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In vivo evolution of antimicrobial resistance in a series of *Staphylococcus aureus* patient isolates: the entire picture or a cautionary tale?

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Objectives: To obtain an expanded understanding of antibiotic resistance evolution *in vivo*, particularly in the context of vancomycin exposure.

Methods: The whole genomes of six consecutive methicillin-resistant *Staphylococcus aureus* blood culture isolates (ST239-MRSA-III) from a single patient exposed to various antimicrobials (over a 77 day period) were sequenced and analysed.

Results: Variant analysis revealed the existence of non-susceptible sub-populations derived from a common susceptible ancestor, with the predominant circulating clone(s) selected for by type and duration of antimicrobial exposure.

Conclusions: This study highlights the dynamic nature of bacterial evolution and that non-susceptible sub-populations can emerge from clouds of variation upon antimicrobial exposure. Diagnostically, this has direct implications for sample selection when using whole-genome sequencing as a tool to guide clinical therapy. In the context of bacteraemia, deep sequencing of bacterial DNA directly from patient blood samples would avoid culture 'bias' and identify mutations associated with circulating non-susceptible sub-populations, some of which may confer cross-resistance to alternate therapies.

Keywords: MRSA, sub-populations, genomics, diagnostics

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia continues to be associated with a high mortality, despite improvements in patient management.[1] While vancomycin remains the treatment of choice the optimal therapy for failing patients is yet to be defined,[2] and it is in these settings that *in vivo* drug resistance is likely to emerge. Multiple studies, particularly since the advent of whole-genome sequencing (WGS), have examined paired isolates (either clinical or laboratory derived) to further elucidate MRSA resistance evolution.[3–5] Although such studies give insights into possible mechanisms of resistance, they are unlikely to provide the entire evolutionary picture. At present, only a limited number of studies have examined a series of MRSA isogenic patient isolates in the context of antibiotic exposure.[6,7] Mwangi *et al.*[6] performed WGS on an initial vancomycin-susceptible *S. aureus* (VSSA) isolate and a vancomycin-intermediate *S. aureus* (VISA) isolate collected after 85 days of extensive (and variable) drug therapy. In addition, they performed PCR amplicon sequencing (directed at identified mutations) for three intervening isolates, and collectively observed that antimicrobial resistance evolution occurred via a step-wise accumulation of mutations. By contrast, Peleg *et al.*[7] detected different daptomycin-associated mutations in a series of isolates and concluded that resistance resulted from the selection of sub-populations. We undertook this study to better understand antibiotic resistance evolution in a single patient over time, particularly in the context of vancomycin exposure.

Methods

DNA manipulations

DNA was extracted from each isolate (via growth from a single colony) using the ISOLATE Genomic DNA Kit (Bioline, London, UK). DNA fragments were amplified by PCR using BioTaq (Bioline) and capillary sequencing was performed by Macrogen Inc. (Seoul, Korea).

Isolates and MIC testing

Clinical and microbiological details have largely been published elsewhere.[8] In summary, a series of six ST239-MRSA-III isolates was recovered over a 77 day period from a patient with persistent bacteraemia (secondary to vertebral osteomyelitis), despite the use of multiple different antibiotics. The initial susceptible isolate was recovered when the bacteraemia developed on day 32 (D32) of the patient's hospital stay. Vancomycin and daptomycin susceptibilities were determined using a combination of broth microdilution and population analysis profiling as appropriate; all isolates were indistinguishable by PFGE. As part of this study, linezolid, quinupristin/dalfopristin and tigecycline MICs were determined by Etest® (bioMérieux, Marcy-l'Étoile, France).

WGS analysis

Following the generation of fragment libraries, five of the isolates (D32, D52, D56, D83 and D109) were sequenced using an Ion Torrent PGM (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, generating on average 261 MB of raw data (range 90–400 MB). The D90 isolate was sequenced by the Wellcome Trust Sanger Institute using an Illumina HiSeq 2000 system (Illumina Inc., San Diego, CA, USA).

