Unravelling the complex interplay between antibiotic consumption and adaptive changes in Methicillin-Resistant *Staphylococcus aureus*.

Sebastiaan J. VAN HAL [1,2,3*], Slade O. JENSEN [3,4], Stephen Y.C. TONG [5,6], Stephen BENTLEY[7], Matthew T. HOLDEN[8]

1 Department of Microbiology and Infectious Diseases, Royal Prince Alfred Hospital, Sydney
2 University of Sydney, Australia
3 Antimicrobial Resistance and Mobile Elements Group, Ingham Institute for Applied Medical Research, NSW, Australia
4 Microbiology and Infectious Diseases, School of Medicine, Western Sydney University, NSW, Australia
5 Victorian Infectious Diseases Service, The Royal Melbourne Hospital, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia
6 Department of Infectious Diseases, The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia
7 Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SA, UK
8 School of Medicine, University of St Andrews, St Andrews, Fife, KY16 9TF

*Corresponding author: Sebastiaan J. van Hal
Email: Sebastiaan.vanhal@health.nsw.gov.au

Running title: Antibiotics and MRSA resistance development

Abstract

Objectives: This study aims to elucidate the genomic dynamics driving the emergence of antimicrobial resistance (AMR), with a specific focus on the interplay between AMR and antimicrobial usage.

Methods: We conducted a comprehensive analysis using a ST239 Methicillin-Resistant Staphylococcus aureus (MRSA) dataset over a continuous 12-year period from a single hospital. Genomic analyses were performed tracking the changes in MRSA populations, particularly the emergence of reduced vancomycin susceptibility, and assessing the impact of glycopeptide use on these emergence events.

Results: Our findings reveal a significant correlation between hospital glycopeptide usage and the selection of MRSA strains with reduced vancomycin susceptibility. Genomic analyses provided insights into the molecular mechanisms driving resistance emergence, including the slowing of the molecular clock rate in response to heightened antimicrobial consumption.

Conclusions: In conclusion, this study the highlights the complex dynamics between AMR and antimicrobial use at the hospital level. The observed correlation between antimicrobial consumption and the development of less susceptible MRSA strains underscores the importance of antimicrobial stewardship programs and the establishment of optimal consumption thresholds for mitigating AMR effectively.

Introduction

The correlation between rates of antibiotic usage and development of antimicrobial resistance (AMR) in bacteria is well-established, making antibiotic overuse a primary driver of AMR. In response, antimicrobial stewardship programs have been established to optimise prescribing. [1] However, the effectiveness of these programs relies on identified antibiotic overuse thresholds. [2]

At a pathogen level, AMR genetic mechanisms are well understood with mutations or horizontal gene transfer, conferring phenotypic resistance. Despite the predictability of these pathways, the genomic dynamics leading to AMR remain less clear. Understanding the complex interplay between usage and genomic adaptation at the population level remains challenging. To address these complexities, we compiled the largest collection of a single methicillin-resistant *Staphylococcus aureus* clone, namely multi-locus sequence type 239 (ST239), from a single hospital over a continuous 12-year period.

ST239 is a multidrug-resistant MRSA clone for which vancomycin remains the mainstay of therapy. [3] Under antimicrobial pressure genomic adaptations lead to reduced glycopeptide susceptibility phenotypes in the form of hetero-resistant vancomycin intermediate *S. aureus* (hVISA) and vancomycin intermediate *S. aureus* (VISA) [4,5]. Due to the difficulties of performing population analysis profiling (PAP) to detect these phenotypes [6] studies have preferentially examined trends in vancomycin minimum inhibitory concentration through time at a population level, also known as MIC "creep". [4] In this study we provide insights into hospital level AMR emergence linking genomic data and phenotypic vancomycin susceptibility testing with institutional glycopeptide consumption.

Materials and Methods

Isolate collection and MRSA rates.

All multi-resistant (i.e., resistance of ≥3 non-β-lactam antibiotic classes such as quinolones, macrolides, lincosamides and folate inhibitors by automated susceptibility testing) *Staphylococcus aureus* blood stream infection isolates obtained from patients admitted to Liverpool hospital, Sydney, Australia between 1/1/1997 to 31/12/2008 underwent whole genome sequencing. In addition to standard susceptibility testing, vancomycin MIC testing by Etest® and PAP was performed on all isolates, with hVISA and VISA status determined as previously described.[5]

Antibiotic usage.

