Vancomycin-intermediate Staphylococcus aureus isolates are attenuated for virulence when compared with susceptible progenitors

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**Abstract**

**Objectives:** Vancomycin-intermediate Staphylococcus aureus (VISA) is associated with genetic changes that may also impact upon pathogenicity. In the current study, we compared the virulence of clinical VISA strains with their isogenic vancomycin-susceptible progenitors (VSSA).

**Methods:** Production of the critical virulence protein, α toxin, was assessed using Western blot analysis and was correlated to agr activity using a bioluminescent agr-reporter. Cytotoxicity and intracellular persistence were compared ex vivo for VSSA and VISA within non-professional phagocytes (NPP).

**Results:** VISA isolates produced up to 20-fold less α toxin compared with VSSA, and this was corroborated by either loss of agr activity due to agr mutation, or altered agr activity in the absence of mutation. VISA were less cytotoxic towards NPP and were associated with enhanced intracellular persistence, suggesting that NPP may act as a reservoir for VISA. Infection with VSSA strains produced higher mortality in a murine bacteraemia model (≥90% 7-day mortality) compared with infection with VISA isolates (20% to 50%, p < 0.001). Mice infected with VISA produced a dampened immune response (4.6-fold reduction in interleukin-6, p < 0.001) and persistent organ bacterial growth was observed for VISA strains out to 7 days.

**Conclusions:** These findings highlight the remarkable adaptability of S. aureus, whereby, in addition to having reduced antibiotic susceptibility, VISA alter the expression of pathogenic factors to circumvent the host immune response to favor persistent infection over acute virulence. **D.R. Cameron, Clin Microbiol Infect 2017;23:767**

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**Introduction**

*Staphylococcus aureus* is a major opportunistic human pathogen that causes a wide spectrum of disease, ranging from mild skin infections to life-threatening diseases such as endocarditis and bacterial sepsis. The clinical significance of *S. aureus* infection is compounded by its propensity to develop antibiotic resistance. Since the emergence of methicillin-resistant *S. aureus*, vancomycin...
has been relied upon as the mainstay treatment for serious S. aureus infections. The in vivo development of strains with reduced vancomycin susceptibility, termed vancomycin-intermediate S. aureus (VISA) has led to treatment failures and prolonged hospitalization [1]. Despite the association with persistent bacteremia, VISA strains are not typically associated with acute clinical instability or lethal sepsis, suggesting that reduced vancomycin susceptibility may impact upon S. aureus pathogenicity [2].

VISA commonly develops from vancomycin-sensitive S. aureus (VSSA) via cumulative mutations in diverse regulatory loci including two-component regulatory systems [3,4]. These mutations contribute to the altered cellular architecture associated with VISA, including thickened peptidoglycan, increased capsule and reduced protein A, each of which probably culminates in an altered host innate immune response [5].

The accessory gene regulator (agr) is an important virulence regulator in S. aureus. RNAIII is the effector of the system, known to up-regulate the expression of toxins, as well as down-regulate genes encoding cell surface-associated proteins [6]. The agrCA mutation is commonly reported for VISA and agr dysfunction in the absence of mutation has also been described, although the molecular mechanism for this down-regulation remains to be elucidated [7].

In the present study, we have examined the consequences of reduced vancomycin susceptibility in S. aureus. To date, studies addressing the virulence of VISA have used the invertebrate Galleria mellonella model [8,9] or have relied on laboratory-generated VISA strains [10]. Here, we found that clinically derived VISA isolates were less virulent than their VSSA progenitors using a murine bacteraemia model, and had the ability to persist in vivo. These data provide insights that help to explain the clinical relationship between VISA and treatment failure (persistent infection) with no increase in mortality [11,12].

Materials and methods

For more detailed methods, see Supplementary material (Text S1).

Strains and growth conditions

Staphylococcus aureus strains used in this study are listed in the Supplementary material (Table S1). The strains were isolated from patients with persistent bloodstream infections during prolonged antibiotic therapy [5,13–15]. Strains of S. aureus were subcultured onto brain–heart infusion agar (Oxoid, Basingstoke, UK) or in heart infusion broth (Oxoid) and were grown at 37 °C to a nal concentration of 10 mg/L. In triplicate. Cell density and bioluminescence were determined at 11.6-minute intervals for 10 hours using FLUOstar OMEGA microplate readers at 37 °C (BMG Labtech, Germany). To examine the impact of exogenous agr inducers on bacterial strains, filter-sterilized supernatants containing autoinducing peptides (AIP) were prepared as described previously and were added at a final concentration of 10% (volume/volume) [18].

