Comparative analysis of phenol-soluble modulin production and *Galleria mellonella* killing by community-associated and healthcare-associated meticillin-resistant *Staphylococcus aureus* strains

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Community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA) have emerged globally and have been associated with more severe disease than healthcare-associated MRSA (HA-MRSA). The purpose of this study was to determine whether laboratory measures of virulence can distinguish dominant CA-MRSA clones from HA-MRSA clones. We compared the production of phenol-soluble modulins (PSMs) and ability to kill *Galleria mellonella* caterpillars for a range of CA- and HA-MRSA strains. Twenty-two HA-MRSA strains [ST22-IV (EMRSA-15), ST36-II (EMRSA-16) and ST239-III] and 26 CA-MRSA strains [ST1-IV (PVL+ USA400), ST1-IV (PVL–), ST8-IV (USA300), ST22-IV (PVL+), ST30-IV, ST59-IV and ST80-IV] were analysed. PSM production was measured using and compared using *t*-tests and ANOVA. A *G mellonella* (caterpillar) pathogenicity model was performed, and differences were compared using survival analysis and the log-rank test. There was no significant difference in overall PSM production between HA and CA strains (*P* = 0.090), but there was significant variation between clones (*P* = 0.003). *G. mellonella* caterpillar killing varied significantly by clone (*P* < 0.001), and overall killing was greater for HA compared with CA clones (*P* = 0.007). The increased acute virulence phenotype of CA-MRSA clones in humans is not associated with increased PSM production *in vitro* or increased killing in an *in vivo* caterpillar pathogenicity model.

**INTRODUCTION**

Community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA) have emerged worldwide over the past 20 years (Chambers & Deleo, 2009; David & Daum, 2010; Otter & French, 2010). CA-MRSA strains are genotypically distinct from dominant healthcare-associated MRSA (HA-MRSA), and there is evidence that CA-MRSA strains have a more virulent clinical phenotype than HA-MRSA strains in being able to cause severe skin and soft tissue and respiratory infections in otherwise healthy individuals (Naimi et al., 2003; Chambers, 2005; Otter & French, 2011; Thurlow et al., 2012; Otto, 2013).

The mechanism underlying an increased acute virulence phenotype of CA-MRSA strains is debated (David & Daum, 2010; Otto, 2010, 2013). Most CA-MRSA strains carry Panton–Valentine leukocidin (PVL) (Otter & French, 2012), and PVL is required for severe disease in some but not all animal models (Labandeira-Rey et al., 2007; Bubeck Wardenburg et al., 2008). PVL carriage by *S. aureus* strains isolated from recurrent severe skin and soft tissue infections or severe pneumonia is also used as a marker of virulence and to guide intravenous immunoglobulin therapy (HPA, 2008). However, the benefit of PVL testing in guiding infection control interventions such as decolonization in hospital and the community has not been defined, and there is interest in identifying alternative correlates of virulence. For example, CA-MRSA strains have also been reported to produce higher levels of a group of core genome-encoded cytolytic peptides called phenol-soluble...
modulins (PSMs) (Wang et al., 2007; Li et al., 2009; Rasigade et al., 2013; Tsompanidou et al., 2013). PSMs have been associated with severe disease in some animal models (Wang et al., 2007; Rautenberg et al., 2011). Although mammalian models are recognized as the ‘gold standard’ models of staphylococcal virulence, these are complex and low throughput. The Galleria mellonella virulence model is relatively quick and easy to perform and has been used to compare S. aureus virulence (Peleg et al., 2009; Chu et al., 2011; Ramarao et al., 2012; Richards et al., 2015). Thus, increased PSM production or rapid G. mellonella killing could be alternative laboratory markers of clones capable of causing severe acute disease.

The purpose of this study was therefore to determine whether increased PSM production or virulence in an in vivo G. mellonella killing model can distinguish dominant CA-MRSA clones known to have an acute virulence phenotype from dominant HA-MRSA clones.

**METHODS**

A range of globally important clones from local collections was assembled and tested comprising 22 HA-MRSA isolates [ST22-IV (EMRSA-15) (n=9), ST36-II (EMRSA-16) (n=9) and ST239-III (n=4)] and 26 CA-MRSA isolates [ST1-IV (PVL-positive USA400) (n=1), ST1-IV (PVL negative) (n=5), ST8-IV (PVL-positive USA300) (n=5), ST22-IV (PVL positive) (n=3), ST30-IV (PVL positive) (n=3), ST59-IV (PVL positive) (n=4) and ST80-IV (PVL positive) (n=5)]. Because ST22 contains common CA and HA lineages, ST22 clones were distinguished based on PVL production (Boakes et al., 2011). Each isolate was tested for PSM production and pathogenicity in a G. mellonella in vivo pathogenicity model.

