High</td>
<td>NA</td>
<td>Male</td>
<td>ICU</td>
<td>61</td>
<td>2005</td>
<td>Intravenous access</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>1.5</td>
<td>1.5</td>
<td>High</td>
<td>CIP, E, R, FA, RIF, TM</td>
<td>Male</td>
<td>ICU</td>
<td>75</td>
<td>2002</td>
<td>Intravenous access</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>1.5</td>
<td>1.5</td>
<td>High</td>
<td>CIP, E</td>
<td>Female</td>
<td>Medicine</td>
<td>79</td>
<td>1999</td>
<td>Endocarditis</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>0.25</td>
<td>0.5</td>
<td>Low</td>
<td>CIP, E, G</td>
<td>Female</td>
<td>Renal</td>
<td>77</td>
<td>2009</td>
<td>Intravenous access</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>0.19</td>
<td>0.5</td>
<td>Low</td>
<td>CIP, E, G</td>
<td>Male</td>
<td>Surgery</td>
<td>40</td>
<td>2006</td>
<td>Urinary tract</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>0.25</td>
<td>0.5</td>
<td>Low</td>
<td>CIP, E</td>
<td>Male</td>
<td>Surgery</td>
<td>64</td>
<td>2006</td>
<td>Foot wound</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t032</td>
<td>IV</td>
<td>CC22</td>
<td>0.25</td>
<td>0.5</td>
<td>Low</td>
<td>CIP, E</td>
<td>Male</td>
<td>Other</td>
<td>82</td>
<td>2004</td>
<td>Intravenous access</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>1</td>
<td>1.5</td>
<td>High</td>
<td>NA</td>
<td>Male</td>
<td>Other</td>
<td>71</td>
<td>2008</td>
<td>Intravenous access</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>1</td>
<td>1.5</td>
<td>High</td>
<td>CIP, E, G, NEO, TE, TM</td>
<td>Male</td>
<td>Surgery</td>
<td>57</td>
<td>2007</td>
<td>Intravenous access</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>1.5</td>
<td>1.5</td>
<td>High</td>
<td>CIP, E, FA</td>
<td>Male</td>
<td>Medicine</td>
<td>38</td>
<td>2004</td>
<td>Intravenous access</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>3</td>
<td>1.5</td>
<td>High</td>
<td>CIP, E, NEO</td>
<td>Female</td>
<td>Medicine</td>
<td>29</td>
<td>2004</td>
<td>Intravenous access</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>0.5</td>
<td>0.38</td>
<td>Low</td>
<td>CIP, E</td>
<td>Male</td>
<td>Medicine</td>
<td>83</td>
<td>2006</td>
<td>Endocarditis</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>0.75</td>
<td>0.38</td>
<td>Low</td>
<td>NA</td>
<td>Male</td>
<td>ICU</td>
<td>70</td>
<td>2001</td>
<td>Chest drain</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>1</td>
<td>0.38</td>
<td>Low</td>
<td>E</td>
<td>Male</td>
<td>Surgery</td>
<td>47</td>
<td>2000</td>
<td>Digestive tract (gut)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>t018</td>
<td>II</td>
<td>CC30</td>
<td>0.5</td>
<td>0.38</td>
<td>Low</td>
<td>CIP, E, G, MUP, NEO</td>
<td>Male</td>
<td>ICU</td>
<td>56</td>
<td>2000</td>
<td>Endocarditis</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*CIP, Ciprofloxacin; E, erythromycin; FA, fusidic acid; G, gentamicin; NEO, neomycin; MUP, mupirocin; RIF, rifampicin; TE, tetracycline; TM, trimethoprim; NA, not available.
uploaded into an ‘in-house’ database. LC-MS/MS was performed using a 4000 QTRAP hybrid quadrupole-linear ion trap mass spectrometer (ABSciex). The enhanced product ion (EPI) mass spectral data were processed into peak lists using Mascot software v. 2.2 (www.matrixscience.com) to determine correct peptide identification. EPI was switched off once retention time and peptide identification were determined to increase the MRM signal. MRM transition ion signals were measured as area-under-curve (AUC) and combined for relative PSM peptide quantitation. ANOVA with post hoc testing was performed to compare PSM production between the four groups as per Joo et al. [21]. One ANOVA was performed to compare total PSM production; a second ANOVA was performed to compare production of the individual PSMs analysed.