Infection of osteoblasts

Human osteoblast-like cells (MG-63) were infected at MOIs of 100: 1 (unless otherwise specified) for 2 h at 37 °C and adhesion and internalization of bacteria were quantified as described previously using a lysostaphin protection assay [19]. To evaluate cytotoxicity, lactate dehydrogenase released from damaged cells was quantified using a Dimension Vista automated clinical chemistry analyser (Siemens Healthcare Diagnostics, Erlangen, Germany). Interleukin-6 (IL-6) was quantified in cell culture supernatants at 72 h post infection (hpi) by ELISA (eBioscience, San Diego, CA) following the manufacturer’s instructions.

Murine virulence model

Female, 6-week-old BALB/c mice were injected intravenously with 1 × 10^8 CFU of S. aureus as described previously [15]. At 15 hpi, five mice were euthanized, and bacterial densities within the liver were quantified [20]. Remaining animals (n = 10) were monitored for illness at least three times daily. Those showing signs of illness were euthanized via CO2 inhalation. After 7 days, surviving animals were euthanized and bacterial densities within the kidney were determined (n = 3). All murine experiments were performed in accordance with the Animal Research Platform Ethics Committees at Monash University and the University of Melbourne, Australia. Blood was collected from infected mice (n = 9) 8 hpi and serum was collected by centrifugation. Interleukin-6, IL-10, monocyte chemotactrant protein-1, interferon-γ, tumour necrosis factor and IL-12p70 levels were compared using a Cytometric Bead Array Mouse Inflammation kit (Becton Dickinson, Franklin Lakes, NJ, USA) as per the manufacturer’s specifications.

Statistical analysis

Two-way analysis of variance, Holm–Sidak multiple comparison tests, unpaired t-tests with Welch’s correction, Kaplan–Meier curves and log-rank tests were performed using GRAPHPAD PRISM v 6.0 (GraphPad Software Inc., San Diego, CA, USA). Differences between VSSA and VISA strains were considered significant when p ≤ 0.05.

Results

Hla production is reduced in VISA compared with VSSA

Hla is an exotoxin that plays an important role in S. aureus pathogenesis [16,21]. We examined the production of Hla in ten VSSA/VISA clinical pairs by Western immunoblotting (Fig. 1a, see Supplementary material, Fig. S1 and Table S1). Overall, Hla levels were significantly lower in VISA strains compared with VSSA (p < 0.001, two-way analysis of variance). However, the magnitude of the difference was not consistent across each clinical pair (p < 0.001 for interaction). When Hla production was compared between individual pairs using the Holm–Sidak multiple comparisons test, significant reductions were observed for five VISA isolates (JKD6008, JKD6023, A5940, A6226, A8094; p < 0.05 for each). Interestingly, only A5940 and A8094 are known to contain an agr
For two strain pairs (JKD6000/JKD6001 and A9635/A9639), Hla production was low for both the VSSA and VISA strains (Fig. 1a).

We next investigated if the decrease in Hla production in VISA isolates was linked to agr dysfunction [6]. We chose a clinical strain pair that contained an agr mutation (A9635/A9639), a pair that had decreased Hla in the absence of a mutation (JKD6009/JKD6008), a pair that showed no decrease in Hla (JKD6052/JKD6051) and a pair that produced low levels of Hla (A9635/A9639). To monitor agr activity, we used a previously described plasmid (pSB2030) containing the RNAIII promoter (agr-P3) fused to a bacterial luciferase reporter [17]. We first compared the agr activity of A8090 (VSSA) to its VISA derivative A8094, a pair that contained an agr mutation (A8090/A8094). For two strain pairs (JKD6000/JKD6001 and A9635/A9639), Hla production was low for both the VSSA and VISA strains (Fig. 1a).

**agr expression in paired VSSA and VISA**

Given that hla is a target of agr, we next investigated if the decrease in Hla production in VISA isolates was linked to agr dysfunction [6]. We chose a clinical strain pair that contained an agr mutation (A8090/A8094), a pair that had decreased Hla in the absence of a mutation (JKD6009/JKD6008), a pair that showed no decrease in Hla (JKD6052/JKD6051) and a pair that produced low levels of Hla (A9635/A9639). To monitor agr activity, we used a previously described plasmid (pSB2030) containing the RNAIII promoter (agr-P3) fused to a bacterial luciferase reporter [17]. We first compared the agr activity of A8090 (VSSA) to its VISA derivative A8094, which has a frameshift mutation in agrC (see Supplementary material, Table S1). As expected, bioluminescence...
on agar plates, which was indicative of agr expression, was high for A8090 and low for A8094 (see Supplementary material, Fig. S2a) and this correlated with their respective Hla production profiles (see Supplementary material, Fig. 1a). Surprisingly, despite high bioluminescence for A8090 on agar, in broth culture the expression of agr was low throughout the growth cycle (Fig. 1b). To determine if agr could be induced by exogenous AIP, we added filter-sterilized supernatants from A8090, A8094, as well as JKD6210, which is a strong AIP producer from the same agr group. For A8090, agr induction was not detected using supernatants from A8090 or A8094; however, exogenous AIP from JKD6210 increased agr expression during the exponential phase (Fig. 1b). Exogenous AIP had no impact on VISA strain A8094 (Fig. 1b) [14].