Sequence reads were mapped to *S. aureus* JKD6008, an Australasian ST239-MRSA-III VISA isolate (GenBank accession CP002120), using CLC Genomics Workbench 5.5 (CLC bio, Katrinebjerg, Denmark); ~99% of reads were successfully mapped (range 98.65%–99.40%) for all isolates. Variant detection was performed using a quality-based algorithm (as implemented in CLC Genomics Workbench) applying an 80% genotype frequency cut-off with a minimum coverage of 10 reads. The remaining variants were curated manually to ensure accurate identification; all variants associated with homopolymers were excluded, as were variants present in all of the isolates. As part of this manual inspection process, and in order to avoid overlooking any mutations present but poorly covered, a relaxed minimum of five reads was applied to any mutation present in any single isolate. Subsequently, identified single nucleotide polymorphisms (SNPs) and insertions/deletions were confirmed via PCR amplification and capillary sequencing. The raw sequence data for the D32, D52, D56, D83 and D109 isolates and the D90 isolate have been uploaded to the Sequencing Read Archive (accession number SRA06042) and the ENA database (accession number ERS055865), respectively.

In order to generate a phylogenetic tree based on SNPs, each dataset was mapped to the JKD6008 reference and SNP positions called using Nesoni (http://www.vicbioinformatics.com/software.nesoni.shtml). Informative SNP positions relative to the reference were output in nexus format using tools included with Nesoni, and subsequent tree manipulation was conducted with FigTree (http://tree.bio.ed.ac.uk/software/figtree).

Results and discussion

In addition to the previously characterized heteroresistant VISA (hVISA)/VISA phenotypes,[8] isolates D83, D90 and D109 also displayed quinupristin/dalfopristin reduced susceptibility (Figure 1; a quinupristin/dalfopristin MIC breakpoint of $\leq$1 mg/L is defined as susceptible by EUCAST); linezolid and tigecycline MICs also increased over time. As a result of variant analysis, 10 mutations were collectively identified in the D52–D109 isolates (that were not present in the susceptible D32 isolate), 8 of which resulted in amino acid changes (Figure 2a). This reflects an increased mutation rate in comparison with recent estimates of the molecular clock rate for *S. aureus*, but is consistent with other studies in the context of

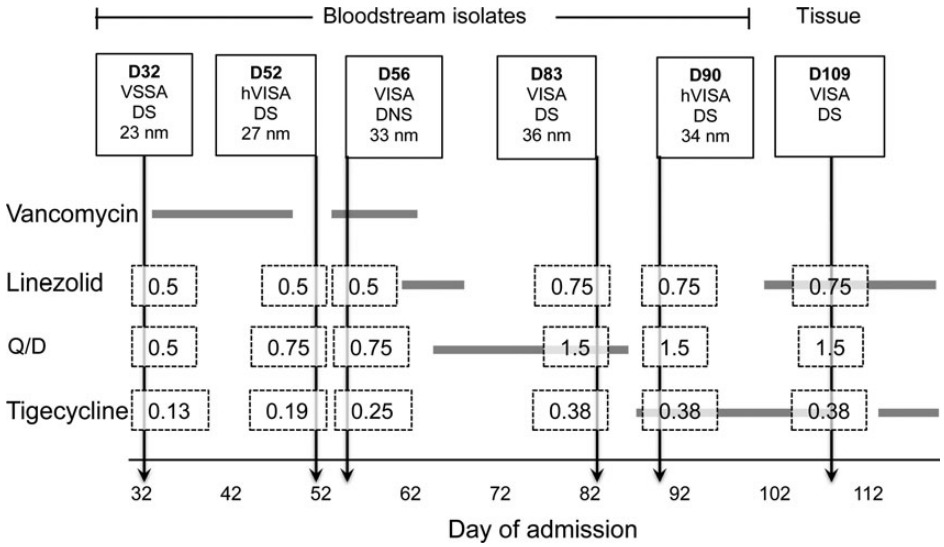

Figure 1. Resistance characteristics of an *S. aureus* isolate series in the context of the patient's antibiotic treatment history (adapted from van Hal *et al*.,[8] with permission). Solid bars represent the length (in days) of indicated antimicrobial therapy (i.e. vancomycin, linezolid, quinupristin/dalfopristin or tigecycline). Isolate names (in bold) are derived from the day of the patient's hospital stay that the blood or tissue sample was collected; names are boxed (with continuous lines) with relevant phenotypic information, including average cell wall thickness (nm) if known.[8] Linezolid, quinupristin/dalfopristin and tigecycline MICs (mg/L) are shown (in boxes with broken lines) for each isolate and were determined as part of this study. D, day; DS, daptomycin susceptible; DNS, daptomycin non-susceptible; Q/D, quinupristin/dalfopristin.

