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Multiple introductions of methicillin-resistant Staphylococcus aureus ST612 into Western Australia associated with both human and equine reservoirs

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Highlights

- ST612 MRSA isolates in Australia form distinct equine/veterinarian-associated and human-associated clades.

- Bacteremia-causing ST612-MRSA isolate in Western Australia is of the equine and veterinarian-associated ST612 clade.

- ST612 MRSA is closely-related to ST8 USA500 and carries conserved insertion sequences in virulence-modulating genes

- The use of rifampicin in equine veterinary practice may encourage colonization of horses by ST612.

Multiple introductions of methicillin-resistant *Staphylococcus aureus* ST612 into Western Australia associated with both human and equine reservoirs

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Abstract

Staphylococcus aureus is a serious human and animal pathogen. Multilocus sequence type 612 (ST612) is the dominant methicillin-resistant *S. aureus* (MRSA) clone in certain South African hospitals and is sporadically isolated from horses and horse-associated veterinarians in Australia. Colonization and infection by ST612-MRSA is increasing in Western Australia. Whole-genome sequencing was performed for 51 ST612-MRSA isolated from Western Australian patients and healthcare workers, South African hospital patients, Australian veterinarians and New South Wales horses. Core-genome phylogenies suggested Australian equine and veterinarian-associated ST612 were monophyletic. Individual Western Australian isolates grouped either with this equine-associated lineage or more diverse lineages related to those in South African hospitals. Bioinformatic analyses of the complete ST612-MRSA reference genome SVH7513 confirmed ST612-MRSA was closely related to ST8 USA500 MRSA. Common use of rifampicin in South Africa and equine veterinarian practice may favor ST612-MRSA in these settings. ST612-MRSA-colonized humans and horses are potential reservoirs for MRSA in Australia.

1. Introduction

Staphylococcus aureus is a versatile and virulent opportunistic pathogen of humans and animals. The organism is increasingly resistant to multiple antimicrobials, which has led to reduced therapeutic options and increased morbidity and mortality. Emergence of methicillin-resistant *S. aureus* (MRSA) within hospitals has prompted increased surveillance and infection control measures. Since the 1990s MRSA has also been increasingly associated with infections acquired outside of the hospital environment and are referred to as community-associated MRSA (CA-MRSA). Although MRSA is associated with human hosts, MRSA carriage, infection and transmission, is also observed in domestic animals such as cats, dogs, horses, and livestock, such as pigs and cows. As such, animals may also act as MRSA reservoirs and may account in part for the rise of CA-MRSA [2].

Expansion of MRSA host range can be attributed to the acquisition of host-specific virulence and colonization factors carried by a variety of mobile genetic elements (MGEs) [3]. Multilocus sequence type 612 (ST612) is a member of clonal complex 8 (CC8), which includes the dominant USA CA-MRSA, USA300, and the closely related USA500 lineage [4]. Although ST612 MRSA is frequently identified in South Africa (SA) [5] where it is over-represented in bacteremia cases [6], it is not a frequently reported cause of human infections elsewhere. In Australia, ST612-MRSA has been isolated from veterinarians and from New South Wales (NSW) horses [7]. Furthermore ST612-MRSA is also increasingly detected in human patients living in Western Australia (WA), with at least one case leading to serious bacteremia [8]. In brief, ST612-MRSA was cultured from the nasal swab of a WA Patient 9. The isolate was resistant to co-trimoxazole, rifampicin, erythromycin and doxycycline. Within a year, the partner of WA Patient 9, WA Patient 1, had a bacteremia with ST612-MRSA. The couple had a close

association with horses. Here, we collected and analyzed the genome sequences of human-associated ST612-MRSA from WA, NSW, South Australia and South Africa, along with horse-associated ST612-MRSA from NSW, in an attempt to identify potential origins of ST612-MRSA in WA.

2. Materials and Methods

2.1 Isolates

The ST612-MRSA (n = 51) isolates used in this study included: isolates from patients (n = 10) and healthcare workers (n = 3) living in WA; patient isolates from Tygerberg hospital (Cape Town, South Africa) (n = 8); isolates from the Scone Veterinary Hospital (Scone, NSW, Australia) isolated from horses (n = 24) and veterinarians (n = 3) [7]; and isolates from South Australian veterinarians attending a series of Australian veterinary conferences in 2009 (n = 3) (previously described, [9]). Isolate details are provided in appendix Table S1.