Despite the absence of an agr mutation in VISA strain JKD6008, Hla production was reduced compared with its parental VSSA strain JKD6009 (Fig. 1c). As a corollary, using our agr reporter, we found that bioluminescence was lower for JKD6008 compared with JKD6009 when grown on solid media (see Supplementary material, Fig. S2b). Although the agr system was functional in JKD6008, agr activation was delayed (Fig. 1c). Each strain was responsive to exogenous AIP induction; however, JKD6008 supernatants produced a reduced response when compared with JKD6009 (Fig. 1c). Hla production correlated with agr expression for the final VSSA/ VISA pairs (JKD6052/JKD6051 and A9635/A9639). JKD6052 and JKD6051 each had functional agr systems and produced similar bioluminescence on agar (Fig. S2c) and throughout the growth cycle (Fig. 1d), which was corroborated by similar Hla production (Fig. 1a). In contrast, both A9635 and A9639 were weak Hla producers, and this correlated with undetectable agr activities using our reporter system (Fig. 1e, and see Supplementary material, Fig. S2d). In addition, neither strain was responsive to the addition of exogenous AIP (Fig. 1e).

VISA strains are less invasive and cytotoxic toward human osteoblasts, but show enhanced intracellular survival when compared with VSSA

VISA clinical isolates are often associated with complex and persistent infections. Internalization of bacteria within host cells, which can act as a sanctuary site, is thought to be a key mechanism to explain persistent infection. As such, we used an osteoblast-like MG-63 cell line model of non-professional phagocytes to compare the adherence, internalization and cytotoxicity of select VISA pairs in vitro. We chose two clinical pairs isolated from patients with complicated bacteraemia and osteomyelitis (A5935/A5940, A9635/ A9639) [8,15]. Each VISA isolate had reduced capacity to adhere to and invade osteoblasts when compared with their susceptible progenitors (Fig. 2a,b). In addition, each VISA strain was less cytotoxic compared with VSSA as determined by reduced lactate dehydrogenase released by damaged osteoblasts (Fig. 2c). To ensure that the observed reduction in cytotoxicity was not due to impaired internalization, we standardized the initial number of internalized bacteria for VSSA- and VISA-infected cells. After standardization, a significant reduction in cytotoxicity for each VISA strain remained (Fig. 2c). VISA clinical isolates also elicited a subdued osteoblast inflammatory response, with significantly lower IL-6 levels compared with infection with matched VSSA clinical strains (Fig. 2d).

Each of the VISA isolates included in this study was associated with persistent bacteraemia (up to 85 days), a common clinical characteristic of VISA infection [5,14]. To determine if VISA isolates caused more persistent infection within osteoblasts compared with VSSA, we performed lysostaphin-protection assays to select for internalized bacteria. Despite a reduced capacity to adhere to and invade osteoblasts and reduced cytotoxicity, each VISA strain had an enhanced capacity to survive intracellularly (Fig. 3).

Clinical VISA isolates are attenuated for virulence in a murine bacteraemia model and show persistent infection in vivo

To corroborate our in vitro findings in an in vivo system, we next assessed the pathogenic consequences of VISA formation using a murine bacteraemia model. We selected the same four clinical strains that were assessed using our agr reporter experiments. In each case, the VISA strain was severely attenuated for virulence compared with its paired susceptible strain (Fig. 4a, p < 0.001 for each). To determine if this difference was attributable to in vivo fitness, we compared organ bacterial densities within the liver at 15 hpi. This time point was chosen, as it was the time when mice infected with VSSA began to succumb to infection. The number of viable bacteria recovered from the liver was similar for all strains, suggesting that virulence attenuation associated with VISA was not due to impaired in vivo fitness (Fig. 4b).