**agr gene expression assay**

Relative expression of *agr* was compared with a constitutively expressed housekeeping gene (*gyrB*) using ΔΔCt quantitative reverse-transcription PCR (qRT-PCR) methodology for one randomly selected isolate from each group. Stationary phase bacterial cells were lysed using 50 mg ml⁻¹ lysozyme (Sigma-Aldrich) and RNA extracted using the RNeasy Mini kit in combination with RNAprotect Bacteria Reagent (QIAGEN). qRT-PCR was performed using the primers and probes outlined in Table 2 and 2 ng RNA for each sample using a Superscript III Platinum One-Step qRT-PCR system with ROX master mix (Invitrogen) on the 7900 Fast Sequencing detector (Applied Biosystems). All qRT-PCR experiments were performed in duplicate on separate days. Ct values were taken at a defined threshold level of 0.05. A validation experiment demonstrated equal amplification efficiency for the target and reference gene. The CC22-high sample was used as the calibrator for the ΔΔCt analysis and all Ct values were normalized to *gyrB*. t-Tests were performed to compare replicate ΔΔCt values for *agr* (target gene) and *gyrB* (reference gene), as described by Yuan [22].

**RESULTS AND DISCUSSION**

Absolute fibrinogen and fibronectin binding varied by clone, V-MIC and growth phase; however, strikingly, there was no significant reduction in binding of CC22-high
isolates to fibrinogen moving from exponential to stationary growth phase ($P=0.562$), whereas a significant reduction in both fibrinogen and fibronectin binding was seen for all other isolate groups (Fig. 1).

Fibrinogen binding plays a role in the development of endocarditis through initial binding to heart valve endothelium [23, 24]. Thus, sustained binding of CC22-high isolates to fibrinogen on endothelium may enhance establishment of the initial infective focus in endocarditis. However, $S. aureus$ from human endocarditis did not exhibit higher absolute in vitro binding to fibrinogen [25]. Thus, sustained fibrinogen binding, rather than peak binding during exponential growth phase, may be important for promoting $S. aureus$ colonization of heart valves.

Caterpillar killing varied by clone, with CC22 isolates producing more rapid caterpillar killing than CC30 isolates [median survival 40 h (95% confidence interval, CI=37.6-42.6) versus 64 h (95% CI=57.2-70.7), $P<0.001$]. However, there was no significant difference between either CC22-high and CC22-low isolates $P=0.137$ or CC30-high and CC30-low isolates $P=0.172$ (Fig. 2). PSMs in stationary growth phase culture supernatants were quantified by MRM measurements in the four isolate groups. Absolute signal intensities for each transition ion were combined to produce a single peptide measurement of relative abundance for each isolate, which could then be compared. Overall PSM production (PSMα1–3, γ-haemolysin and PSMβ1–2) was significantly higher in CC22-high isolates compared with CC22-low isolates ($P=0.034$), whereas overall PSM production by CC30 isolates did not vary by V-MIC ($P=0.914$) (Fig. 3a). There was notably lower combined PSMβ production by high V-MIC isolates compared with low V-MIC isolates in both the CC30 ($P=0.030$) and CC22 ($P=0.001$) clone groups (Fig. 3b). This was accomplished by a significant increase in PSMα1 production specifically in the CC22-high isolates but not CC30-high isolates [CC22-high versus CC22-low ($P=0.001$) and CC22-high versus CC30-high ($P=0.003$)] (Fig. 3b). The increased PSMα1 production by CC22-high isolates accounted for the increased overall PSM production in this clone group. agr expression was higher for CC22-low versus CC22-high isolates (1.4-fold, $P=0.021$) and for CC30-low versus CC30-high isolates (2.2-fold, $P<0.001$) (Fig. 4). The expression of agr was higher in all CC22 isolates versus all CC30 isolates ($P<0.001$).