Nano-electrospray liquid chromatography and a multiple reaction monitoring (MRM) method of MS were used to measure relative production of PSM α1–4, β1 and 2 and γ-haemolysins (Anderson & Hunter, 2006; Wang et al., 2007; Joo et al., 2011). MRSA were cultured in tryptic soy broth (Oxoid) at 37 °C with shaking at 180 r.p.m.; heat-treated MRSA strains were measured for total protein content using the Bradford method (Bradford, 1976) at OD595. A C18 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 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 uploaded into an in ‘house’ database. Liquid chromatography–tandem MS was performed using a 4000 QTRAP hybrid quadrupole/linear ion trap mass spectrometer (AB Sciex). The enhanced product ion mass spectral data were processed into peak lists using Analyst version 1.5. The peak list was searched against an ‘in-house’ database containing the target peptides using Mascot software v2.2 (http://www.matrixscience.com) to determine correct peptide identification. Enhanced product ion 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 and combined for relative PSM peptide quantitation. PSM production from each strain was tested in triplicate. t-Tests were performed to compare mean overall and individual PSM production in HA versus CA strains. ANOVA with post-hoc tests as per Joo et al. (2010) was performed to assess overall and individual PSMs for all clonal groups. ST8 (USA300) was selected as a reference for the post-hoc testing because of its relatively high PSM production.

A G. mellonella (wax moth) larvae in vivo pathogenicity model was employed (Peleg et al., 2009). Larvae (Livefoods) were used within 7 days of purchase and checked to ensure that they had normal colour, size and mobility. Tryptic soy broth was inoculated with five pure colonies of each MRSA isolate from blood agar plates (Oxoid) and incubated overnight at 37 °C at 180 r.p.m. Overnight cultures were adjusted to an OD600 of 0.150, and then 1.5 ml of adjusted cultures was spun at 14000 r.p.m. and re-suspended in 1 ml saline; centrifugation and re-suspension were repeated three times. Serial dilutions from each adjusted overnight culture were performed and plated, identifying a mean inoculum of 8.3×10^12 c.f.u. ml^{-1} (range, 6×10^11–1.1×10^13 c.f.u. ml^{-1}). A 25 µl glass syringe (Hamilton) was disinfected with 100 % ethanol, washed with sterile saline and used to inject 10 µl of the washed culture into each caterpillar (Peleg et al., 2009). 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 of 12 caterpillars for each batch. Each caterpillar was scored as dead/alive 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 all HA-MRSA versus CA-MRSA strains and for all clonal groups.

**RESULTS AND DISCUSSION**

Our PSM measurement methods provide a relative rather than absolute measure of PSM production, which is useful for comparing levels between different strains. There was no significant difference in overall PSM production between HA and CA isolates (mean relative production, 11 538 for HA vs 14 696 for CA; P=0.090), although there were differences between clones (P=0.004) (Fig. 1). PSM production was highest in ST8-IV (USA300) and significantly more than ST36 (EMRSA-16) (P=0.001) and ST59 (P=0.010), but not other clones [mean relative production, 22 114 for ST8-IV, 6722 for ST36 (EMRSA-16) and 6348 for ST59].

There was considerable variation by clone in the production of individual PSMs, with production of PSMα1, PSMα2, PSMβ1 and PSMβ2 being generally greater than PSMγ3, PSMγ4 and γ-haemolysin peptides (Fig. 1). CA strains produced significantly more γ-haemolysin (P=0.001) and PSMβ2 (P=0.003) than HA strains, but there were no significant differences in production of other individual PSMs between CA and HA strains (Fig. 1). Using ST8-IV (USA300) isolates as a reference, ST1-IV (PVL negative) (P=0.046) produced significantly less PSMα2, ST36 (EMRSA-16) (P=0.027) and ST22-IV (PVL positive) (P=0.049) produced significantly less PSMα3 and PVL+ ST1 (USA400) (P=0.001) and ST59-V (P=0.009) produced significantly more γ-haemolysin.