![Fig. 2. Intracellular passage and cytotoxicity of vancomycin-susceptible (VSSA) and vancomycin-intermediate (VISA) Staphylococcus aureus isolates within infected osteoblasts. Two independent vancomycin-exposed clinical pairs (A9635/A9639 and A937/A939) were used to inoculate MG-63 osteoblast cells at MOI of 100:1 unless otherwise stated. Osteoblasts inoculated with VSSA are represented by black circles and those infected with VISA are represented by grey squares for each panel. Error bars represent SEM and significance was determined using unpaired t tests with Welch’s correction, *p < 0.05, *p < 0.01, ***p < 0.001. Adhesion was assessed at 2 h post infection (a). Following lysostaphin treatment, viable intracellular bacteria were quantified 3 h after inoculation (b). Relative cytotoxicity was determined by quantifying lactate dehydrogenase released by damaged MG-63 cells 72 h post infection (c). Interleukin-6 (IL-6) release was determined using ELISA at 72 h post infection (d). To control for initial internalized bacteria, the MOI for A9639 and A5940 were increased to 250:1 and 500:1, respectively. This experimental condition is indicated by # and grey triangles (c and d).](image-url)
We have demonstrated that in comparison to VSSA, VISA induces an attenuated pro-inflammatory response in osteoblasts (Fig. 2d). To confirm these data in vivo, we determined cytokine stimulation profiles in blood collected from VSSA- and VISA-infected mice 8 hpi (Fig. 4c). When individual cytokines were compared, IL-6 levels were significantly lower in the sera of mice infected with VISA (p < 0.001, Holm–Sidak test). To assess for prolonged bacterial survival in vivo, we determined the bacterial burden of the kidneys 7 days post infection. In each case, surviving mice infected with VISA showed high bacterial loads (Fig. 4d).

Discussion

The evolution of vancomycin-intermediate *S. aureus* is linked to diverse cellular changes, which appear to provide a selective advantage for the pathogen in the face of antibiotic therapy and the host immune response. In addition to vancomycin therapeutic failure, VISA is associated with prolonged bacteraemia and this relationship is poorly understood [1]. In the current study, we have shown using infected non-professional phagocytes and a murine model of septicaemia that VISA strains are consistently less virulent...
compared with their susceptible, antibiotic naive progenitors, and that VISA are well equipped to cause persistent infection in vivo, mimicking what is observed clinically.

To understand the molecular mechanisms that contribute to virulence attenuation for these strains, we focused on agr. Multiple reports have highlighted agrCA loss-of-function mutations in diverse VISA isolates [7,14]. In support of this, the agr system of VISA strain A8094 was non-functional due to an AgrC truncation and this correlated with low Hla production and virulence attenuation in vivo. Intriguingly, recent reports have also described agr dysfunction for VISA in the absence of agrCA mutation [22]. In the current study, we have shown that VISA strain JKD6008, which does not harbour a mutation in the agr locus, produced less Hla when compared with its VSSA progenitor JKD6009, suggesting impaired agr function. Reduced Hla production appeared to occur as a result of delayed agr activation, which has been shown previously to impact on agr-regulated Hla translation [23]. We initially proposed that delayed agr activation was the result of alterations in the VISA cell wall. In contrast, the agr expression profiles were similar for JKD6052 (VSSA) and JKD6051 (VISA), despite reduced virulence for the latter. This suggests that virulence attenuation for VISA is strain and mutation specific and involves pathways in addition to agr.

The VSSA strain A9635, which had a non-functional agr locus (Fig. 1e) was equally virulent when compared with agr-positive isolates including JKD6052 and JKD6009. These data indicate that agr expression is not essential for virulence and in vivo fitness in this murine bacteraemia model. Despite agr dysfunction and low Hla production for both isolates, A9639 was highly attenuated for virulence when compared with A9635, further suggesting that other virulence regulators are involved. Collectively, our data indicate that agr expression is not a consistent predictor of pathogenicity in a murine bacteraemia model, particularly in the context of VISA.

We observed an attenuated pro-inflammatory response for osteoblasts infected with VISA and this correlated with an altered host immune response in mice through a decrease in IL-6. Interleukin-6 is a pro-inflammatory cytokine that is important for both innate and adaptive immunity and is induced by surface protein A [24]. In contrast, capsule has been shown to suppress IL-6 in bacterial pathogens [25]. As such, decreased protein A and enhanced capsule associated with VISA [26] may act synergistically to dampen the IL-6 response. In addition, capsule overproducing strains of S. aureus have been associated with impaired bacterial clearance in a murine model [27], and it is plausible that enhanced capsule with an associated dampened host immune response contributes to the persistent infection observed for VISA in this study. We also showed that once internalized by osteoblasts, VISA are well-adapted to the intracellular compartment, suggesting that non-professional phagocytes may act as a bacterial reservoir that could contribute to the chronicity of VISA infection.

This study has important limitations. First, although we observed a clear association between VISA and reduced pathogenicity, we have not addressed the precise contribution of individual genetic mutations. Like the genetic mechanisms underlying reduced vancomycin susceptibility, it is apparent that virulence attenuation is multifactorial and involves diverse pathways in addition to agr. Second, given the relative virulence of VSSA in our acute infection model, animals succumbed to disease rapidly and bacterial burden could not be assessed over time. Future experiments should assess the persistence of VSSA using chronic infection models such as those mimicking osteomyelitis [28].

In summary, we have described the pathogenic consequence of VISA formation. It is apparent that S. aureus alters the expression of virulence factors in the face of vancomycin treatment, which impacts host immune responses and promotes persistent infection over virulence.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2017.03.027.

Transparency declaration

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