Glycopeptide dispensing data between 1999-2008 was standardised using the World Health Organisation defined daily dose (DDD) guidelines and calculated by the equation:

where 1 DDD for vancomycin (J01XA01) is equivalent to 2 grams dispensed.
During the study period, alternative MRSA ST239 active agents (e.g., linezolid and daptomycin) were not prescribed for any patient due to lack of availability in Australia. Teicoplanin was available but due to its expense was limited to infrequent outpatient treatment only and therefore was not included in the analysis.

Sequencing and Analysis.

Genomic DNA samples were sequenced on the Illumina HiSeq 2500 platform using 151b paired end TruSeq chemistry. Reads were mapped to the TW20 reference genome (GenBank: NC017331) [7] using SMALT. Variants were called using a combination of samtools mpileup [8] and bcftools.[9] To ensure high-quality SNPs, variants were filtered for read depth (>20), base quality (>13) and if they occurred within either indels, mobile elements or recombinant tracts as detected by Gubbins. [10] The filtered SNPs were subsequently used to cluster isolates using hierBAPs [11] and to create a Maximum Clade Credibility tree under the GTR substitution model with gamma correction in BEAST2 v2.01. [12] All combinations of strict, relaxed and random clock models were evaluated in combination with constant, exponential and skyline population models. Each model was run for 100 million generations sampling every 1000 generations with the optimal model (i.e., strict clock model with a constant population) selected based on convergence, effective sample size (ESS) and harmonic mean estimates within Tracer v1.4.[13]
When molecular clock assumptions are not violated,

phylogenetic Using this data, mapped to each branch in the final MCC tree, annual evolutionary rates was calculated using R package treeio v1.8.2. [14]

Ancestral state reconstruction was employed to determine node sequences, utilizing RAxML v.8.2.12.[15] The likely phenotype was inferred based on the status of the closest phylogenetic tip sequence.

Global ST239 phylogeny.

To places our isolates into a global context, 454 short read sequence data was downloaded and analysed using the above parameters. A maximum likelihood phylogeny was then generated for all 787 sequences using RAxML under GTR model with gamma correction.

Pangenome and Genome Wide Association (GWAS) analysis

Isolate assemblies were generated using SPades v.3.15.3 and annotated using prokka v.1.12 following filtering of contigs <1000 bps in length. [16, 17] The pangenome was subsequently constructed using Roary v3.8.0.[18] To investigate pangenome dynamics a Finitely Many Genes (FMG) which allows for the same gene to be gained and lost multiple times was implemented.[19]

To identify possible adaptative events leading to ST239 clades or hVISA phenotype a 2-step GWAS approach was taken. The first, used the gene presence-absence matrix of the identified pangenome. Associations between genes with traits of interest were examined using Scoary v1.6.16.[20] Only associations with a Bonferroni p-value correction for multiple comparisons of less than 5e-25 were examined further. Multiple candidate genes with similar statistical profiles were observed suggestive of larger structures and/or mobile genetic elements (MGE). These regions were collapsed into larger elements provided that 1) >50% of the constituent genes were present and shared >95% similarity with corresponding ones in the TW20 reference and ii) >75% coverage of the MGE was obtained using progressive Mauve when aligning isolate contigs to the TW20 sequence fragment.

The second GWAS approach scanned for possible causal variant(s) relative to traits of interest implemented through plink v1.09.[21] Identified SNPs were then mapped back to the phylogeny to identify possible candidate regions selecting the hVISA cluster.

Statistical analysis. All comparisons were performed using R v4.2.3 [22] with correlations determined through the Hmisc v5.1-1 package,[23] and trend data assessed using the linear model's function.

Availability of data and materials:
All current short read sequence data was deposited to ENA under Project Number PRJEB2561. The remaining (n=454) sequences were downloaded ENA with accession numbers and associated metadata provided in **Supplementary Table S1**.