A commonly reported phenotype of CA-MRSA is the ability to produce more toxins than HA strains, especially PSM and PVL (Wang et al., 2007; Cheung et al., 2011; Otto, 2013). We did not evaluate the production of PVL or evaluate the potential role of PVL in the virulence process; PVL was used purely as an epidemiological marker in this study. However, we identified no significant difference in overall PSM production between CA and HA strains, although CA strains did produce more γ-haemolysin and PSMβ2. USA300, which has been the main clone investigated and compared with others for PSM production, did
produce significantly more PSM overall, and particularly PSM2 and 3, but produced less γ-haemolysin when compared with some other clones. PSMα peptides have a cytolytic effect on neutrophils and monocytes that is not seen with other PSMs (Wang et al., 2007), so the increases in PSM production in USA300 compared with some other clones may be linked with the increased virulence associated with USA300 in particular (Tenover & Goering, 2009; Otto, 2013). However, the relatively high production of PSMs by HA strains was unexpected and in contrast to other studies (Wang et al., 2007; Li et al., 2009; Cheung et al., 2011). We believe that ours is the first study to report comparable levels of PSM production in HA and CA clones, which suggests that previous reports of much higher PSM production in CA strains may have been explained, at least in part, by strain selection (Wang et al., 2007; Li et al., 2009; Cheung et al., 2011). However, it is important to note that PSM production in vitro may be distinct from PSM production in vivo given the complex gene regulatory systems in S. aureus, especially the function of agr (Cheung et al., 2011; Otto, 2013).

Surprisingly, HA-MRSA strains killed G. mellonella larvae more quickly than CA strains (median survival time, 40 h for HA strains vs 64 h for CA strains; P=0.007). This reflected a significant variation in G. mellonella killing between clones [range: median 16 h for ST22-PVL+ to 96 h for ST1-IV (PVL+), P<0.001] (Fig. 2). The G. mellonella virulence model was chosen as a relatively quick and potentially high-throughput in vivo assay compared with mammalian models and has been used to investigate S. aureus virulence (Peleg et al., 2009; Chua et al., 2011; Ramarao et al., 2012; Richards et al., 2015).

The purpose of this study was to assess whether dominant CA-MRSA clones produced more PSMs or resulted in more rapid G. mellonella killing compared with dominant HA-MRSA clones, and therefore, whether these assays could be used to guide development of a rapid screening test for CA-MRSA isolates to identify those capable of causing acute severe disease. However, our findings suggest that these laboratory measures of virulence would not be a basis for a useful rapid screening test. Furthermore, our laboratory investigations suggest that the difference in epidemiological behaviour between HA and CA clones is not explained by the measures of virulence that we examined – and may not be virulence related.

Most studies of CA-MRSA virulence have compared one MRSA lineage with another, for example, USA300 with USA400 or USA300 with a small number of HA clones (Montgomery et al., 2008; Cheung et al., 2011; Otto, 2013). Our findings support the inclusion of comparator strains from the same genetic background when investigating virulence mechanisms of epidemiologically defined clones such as CA-MRSA (Montgomery et al., 2008; Cheung et al., 2011; Otto, 2013).

Our study has several strengths. We included multiple isolates and a range of clones of international importance, whereas
other studies have included a single strain from each clone (Wang et al., 2007; Cheung et al., 2011). This provides a more representative insight into virulence of each clone. Limitations include restricting our analysis to only two laboratory assays, PSM production and G. mellonella killing, whereas S. aureus are known to produce a wide range of toxins and have multiple virulence mechanisms. Mammalian models are also recognized as the 'gold standard' for investigating staphylococcal virulence.

In summary, increased PSM production and G. mellonella killing were not identified as laboratory markers of known virulent CA-MRSA clones compared with dominant HA clones, although there was significant inter-clone variation in both PSM production and G. mellonella killing. Our findings do not support the hypothesis that CA strains are more virulent than HA clones, and this highlights the difficulty in extrapolating clinical phenotypes from laboratory assays of virulence. Further comparative *in vitro* studies of virulence in dominant, sporadic and emerging CA-MRSA clones should include intra-clone as well as inter-clone comparators. The molecular mechanism of severe acute disease associated with CA-MRSA clones remains unclear.

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**REFERENCES**


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**Fig. 2.** Survival of *G. mellonella* larvae injected with MRSA. There was significant variation in *G. mellonella* killing between clones (log-rank test, P<0.001). The y-axis shows the cumulative survival as a proportion of the total. The x-axis shows the time in hours. A total of 48 strains were tested, and each clone group was repeated three times.