2.2 Genome sequencing and assembly

All isolates were grown in overnight cultures of tryptic soy broth media, with shaking at 37 ˚C. Whole DNA extractions were performed as described previously [10]. Genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina) and were sequenced on the Illumina MiSeq platform. Reads were cleaned, assembled and annotated using the Nullarbor bioinformatic pipeline software package (github.com/tseemann/nullarbor). Isolate SVH7513 from the Scone Veterinary Hospital collection, was sequenced using long-read SMRT-cell sequencing to produce a finished high-quality reference genome, as previously described [10]. Genome sequence assemblies have been deposited in Genbank under BioProject accession PRJNA558684.

2.3 Typing and identification of mobile genetic elements

Multi-locus sequence typing was performed with MLST v 2.10 (github.com/tseemann/mlst). *spa* and SCC*mec* typing were performed in silico using spaTyper 1.0 (https://cge.cbs.dtu.dk/services/spatyper) and SCCmecFinder (https://cge.cbs.dtu.dk/services/SCCmecFinder/) software, respectively. Plasmids were detected using PlasFlow [11]. Antimicrobial-resistance and virulence genes were detected using abricate (v 0.8, github.com/tseemann/abricate) to query the Resfinder (https://bitbucket.org/genomicepidemiology/resfinder_db.git) and Virulence Factor Database (VFDB) databases, respectively. SaPI elements were identified manually with BLASTn by searching genomes for previously described att sites [12], while comparisons of SaPI were made with a BLASTn library of SaPI elements retrieved from Genbank (Table S2) and visualized in Ugene v1.3.

2.4 Comparison of genomes and phylogenetics

Reference ST612-MRSA genome SVH7513 (CP029166.1) was aligned with USA500 (CP007499) using BRIG (v 0.95, [13]), as were their associated plasmids pSVH7513a (CP029167.1) and pUSA500 (CP007500.1), respectively. Core-genome alignments were produced using Snippy v 3.2 (github.com/tseemann/snippy). Approximately-maximum-likelihood phylogenetic trees were computed using Fasttree v 2.1.10 [14] with the generalized-time reversible substitution model. Trees were visualized with the interactive Tree of life (iTol v 3, https://itol.embl.de/).

3. **Results**

3.1 Genome composition of the ST612-MRSA reference genome SVH7513.

SVH7513, isolated from an Australian horse in 2008, was selected as a reference genome. Initial queries of the NCBI database confirmed SVH7513 was closely related to the ST8 strain USA500. The closest related USA500 strains were from CC8-USA500 clade I, the same clade as the USA500 clinical isolate reference genome 2395 USA500 [15, 16]. Whole-genome comparisons revealed SVH7513 and USA500 2395 (Accession CP007499) shared 99% identity and over 97% coverage of the genome. USA500 is a highly virulent hospital and CA-MRSA strain, whose virulence has been attributed to the modulation of virulence gene expression through the acquisition of insertion sequences (IS) within virulence-associated gene-regulatory regions. Sixteen copies of IS*256* are found throughout the USA500 chromosome, while fourteen copies were found in the SVH7513 chromosome. Seven copies of IS*256* were located in identical positions to those in USA500 2395 (Table S3), including a copy located upstream of the virulence-associated fibrinogen-binding gene *sdrD* and one copy interrupting the 'repressor of toxin' gene *rot*, which is attributed to the enhanced virulence of USA500 [16]. Of the seven unique IS*256* disruptions in SVH7513, five were within intergenic regions, while two occurred within coding sequences for predicted genes with no homologues (Table S3).

Sequence queries of SVH7513 and USA500 2395 genomes with the Virulence Factor Database revealed both lineages share the same number of known virulence-associated genes (*n*=68), including leukotoxin *lukEv-lukDv* and cytotoxin genes (*hla*, *hld*, and *hlgABC*). Like USA500, SVH7513 carried a Type IVd SCC*mec* element and a 27,887-bp multi-resistance plasmid dubbed pSVH7513a (CP029167), which was 99% identical (98% coverage) to pUSA500. pSVH7513a carried resistance genes for trimethoprim (*dfrC*), cadmium (*cadC*), aminoglycosides (*aac6-aph2*)

and beta-lactams (*blaZ*). Additionally, SVH7513 carried a small 2,496-bp plasmid, dubbed

pSVH7513b (CP029165.1), which encoded the inducible erythromycin resistance gene *ermC* and

repL-family rolling-circle replication initiation gene. USA500 2395 and SVH7513 shared several

prophage and pathogenicity islands, including *hlb*-converting prophage φSA3, which carried the

human immune evasion genes *sak* and *scn* and enterotoxin *sea*, and the Staphylococcal

Pathogenicity Island SaPI3, encoding enterotoxin genes *seb*, *selk* and *selq*. The SVH7513

genome was 40.3 kb smaller than the USA500 2395 genome, primarily due to variations within

SaPI elements and prophage φSA2 and the absence of prophage φSA7. SVH7513 contained a

previously unidentified SaPI, named here SaPIsvh7513 (Figure 1).