Results.
During the 12-year period, our institution recorded a total of 406 methicillin-resistant *Staphylococcus aureus* bacteraemic (MRSA-B) episodes with an overall incidence of 0.3 (IQR 0.29-0.31) per 1000 occupied bed days (OBD). This rate exceeded the accepted quality benchmark of 0.2 per 1000 OBD for this specified period. [24] Among these 406 episodes, only 333 were multi-drug resistant with all confirmed as ST239 MRSA on in silico multi-locus sequence typing. It is worth noting that this timeframe (1997-2008) captured

the first MRSA-B episode at our institution and concludes at the end of 2008 when the proportion of non-multi-resistant ST22 MRSA replaced ST239 MRSA as the most encountered MRSA-B isolate, a trend consistent with reports elsewhere. [25]

The ST239 population within the hospital was split into two distinct clusters, Cluster A (n=184) and Cluster B (n=149) based on 3442 core genome single nucleotide polymorphisms. The maximum Cluster credibility (MCC) tree confirmed this split with Cluster B being monophyletic and arising from Cluster A around 1996 (**Figure 1A**). When viewed within the context of all other published ST239 MRSA sequences, cluster B sequences maintain their distinct grouping confirming this event appears to be predominantly confined to our hospital (**Figure 1B and Supplementary Figure 1**).

Using a pangenome analysis approach, we identified several genetic acquisitions and losses associated with the emergence of Cluster B. The most notable, was the chromosomal integration of the Arginine Catabolic Mobile Element (ACME), a genetic element known for its association with biofilm formation and host immune responses. [26] Cluster B isolates also gained an additional copy of *murF* and *gtfA* two genes associated with peptidoglycan synthesis and cell division [27, 28] as well as a *Staphylococcus* bacteriophage (resembling PT1028; GenBank NC_007045). In contrast, the *Staphylococcus aureus* Pathogenicity Island which carried staphylococcal enterotoxin Q and enterotoxin SEA was lost (**Figure 1A**). Using a finitely many genes model, we calculated the ratio of gene gain to loss events as 11.9 indicative of ongoing adaptation within the hospital environment. [19]

A Bayesian skyline analysis, which infers trends in the Effective Population Size (EPS) based on estimates of genetic diversity in relationship to mutation rate, confirmed the emergence of clade B in 1996 followed by rapid expansion resulting in a similar population size to clade A within the hospital, by the end of the study period (**Figure 1C**). This excludes overall declining ST239 subpopulation or the gradual increase of ST22 MRSA population as an explanation for gene associations. Of interest this analysis also gives a reliable estimate of the time lag (of approximately 4 years) between emergenceof clade B ST239 isolates and the occurrence of the first positive Clade B blood stream infection.

Using ancestral state reconstruction and sequence similarity, Cluster B emerged from a hVISA ancestor (indicated by the asterisks in **Figure 1**). However, it should be noted that this method is contingent on the phylogeny and therefore does not exclude an alternative explanation where Clade B emergedfrom the unsampled MRSA ST239 pool of non-BSI isolates.

A significant increase in the vancomycin MIC over time was observed (p<0.001) not only within the entire ST239 population but also separately in both Clusters (**Figure 2B**). We hypothesised that vancomycin use at our hospital may have contributed to ongoing selection. When examining the glycopeptide usage data, we noted an overall consumption density of 900 DDD per 1000 OBD, consistent with usage in other Australian principal referral hospitals. A significant and gradual increase of usage over time was apparent (p=0.002) with a strong temporal association between usage and vancomycin MIC (R=0.71, p=0.008) (**Figure 2C**). In our quest to identify genetic markers for increased MIC, we first examined mutations in isolates with the hVISA (n=49) and VISA (n=3) phenotype in genes previously associated with reduced vancomycin susceptibility (*e.g., walRK*, *vraSR,* graSRoperons and *rpoB* gene). [29] Only seven non-synonymous mutations were detected (Supplementary Figure 2). Several possible novel candidate mutations in the *menH*, *graX* and *cap8G* genes associated with the small colony variant (SCV) phenotype, acetyltransferase and capsular biosynthesis respectively were linked to the hVISA phenotype on genome wide association analysis (Supplementary Figure 3). Although specific mutations linked to high vancomycin MIC susceptible phenotypes were not identified, the dN/dS ratio of 2.5, provides evidence for the presence of selection as did the molecular clock rate which showed a significant slowing with increased usage (R= -0.65, p=0.043) (**Figure 2D**).[30]

MIC creep and molecular clock associations remained when excluding hVISA/VISA isolates suggesting that the observed effects were due to changes within the VSSA population. Despite this subgroup analysis, a genotype correlate with respect to MIC was not found suggestive of multiple resistance pathways and pathogen redundancies.