Attenuated virulence is an established phenotype of VISA isolates linked with reduced expression of agr [9, 10, 13]. PSM production and caterpillar killing were used as measures of the effector arm of $S. aureus$ virulence. PSMβ production was significantly lower in CC22-high and CC30-high isolates compared with their low V-MIC counterparts, consistent with a reduction in effector virulence function; however, interestingly, CC22-high isolates showed high PSMα production, predominantly PSMα1, resulting in significantly higher overall PSM production. The increased production of PSMα in CC22-high isolates despite lower agr expression is somewhat paradoxical since PSMs are generally regulated by agr [21]. However, agr-independent regulation of PSMα has been reported [17]. This study found limited correlation between PSMα and measured production of RNAIII, the regulatory molecule of agr. Further experiments on these isolates to investigate the increased production of PSMα seemingly independent of agr are warranted. PSMα peptides have a cytolytic effect on neutrophils and monocytes that is not seen with other PSMs [17], so increased PSMα1 production may protect the bacteria from immune attack during the initial colonization phase of endocarditis. PSMs are also associated with both biofilm stabilization and detachment, depending on their concentration [26, 27], and endocarditis is associated with $S. aureus$ biofilms [28, 29]. In contrast to studies on VISA isolates [13], there was no reduction in virulence in the $G. mellonella$ model indicating that although agr activity was reduced and there were changes in PSM production, this was not associated with reduced acute virulence.

We have identified a distinct phenotype for CC22-high isolates that were strongly associated with endocarditis in an epidemiological study [2]. CC22-high isolates showed sustained binding to fibrinogen and increased production of PSMα peptides. These differences were not identified in
comparator CC30 isolates with a high and low V-MIC obtained from the same collection. Our findings suggest that CC22-high and CC30-high isolates have undergone comparable molecular changes, linked with their reduced vancomycin susceptibility, resulting in an intermediate phenotype between low V-MIC VSSA isolates and VISA isolates. There is reducedagr activity and reduced PSMβ production, but not an overall reduction in virulence that has been reported for VISA isolates. Additionally, CC22-high isolates have sustained fibrinogen binding and produce high levels of PSMα1, both of which could plausibly enhance their ability to cause endocarditis. The identification of these two changes within the same clone type (CC22), but not within another clone type (CC30), is compelling evidence that bacterial factors contribute to this association with endocarditis. It remains unclear whether vancomycin treatment parameters, either delays to starting therapy or inadequate serum levels on therapy, further contribute to the association with endocarditis.

This study has several limitations. Firstly, these high-V MIC isolates are likely to have undergone other molecular changes, so a more comprehensive genotypic and phenotypic analysis of these isolates will now be required to

---

**Fig. 3.** PSM production. To measure reproducibility, we calculated the coefficient of variation (CV) for the peak intensities of all spectra in each sample in the data set. The error bars represent plus one mean CV for each data point across the replicates. (a) Overall mean relative PSM production. (b) Production of individual PSMs.

**Fig. 4.** Mean expression of theagr regulator RNAIII at stationary phase. Error bars represent plus one standard deviation.
extend these findings [8, 9]. Specifically, we did not use an established in vitro or in vivo endocarditis model, or a measure of growth rate, which we plan to do in future studies. Secondly, we performed phenotypic and genotypic evaluation of a limited number of isolates from each clone so it is possible that results do not reflect the whole population.

The study does however have important strengths. Most notably, the clonal group under analysis (CC22-high) had a strong epidemiological association with endocarditis derived from a large study [2]. The analysis included a CC30 comparator group with comparable V-MICs derived from a large study [2]. The analysis included a CC30 comparator group with comparable V-MICs derived from the same collection, which has previously been associated with a small but significant increase in endocarditis [3, 4]. This indicates that the CC22-high isolates analysed here, although uncommon (about 2% of the MRSA BSI collection [2]), have a clear phenotype (30% of cases developing endocarditis [3, 4]). This indicates that the CC22-high isolates analysed here, although uncommon (about 2% of the MRSA BSI collection [2]), have a clear phenotype (30% of cases developing endocarditis [3, 4]).

In summary, this study provides data supporting the hypothesis that inter- and intra-clone differences influence the ability of S. aureus to cause endocarditis. Although V-MIC varies within a clone, the association between higher V-MIC isolates and endocarditis during an MRSA BSI cannot be explained by pharmacokinetic and pharmacodynamic parameters related to vancomycin therapy alone.

Funding information

This study was supported by research grants from Pfizer and the Guy’s and St Thomas’ Charity; no authors have a financial relationship with the sponsors of the study. J. A. O. would like to acknowledge support from the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Healthcare-Associated Infections and Antimicrobial Resistance at Imperial College London in partnership with Public Health England (PHE), and the Imperial College Healthcare Trust NIHR Biomedical Research Centre (BRC).

Conflicts of interest

J. A. O. was employed part-time by Bioquell at the time this work was performed. He is now a consultant to Gama Healthcare. All other authors have no conflicts to declare.

References