3.2 Sequencing and whole-genome comparisons of ST612-MRSA isolated from humans and

horses in Australia and humans in South Africa.

To determine the origin of ST612-MRSA in WA, 51 ST612-MRSA genomes were sequenced

with an average 56-fold depth of coverage**.** The majority of isolates 42/51 (82.3%) were *spa* type

t064. The remaining isolates were t1257 (6/51=11.8%), t723 (2/51=3.9%) and a single t7571, all

of which are closely related to t064, containing either insertions, rearrangements or deletions of

spa repeat sequences. Overall, there was little variation of the core genome between ST612-

MRSA genomes. All isolates harbored SCC*mec* IVd, *lukEv-lukDv* and carried a tetracycline-

resistance gene (*tetM*) on an integrative and conjugative element (ICE) related to Tn*5801*,

integrated at the 3' end of the GMP-synthase gene *guaA*. The isolates shared identical RNA

polymerase subunit B (*rpoB*) genes, conferring rifampicin resistance (His481->Asn, Ile527-

>Met). All isolates carried pSVH7513a (described above), while the erythromycin-resistance

plasmid pSVH7513b was sporadically present in 58.8% of isolates (30/51). The beta-lactamase

gene *blaZ* was present in all isolates, but was disrupted by an IS*256* insertion in all but three

isolates (NSW Horse 18 and 21, and South African Patient 4). Some resistance genes were

present only sporadically, such as macrolide-streptogramin resistance (*msrA*, *n*=3), quaternary

ammonium disinfectant resistance (*qacB,* *n*=1), chloramphenicol (*catA7*) and streptomycin (*str*)

resistance (*n*=1). In two isolates, the loss of prophage φSA3 resulted in the restoration of the β-

hemolysin gene*, hlb*. Two unique SaPIs were identified among the South African isolates,

dubbed SaPItkn92 and SaPIthw70 (Figure 1). Differences in virulence-associated and

antimicrobial resistance genes are depicted in Figure 2.

3.3 Equine-associated ST612-MRSA form a distinct clade and both human and equine-

associated lineages are both possible sources of WA ST612-MRSA.

A Maximum-Likelihood phylogenetic tree was constructed using the core genomes of the 51

ST612-MRSA and represented a total of 3,988 single-nucleotide polymorphisms (SNPs) across a

core genome of 2,915,384 bp. All horse and veterinarian-isolated ST612-MRSA grouped within

a single clade together with several WA patient isolates (Figure 2). The greatest SNP differences

were between WA Patient 2 and South African Patient 3 (*n*=134 bp), while the minimum number

of SNP differences were between NSW Horses 16, 18 and 22 (*n*=0 bp) and NSW Horses 1 and

15 (*n*=0 bp). Five of the Western Australian human isolates grouped with the equine-associated

clade, while the remaining eight clustered with various more diverse South African ST612-

MRSA isolates.

4. Discussion

This study aimed to identify the origins of ST612 in WA. Characterization of ST612-MRSA was performed by comparison with a high-quality ST612-MRSA genome and closely-related and well-characterized USA500 MRSA genome (Accession CP007499, [16]). Except for variation in prophage and SaPI, ST612-MRSA SVH7513 shared many MGEs and an identical suite of characterized virulence-associated genes.

'Hyper virulence' of USA500 has been attributed to the increased expression of virulence genes, mediated by chromosomal insertions of IS*256*. Many IS*256* sites of USA500 were shared with SVH7513, including a copy inserted into the repressor of toxins gene, *rot.* The insertion of IS*256* in *rot* results in increased toxin production, resulting in enhanced spleen colonization and survival in the presence of neutrophils [16]. Disruption of *rot* suggests SVH7513 may share a similar virulence-gene expression profile as the hyper-virulent USA500. Variations in IS*256* distribution amongst ST612-MRSA genomes, such as the absence of a disruptive IS*256* insertion in the *blaZ* genes of three isolates, as well as the different distributions of seven IS*256* in SVH7513 relative to USA500, demonstrates the mobility of IS*256* in these isolates.