Discussion

This study provides important insights into the dynamic relationship between environmental pressures and adaptive changes in a significant hospital pathogen, ST239 MRSA. Furthermore, it highlights the adaptability of MRSA in response to antimicrobial pressure and demonstrates that resistance pathways are complex and multifactorial. [31]

The most notable finding is the intricate relationship between glycopeptide consumption and MRSA population resistance selection. First, our study sheds light on the complexity of MIC creep with no genetic correlates of MIC found and explains the diverse findings of previous studies contributing to the controversy surrounding MIC creep. One possible reason for this variability includes treating MRSA as a homogenous population by combining different MRSA clones including non-multi-resistant MRSA with multi-drug resistant ones and/or community clones with hospital clones. Failure to differentiate between these distinct MRSA populations using typing and/or sequencing in the context of well-chosen time frames may significantly impact the interpretation and accuracy of the results. [32]

Second, isolates with reduced glycopeptide susceptibility, hVISA and VISA phenotypes, emerged through both known and novel genetic pathways. These phenotypes are known to be less fit and thus serve as evolutionary bottlenecks for compensatory adaptive changes to occur, as observed with theemergence of Cluster B from a Cluster A hVISA ancestor through the acquisition of several mobile genetic elements. This overall genetic gain led to restoration of vancomycin susceptibility and likely increased fitness due to the greater propensity for growth and biofilm formation with ACME acquisition. [26, 33] However, following the emergence of Cluster B, the cluster B population demonstrated subsequent increases in vancomycin MIC.

Thirdly, evidence for selection at genome scale was observed with a dN/dS ratio >1, as well as changes in the overall molecular clock rate.[30] These changes were independent of the hVISA/VISA phenotype probably as these isolates are less fit and represent a genomic bottleneck.[34] Consequently, we observed both a reduction in genetic diversity anthe preferential selection of antibiotic-resistant phenotypes within bacterial populations. These findings should be considered in the context of antimicrobial consumption which mirrored other principal referral hospitals in Australia. As a result, while the study's findings are confined to a specific setting, they offer a valuable model for understanding the emergence of AMR.

Our study benefits from the “isolated” nature of our hospital, a generally healthcare restricted bacterial clone and an antimicrobial agent that is limited in its spectrum of activity and therapeutic indication. For these reasons, replicating these findings will remain a challenge and underscores the complexity of antibiotic consumption.

In conclusion, our study highlights the dynamic interplay between antimicrobial consumption and adaptive changes. These findings advance our understanding of AMR dynamics and provide a valuable framework for understanding AMR emergence across

diverse healthcare settings.

Declarations:

Ethics approval and consent to participate: Not applicable.

Consent for publication: not applicable.

Availability of data and materials:

All current short read sequence data was deposited to ENA under Project Number PRJEB2561. The remaining (n=454) sequences were downloaded ENA with accession numbers and associated metadata provided in **Supplementary Table S1**.

Transparency declarations:

The authors declare that they have no competing interests.

Funding sources:

This study was supported by internal funding.

References

1. Barlam TF, Cosgrove SE, Abbo LM *et al*. Implementing an Antibiotic Stewardship Program: Guidelines by the Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America. *Clin Infect Dis* 2016; **62**: e51-77.
2. Lopez-Lozano JM, Lawes T, Nebot C *et al*. Author Correction: A nonlinear time-series analysis approach to identify thresholds in associations between population antibiotic use and rates of resistance. *Nat Microbiol* 2020; **5**: 1449.
3. Liu C, Bayer A, Cosgrove SE *et al*. Clinical practice guidelines by the infectious diseases society of america for the treatment of methicillin-resistant Staphylococcus aureus infections in adults and children. *Clin Infect Dis* 2011; **52**: e18-55.
4. van Hal SJ, Barbagiannakos T, Jones M *et al*. Methicillin-resistant Staphylococcus aureus vancomycin susceptibility testing: methodology correlations, temporal trends and clonal patterns. *J Antimicrob Chemother* 2011; **66**: 2284-7.
5. Howden BP, Davies JK, Johnson PD *et al.* Reduced vancomycin susceptibility in Staphylococcus aureus, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin Microbiol Rev* 2010; **23**: 99-139.
6. Walsh TR, Bolmstrom A, Qwarnstrom A *et al.* Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides. *J Clin Microbiol* 2001; **39**: 2439-44.