(a)

D32 (initial susceptible isolate)

		Locus	Gene	Function	Type	AA change
D56	VISA, DNS	00019	*walK*	Regulation of cell wall autolysis	SNP (A1106G)	Q369R
D52	hVISA	00019	*walK*	Regulation of cell wall autolysis	SNP (G660A)	M220I
D83	VISA, Q/DNS	00019	*walK*	Regulation of cell wall autolysis	SNP (G660A)	M220I
D109	VISA, Q/DNS	00678	*vraG*	Regulation of cell wall 'stimulon'	Deletion (880-885; TTAAAC)	Δ294-295LN
		00742	-	Oligopeptide transport	SNP (T614C)	V205A
		intergenic	-	-	SNP (G to A)	-
		02287	*rplC*	50S ribosomal protein L3	SNP (C470T)	S158P
D90	hVISA, Q/DNS	00019	*walK*	Regulation of cell wall autolysis	SNP (G660A)	M220I
		00678	*vraG*	Regulation of cell wall 'stimulon'	Deletion (880-885; TTAAAC)	Δ294-295LN
		intergenic	-	-	SNP (G to A)	-
		00482	*dnaX*	DNA replication	SNP (A947G)	Y316C
		00544	*rplA*	50S ribosomal protein L1	SNP (T507C)	Silent
		01173	*rlmN*	Ribosomal RNA methyltransferase	SNP (T2C)	M1T
		02287	*rplC*	50S ribosomal protein L3	SNP 1 (T472C)	A157V

JKD6008 (reference)

(b)

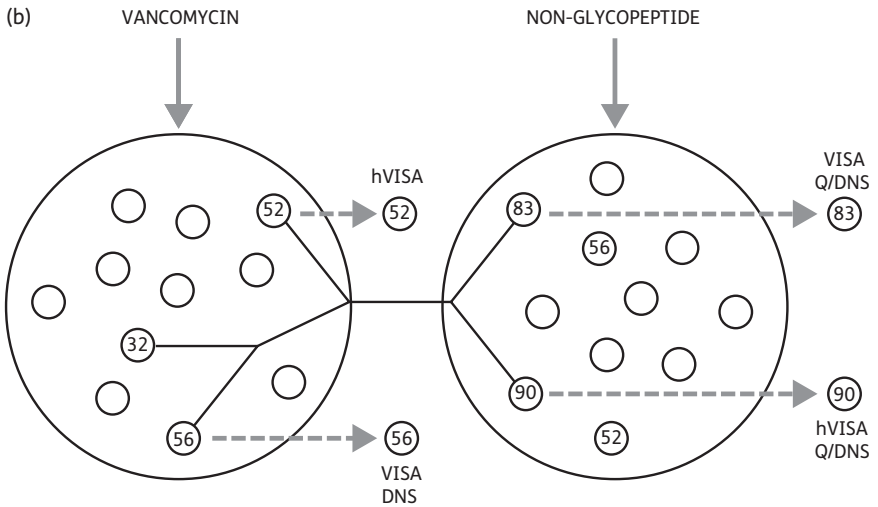

Figure 2. *In vivo* resistance evolution of an *S. aureus* isolate series. Phylogenetic relationships were generated as described in the Methods section. (a) Mutations in isolates D56–D109, not present in the initial susceptible D32 isolate, are indicated by location (using *S. aureus* JKD6008 loci; GenBank accession CP002120), gene name/function, mutation type and resultant amino acid change (where relevant). The cladogram is rooted at the midpoint. (b) SNP tree-based model of resistant sub-populations (small circles) emerging from clouds of variation (large circles) upon antibiotic exposure. Note that the D83 and D90 isolates could have existed in the variant cloud prior to vancomycin exposure. The radial tree has equal branch lengths and is not rooted (D109 isolate and JKD6008 reference not shown). AA, amino acid; DNS, daptomycin non-susceptible; Q/DNS, quinupristin/dalfopristin non-susceptible.