The ST612-MRSA core-genome phylogeny was consistent with multiple introductions of ST612-MRSA into WA. The phylogenetic tree formed a single low-diversity clade containing all horse and veterinarian-associated ST612-MRSA along with several WA ST612-MRSA isolated from humans. The remainder of Western Australian and South African ST612-MRSA were relatively more diverse, but groupings suggested multiple exchanges of distinct ST612-MRSA. WA has a significant South African population (1.7% of the WA population, [*Population census*

- *country of birth,* ABS 2016, [17]]), so the potential for direct introductions of ST612-MRSA from South Africa by travel is not unreasonable.

The monophyletic grouping of equine and veterinarian-isolated ST612-MRSA in this study is unsurprising, as previous studies have identified an increased risk of carriage of ST612 MRSA for equine veterinarians [9]. It is possible that this lineage of ST612-MRSA has adapted to persistently colonize horses and may therefore present a reservoir of MRSA for human infections. Indeed, the most serious case of ST612-MRSA infection in WA (WA Patient 1) occurred within a patient who had direct contact with horses, suggesting transmission of virulent ST612 MRSA to and from horses. However, we additionally sampled thirty-nine horses on the property of WA Patient 1 by nasal swab in March 2016 in an attempt to isolate ST612 MRSA, but no MRSA were detected. Thus, the source may have been a different stable, introduced for example from personnel working between stables or following interstate travel.

The equine and veterinarian-associated ST612-MRSA in this study were isolated in 2008-2009, while the most recent and likely zoonotic infection from a member of this clade occurred in 2016, suggesting ST612-MRSA has been circulating in Australian horse populations for at least seven years with little genetic change occurring during that time. The presence of genetically close ST612-MRSA in horses and humans, over a wide span of years and geographic range, suggests ST612-MRSA may have a combination of virulence factors and antimicrobial-resistance determinants that allow it to persist in at least one of these hosts. The equine-adaptation phage ΦSaeq1 was not identified in any of the isolates here and therefore is not a requisite for the colonization or infection of horses. The presence of a φSA2-type prophage,

φSA2svh7513, and SaPIsvh7513, all of which encode genes of unknown functions within accessory gene regions, could contribute to equine host adaptation. The ability of ST612-MRSA to persist in South Africa and the Australian horse population however may be an unfortunate consequence of antimicrobial selection favoring ST612-MRSA. All ST612-MRSA in this study had an identical mutant *rpoB* gene, which provides constitutive rifampicin resistance. The mutations in the *rpoB* genes are consistent with those previously identified in ST612-MRSA from South Africa [5]. Additionally, all ST612-MRSA carried the trimethoprim-resistance plasmid pSVH7513a. Jansen van Rensburg et al (2011) [18] suggested the prevalence of ST612-MRSA in South Africa could be the result of selection by the use of rifampicin and trimethoprim/sulfamethoxazole. Rifampicin is used in South Africa for the treatment of tuberculosis [5], while trimethoprim/sulfamethoxazole is used prophylactically for the control of bacterial and *Pneumocystis* infections in HIV infected patients. Rifampicin is uncommonly prescribed in Australia with only 6,020 prescriptions recorded in 2015 by the national Prescribing Benefit Scheme as compared to 5.6 million prescriptions for the commonly prescribed antibiotic cephalexin [19]. However, Saputra et al [20] suggest the prevalence of rifampicin-resistant ST612-MRSA in Australian horses is the result of the treatment of foals with rifampicin, in combination with macrolides, in preventing *Rhodococcus equi* infections. Trimethoprim has been used in horses, orally, for the treatment of respiratory infections, which would further select for ST612-MRSA in Australian horses.

Our findings support current local policy of MRSA screening at time of hospital admission for patients recently treated in overseas health facilities, but a targeted campaign raising awareness among equine veterinarians may also be warranted. The zoonotic potential of ST612-MRSA also

raises the question of whether the problem could be greater in nations with larger populations of both HIV infection and domesticated horses.

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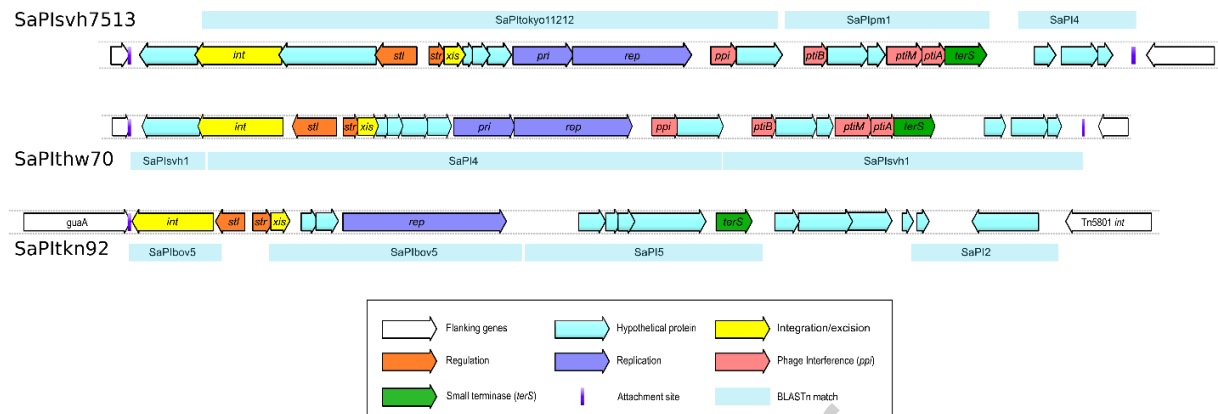
Tables and Figures