7. Holden MT, Lindsay JA, Corton C *et al*. Genome sequence of a recently emerged, highly transmissible, multi-antibiotic- and antiseptic-resistant variant of methicillin-resistant Staphylococcus aureus, sequence type 239 (TW). *J Bacteriol* 2010; **192**: 888-92.
8. Li H, Handsaker B, Wysoker A *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; **25**: 2078-9.
9. Narasimhan V, Danecek P, Scally A *et al.* BCFtools/RoH: a hidden Markov model approach for detecting autozygosity from next-generation sequencing data. *Bioinformatics* 2016; **32**: 1749-51.
10. Croucher NJ, Page AJ, Connor TR *et al.* Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 2015; **43**: e15.
11. Cheng L, Connor TR, Siren J *et al*. Hierarchical and spatially explicit clustering of DNA sequences with BAPS software. *Mol Biol Evol* 2013; **30**: 1224-8.
12. Bouckaert R, Heled J, Kuhnert D *et al*. BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Comput Biol* 2014; **10**: e1003537.
13. Rambaut A, Drummond AJ, Xie D *et al*. Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. *Syst Biol* 2018; **67**: 901-4.
14. Wang LG, Lam TT, Xu S *et al*. Treeio: An R Package for Phylogenetic Tree Input and Output with Richly Annotated and Associated Data. *Mol Biol Evol* 2020; **37**: 599-603.
15. Stamatakis A. Using RAxML to Infer Phylogenies. *Curr Protoc Bioinformatics* 2015; **51**: 6 14 1-6
16. Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455-77.
17. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068-9.
18. Page AJ, Cummins CA, Hunt M *et al*. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015; **31**: 3691-3.
19. Zamani-Dahaj SA, Okasha M, Kosakowski J *et al*. Estimating the Frequency of Horizontal Gene Transfer Using Phylogenetic Models of Gene Gain and Loss. *Mol Biol Evol* 2016; **33**: 1843-57.
20. Brynildsrud O, Bohlin J, Scheffer L *et al*. Rapid scoring of genes in microbial pan-genome-wide association studies with Scoary. *Genome Biol* 2016; **17**: 238.
21. Purcell S, Neale B, Todd-Brown K *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81**: 559-75.
22. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. 2023.
23. Harrell FE. Hmisc: Harrell Miscellaneous. URL https://CRAN.R-project.org/package=Hmisc. 2023.
24. Australian Commission on Safety and Quality in Health Care. Antimicrobial Use in Australian Hospitals. 2016 annual report of the National Antimicrobial Utilisation Surveillance Program. Last accessed in September 2021 at https://www.safetyandquality.gov.au/publications-and-resources/resource-library/2016-annual-report-national-antimicrobial-utilisation-surveillance-program. 2018.
25. Hsu LY, Harris SR, Chlebowicz MA *et al*. Evolutionary dynamics of methicillin-resistant Staphylococcus aureus within a healthcare system. *Genome Biol* 2015; **16**: 81.
26. Fey PD, Olson ME. Current concepts in biofilm formation of Staphylococcus epidermidis. *Future Microbiol* 2010; **5**: 917-33.
27. Li Y, Huang X, Li J *et al*. Both GtfA and GtfB are required for SraP glycosylation in Staphylococcus aureus. *Curr Microbiol* 2014; **69**: 121-6.