selection pressure and *in vivo* (within-host) evolution.[9–11] Note that no other genetic changes were detected and, based on previous studies, the non-synonymous changes are likely to be directly associated with the type and duration of antimicrobial therapy, as discussed below.

In the context of vancomycin exposure, mutations were detected in genes previously associated with the hVISA/VISA phenotype.[5] The D52 and D56 isolates had different mutations in WalK (M220I and Q369R, respectively), a sensor histidine kinase involved in the control of cell wall metabolism.[5] This highlights the potential importance of resistant sub-populations, as the Q369R mutation also resulted in daptomycin cross-resistance in D56, despite the absence of daptomycin exposure. In addition to the WalK M220I mutation, the D83/109 isolates (identical genotypes; see Figure 2a) and the D90 isolate also had a two amino acid deletion in VraG (Δ294–295LN), an ABC transporter permease;[12] mutations in both WalK and VraG have previously been observed in hVISA/VISA isolates.[6,12] Furthermore, as the D90 isolate is hVISA (and the phenotypes of all isolates appear to be stable), it is reasonable to speculate that a mutation unique to the D83/109 isolates (in comparison with the D90 isolate) is contributing to the VISA phenotype. Although not previously associated with intermediate vancomycin resistance, the V205A mutation in the putative peptide transporter is a likely candidate, as such transporters are known to play a role in the recycling of cell wall peptides.[13]

Following vancomycin therapy, various non-glycopeptide antibiotics were used that inhibit protein synthesis by targeting the ribosome (linezolid, quinupristin/dalfopristin and tigecycline; Figure 1). Consistent with such exposure, the D83/109 isolates and the D90 isolate had different mutations in RplC (S158P and A157V, respectively), a 50S ribosomal protein (L3),[14] which again indicated the presence of sub-populations with reduced susceptibility (in the bloodstream). Additionally, the D90 isolate also had a mutation in the ribosomal RNA methyltransferase RlmN (M1T), and as the next recognized start codon occurs at amino acid position 29, a possible consequence of this mutation is protein truncation. While mutations in RlmN and RplC have previously been associated with increased linezolid and linezolid/quinupristin/dalfopristin MICs, respectively,[14–16] they have not been implicated in reduced tigecycline susceptibility. In any case, as the linezolid, quinupristin/dalfopristin and tigecycline MICs are identical for the D83/109 isolates and the D90 isolate, it is likely that their respective RplC mutations are driving these MICs in combination with increased cell wall thickness (see Figure 1). Note that the potential role of the DnaX mutation in the D90 isolate (Figure 2a) is presently not clear.

In summary, tracking *in vivo* resistance evolution (in a single patient) using WGS revealed that non-susceptible sub-populations (representing different mutations) could emerge from an initial cloud of variation upon antimicrobial exposure (see Figure 2b). This is most likely to occur in the case of high-burden infections and highlights the importance of sequencing *multiple* patient isolates (if available) when investigating resistance emergence. This has direct implications with respect to sample selection and the potential use of genomics as a diagnostic tool to guide clinical therapy. In this respect, deep sequencing of bacterial DNA isolated from patient blood samples would overcome the selective culture 'bias' of isolated colonies and provide a more representative snapshot of circulating mutations. For example, in the context of vancomycin therapy, the availability of metagenomic information would allow the identification of *walK* and/or other mutations that also result in cross-resistance (or decreased susceptibility) to alternative antimicrobials, thus preventing additional clinical failures. However, the utility of such an approach would require a more detailed understanding of resistance mechanisms (including the role of the broader genetic background) and the development of new technologies that facilitate the adequate recovery of bacterial DNA (from blood/tissue samples) for WGS.

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Transparency declarations

None to declare.

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