Figure 1. Unique SaPI elements found in ST612 genomes. BLASTn matches represent regions of >95% nucleotide identity to the indicated SaPI elements. SaPIsvh7513 and SaPIthw70 both integrate into the 30S ribosomal S18 gene, while SaPItkn92 has displaced Tn*5801* at the *guaA* GMP synthase gene site. Distribution of these SaPI among ST612 isolates is indicated in Figure 2.
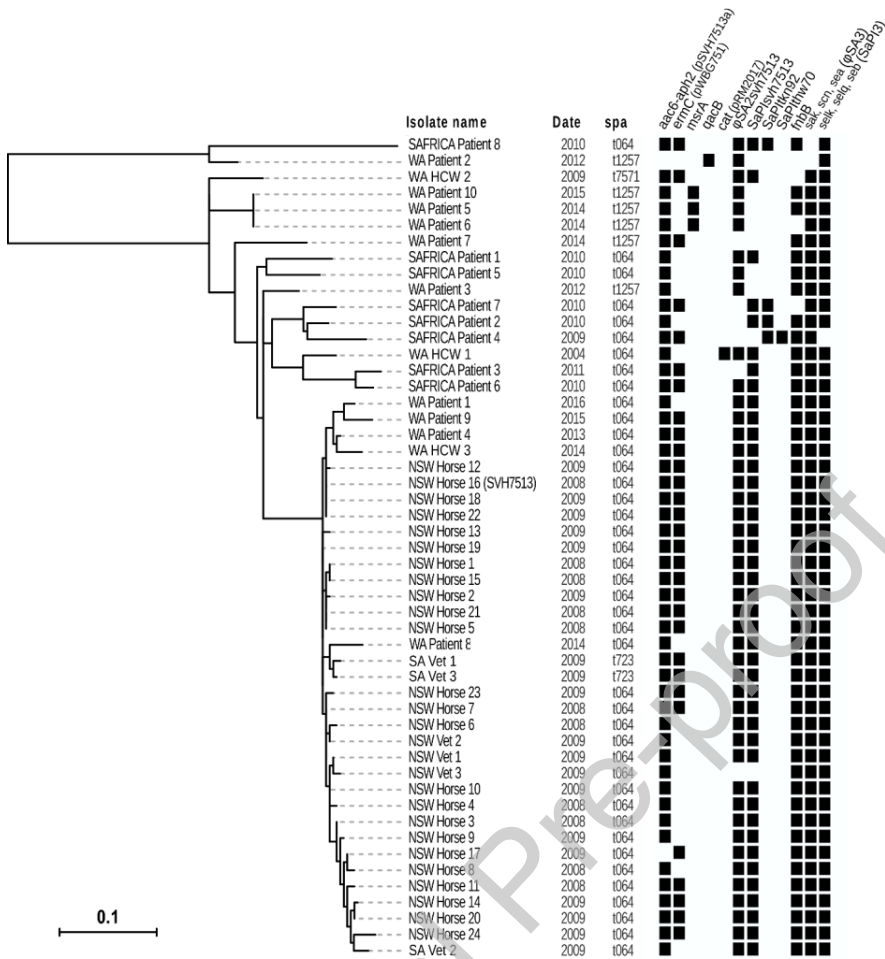
Figure 2. Maximum-Likelihood phylogenetic tree of core genomes from 43 Australian and 8 South African ST612 *S. aureus.* Isolate names indicate origins of the represented genomes: NSW (New South Wales), WA (Western Australian), SA (South Australian), and SAFRICA (South African). HCW denotes a healthcare worker. Year of isolation (Date) and *spa* type are shown. Scale bar indicates average substitutions per site. Presence of resistance and virulence genes, prophage and SaPI is indicated by filled squares. Names of associated mobile genetic elements are bracketed if known.