28. Sobral RG, Ludovice AM, de Lencastre H *et al*. Role of murF in cell wall biosynthesis: isolation and characterization of a murF conditional mutant of Staphylococcus aureus. *J Bacteriol* 2006; **188**: 2543-53.
29. Hafer C, Lin Y, Kornblum J *et al*. Contribution of selected gene mutations to resistance in clinical isolates of vancomycin-intermediate Staphylococcus aureus. *Antimicrob Agents Chemother* 2012; **56**: 5845-51.
30. Neilsen R. Molecular signatures of natural selection. *Annual Review of Genetics* 2005.
31. Baym M, Lieberman TD, Kelsic ED *et al*. Spatiotemporal microbial evolution on antibiotic landscapes. *Science* 2016; **353**: 1147-51.
32. van Hal SJ, Paterson DL. Systematic review and meta-analysis of the significance of heterogeneous vancomycin-intermediate Staphylococcus aureus isolates. *Antimicrob Agents Chemother* 2011; **55**: 405-10.
33. Hon PY, Chan KS, Holden MT *et al*. Arginine catabolic mobile element in methicillin-resistant Staphylococcus aureus (MRSA) clonal group ST239-MRSA-III isolates in Singapore: implications for PCR-based screening tests. *Antimicrob Agents Chemother* 2013; **57**: 1563-4.
34. Rishishwar L, Kraft CS, Jordan IK. Population Genomics of Reduced Vancomycin Susceptibility in Staphylococcus aureus. *mSphere* 2016; **1**.

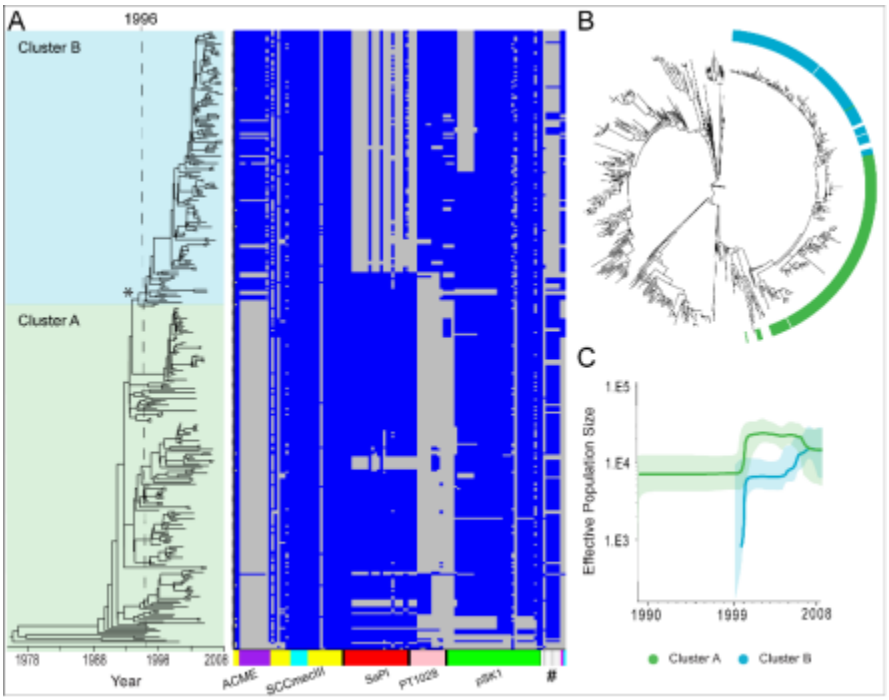

Figure 1

Figure 1 A: Maximum Clade Credibility tree of 333 Australian ST239 isolates with clades (A and B) indicated by the coloured boxes. Clade B emerged around 1996 with the ancestral sequence indicated by the asterisk. A heatmap of genes associated with either clade (see text for details) is depicted by blue (present) or grey (absent) to the right of the tree. Genes were collapsed into larger structures including mobile genetic elements (MGEs) relative to TW20. Larger identified structures include the Arginine Catabolic Mobile Element (ACME; purple) within the SCC*mec* III element; a Staphylococcal pathogenicity island (SaPI; red); a pSK1 plasmid (green) and a bacteriophage (PT1028; pink). The # section of the heatmap indicates individual genes with the *murF* and *gtfA* genes shown by the coloured bars respectively.
B. ST239 Global Phylogeny. A circular maximum likelihood phylogeny of 787 ST239 sequences with the coloured ring indicating the current clade A and B sequences. The unbalanced phylogeny is suggestive of directional selection predominantly at a local level with limited introductions of other ST239 subclones.
C: A Bayesian skyline analysis. The skyline plot shows the effective population size of Clade B expanding rapidly and reaching a size similar to Clade A by the end of the study period following its emergence around 1996.

