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Original article

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

D.R. Cameron [1, 8], Y.-H. Lin [2, 8], S. Trouillet-Assant [3], V. Tafani [3], X. Kostoulias [1], E. Mouhtouris [4], N. Skinner [5], K. Visvanathan [5], S.L. Baines [2], B. Howden [2], I.R. Monk [2], F. Laurent [3], T.P. Stinear [2], B.P. Howden [2, 6, *], A.Y. Peleg [1, 7, *]

[1] *Infection and Immunity Program, Monash Biomedicine Discovery Institute and Department of Microbiology, Monash University, Australia*
[2] *Department of Microbiology and Immunology, The University of Melbourne at the Doherty Institute of Infection & Immunity, Australia*
[3] *Department of Microbiology, French National Reference Centre for Staphylococci, Hospices Civils de Lyon, International Centre of Infectiology Research, France*
[4] *Department of Surgery, The University of Melbourne, Australia*
[5] *Department of Medicine, The University of Melbourne, Australia*
[6] *Infectious Diseases and Microbiology Departments, Austin Health, Australia*
[7] *Department of Infectious Diseases, The Alfred Hospital and Central Clinical School, Monash University, Australia*

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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). Virulence and host immune responses were further explored *in vivo* using a murine model of bacteraemia.

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 favour persistent infection over acute virulence. **D.R. Cameron, Clin Microbiol Infect 2017;23:767**

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

* Corresponding authors: B. Howden, Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, University of Melbourne, Victoria, 3010, Australia and A.Y. Peleg, Department of Infectious Diseases, The Alfred Hospital and Central Clinical School, Monash University, Melbourne VIC 3004, Australia.
E-mail addresses: bhowden@unimelb.edu.au (B.P. Howden), anton.peleg@monash.edu (A.Y. Peleg).
[8] D.R. Cameron and Ya-Hsun Lin are joint first authors of this article.

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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 bacteraemia, 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. When required, chloramphenicol was added to a final concentration of 10 mg/L.

α toxin Western immunoblotting and agr induction assays

α toxin (Hla) Western immunoblotting was performed in biological triplicate using a polyclonal rabbit anti-Hla antibody (Sigma-Aldrich, St Louis, MO, USA) as described previously [16]. Blots were quantified using a GS800 Calibrated Densitometer (BioRad, Hercules, CA, USA) and ImageJ. The *S. aureus* isolates harbouring a plasmid reporter (pSB2035) containing an *agr*-P3 promoter fused to a bacterial luciferase operon (*luxABCDE*) [17] were first assessed on brain–heart infusion agar (chloramphenicol 10 mg/L) after overnight incubation. Bioluminescence was captured using an *in vivo* imaging system (IVIS100; Perkin Elmer, Waltham, MA, USA) with 2-minute exposure. To assess *agr* activity throughout the growth cycle, cells were grown to an optical density at 600 nm of 0.2, washed and resuspended in heart infusion broth, then 180-μL aliquots were placed into 96-well flat-bottomed plates 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, Ortenberg, 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 CO_2 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 chemoattractant 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 $p \leq 0.05$.

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*