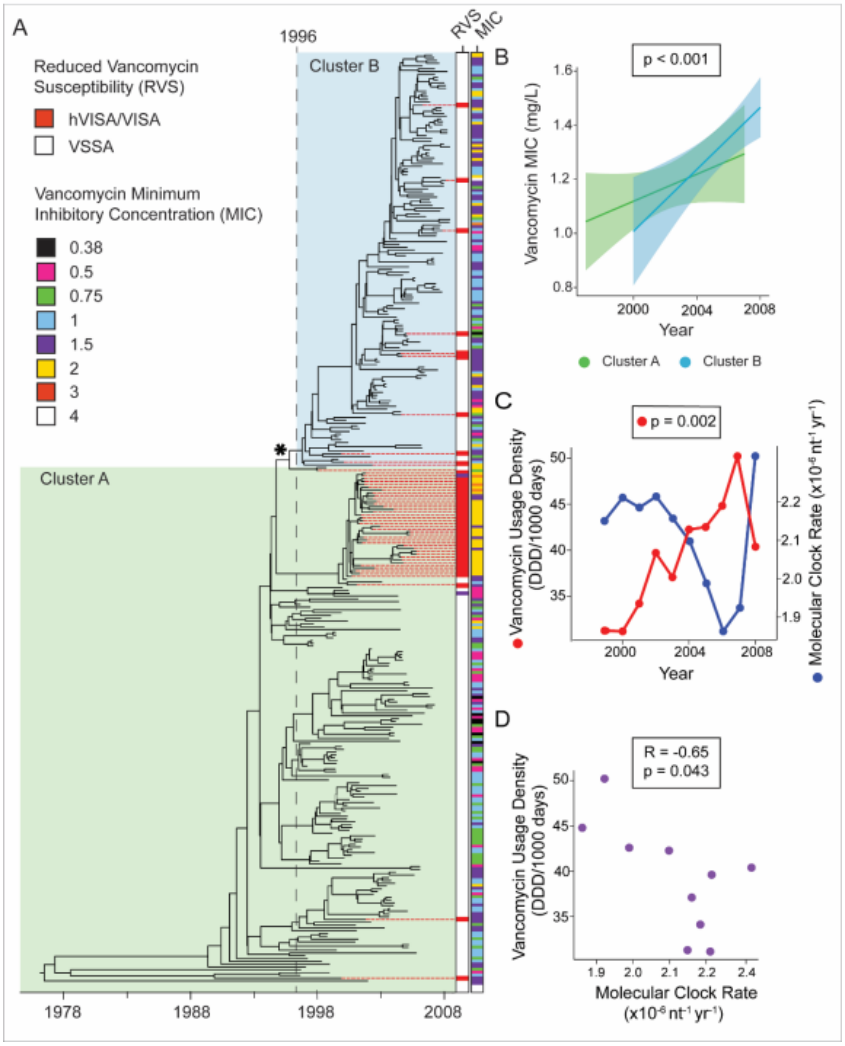

Figure 2

Figure 2 A: Maximum Cluster Credibility Tree. MCC tree of 333 Australian ST239 isolates with Clusters (A and B) highlighted by coloured boxes. Clade B emerged around 1996 with the ancestral sequence indicated by the asterisk. Associated metadata is depicted to the right of the tree with reduced vancomycin susceptibility (RVS) and vancomycin MIC (mg/L) determined by Etest® shown are in the columns to the right of the tree. RVS was defined by the presence of hVISA (heterogenous Vancomycin Intermediate *Staphylococcus aureus*) and VISA (Vancomycin Intermediate *Staphylococcus aureus*) detected by population analysis profiling. VSSA = Vancomycin susceptible *Staphylococcus aureus*.

B: Vancomycin Minimum Inhibitory Concentration Creep. Vancomycin MIC values by time grouped by cluster showing increasing MIC (mg/L) over time.

C: Vancomycin Usage Density over time. Line graph of Annual Vancomycin usage per 1000 occupied bed days and median molecular clock rate (x10^{-6} per nucleotide per year) on the secondary axis.

D: Relationship between Vancomycin Usage and Molecular clock Rate. Scatterplot of vancomycin usage per 1000 occupied bed days and the median molecular clock rate (x10^{-6} per nucleotide per year).