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Whole genome sequencing and molecular epidemiology of paediatric *Staphylococcus aureus* bacteraemia

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ABSTRACT

Objectives: The role *Staphylococcus aureus* antimicrobial resistance genes and toxins play in disease severity, management and outcome in childhood is an emerging field requiring further exploration.

Methods: A prospective multisite study of Australian and New Zealand children hospitalised with *S. aureus* bacteraemia (SAB) occurred over 24 months (2017–2018). Whole genome sequencing (WGS) data were paired with clinical information from the ISAIAH cohort.

Results: 353 SAB isolates were sequenced; 85% methicillin-susceptible *S. aureus* ([MSSA], 301/353) and 15% methicillin-resistant *S. aureus* ([MRSA], 52/353). There were 92 sequence types (STs), most commonly ST5 (18%) and ST30 (8%), grouped into 23 clonal complexes (CCs), most frequently CC5 (21%) and CC30 (12%). MSSA comprised the majority of healthcare-associated SAB (87%, 109/125), with principal clones CC15 (48%, 11/21) and CC8 (33%, 7/21). Panton-Valentine leukocidin (PVL)-positive SAB occurred in 22% (76/353); predominantly MSSA (59%, 45/76), community-onset (92%, 70/76) infections. For community-onset SAB, the only microbiological independent predictor of poor outcomes was PVL positivity (aOR 2.6 [CI 1.0–6.2]).

Conclusion: From this WGS paediatric SAB data, we demonstrate the previously under-recognized role MSSA has in harbouring genetic virulence and causing healthcare-associated infections. PVL positivity was the only molecular independent predictor of poor outcomes in children. These findings underscore the need for further research to define the potential implications PVL-producing strains may have on approaches to *S. aureus* clinical management.

1. Introduction

Staphylococcus aureus is a common and important human pathogen, with a broad repertoire of virulence factors and clinical spectrum of disease [1]. It is one of the most frequent causes of bacteraemia in the post-conjugate pneumococcal vaccine era in Australian and New Zealand children (incidence rate: 4.4/100 000/y [95% confidence interval (CI) 2.2–8.8]) [2] and the principal cause of childhood skin and soft tissue infections (SSTIs), osteomyelitis and infective endocarditis [2–4].

S. aureus antimicrobial resistance has varied significantly over time and by geographical location [5–8]. Although community and hospital clones of methicillin-resistant *S. aureus* (MRSA) have emerged in Australia; methicillin-susceptible *S. aureus* (MSSA) remains the major cause of bacteraemia [5,6]. Key knowledge gaps exist in MSSA molecular epidemiology and virulence factors in children [8]. This evidence deficit highlights the importance of ongoing national molecular surveillance systems for *S. aureus* bacteraemia (SAB) across the life course akin to the government-funded Australian Staphylococcal Sepsis Outcome Program (ASSOP) performed by the Australian Group on Antimicrobial Resistance (AGAR) [7].

The role of *S. aureus* toxins in SAB disease severity, management and outcome is an emerging field requiring further exploration [9–11]. Controversies exist regarding the most widely studied *S. aureus* toxin, Panton-Valentine leukocidin (PVL), given it is frequently more prevalent in non-invasive compared with invasive *S. aureus* isolates and does not appear to affect outcomes for all clinical phenotypes [11]. Additionally, there are limited studies examining other virulence factors for childhood SAB [12,13], highlighting the need for further paediatric-specific *S. aureus* molecular analyses to improve our understanding of complex host-pathogen dynamics.

Given the burden of disease, changing antimicrobial resistance patterns and uncertainties regarding the prevalence and impact of specific virulence factors, we conducted a prospective genomic analysis of paediatric SAB. We aimed to identify the molecular epidemiology of SAB in childhood and explore associations of antimi-

crobial resistance genes and virulence factors with clinical phenotypes, severity and outcome.

2. Methods

This was a prospective, multisite, cross-sectional study of paediatric SAB in Australia and New Zealand, involving seven tertiary and one secondary paediatric hospital and ten neonatal intensive care units (NICUs), from the Invasive *Staphylococcus aureus* infections and hospitalisations (ISAIAH) cohort [2]. Clinical outcomes examined included 90-day all-cause mortality, or a composite outcome (defined a priori as 90-day all-cause mortality, 90-day relapse, ICU admission or length of stay [LOS] >30 days). Further information on the methods for the ISAIAH dataset are outlined below and have previously been described [2].

2.1. Isolate collection

Children ≤18 years with a positive blood culture for *S. aureus* presenting to study hospitals were prospectively recruited. Episodes were identified by local microbiology or infectious diseases services over 24 months (2017–2018). Children with SAB transferred from peripheral hospitals to study sites were also included. Polymicrobial blood cultures were excluded from the study.

2.2. Isolate identification and antimicrobial susceptibility testing

Laboratories used standard commercial blood culture systems (BACTEC™, BD Diagnostics); bacterial identification (MALDI-TOF; Bruker Daltonics, Bremen, Germany or Vitek® MS; bioMérieux, France) and semi-automated susceptibility platforms (Vitek® 2; bioMérieux, France or BD Phoenix™; Becton Dickinson, USA). CLSI M100 minimum inhibitory concentration (MIC) breakpoints [14] were used with each laboratory reporting their own antimicrobial susceptibility data. Resistant and intermediate susceptibility isolates were classified as non-susceptible. Multi-resistant MRSA (mrMRSA) isolates were classified as those resistant to ≥3 non-beta-lactam antimicrobial classes.

2.3. Whole genome sequencing, genome assembly and phylogenetic analysis

All isolates were transferred to the national AGAR *S. aureus* reference laboratory for whole genome sequencing (WGS). Sequencing was performed on a NextSeq® platform (Illumina Inc., San Diego, CA, USA) using 150-bp paired-end chemistry. Genomic DNA was extracted using the MagMAX™ Express-96 deep well magnetic particle processor (Thermo Fisher Scientific, 4400077) and DNA quantification was performed using a Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Q33216). DNA libraries were prepared and normalised using the Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) as recommended by the manufacturer. Each genome was sequenced with an expected coverage of >60 × .

Raw sequence reads were assembled de novo using SPAdes version 3.14.1 [15], and contigs <200 bp were discarded. The genome assembly quality statistics are provided in Supplementary Appendix S1. The assembled contigs for each isolate were submitted to the Bacterial Isolate Genome Sequence Database (BIGSdb) on the PubMLST *S. aureus* website (www.pubmlst.org/saureus) and are accessible using a unique identifier for each isolate (id: 37650–38002). Genomes were annotated with Prokka version 1.14.6 [16] using default parameters. Core genes (n=1269) from this set were concatenated and aligned using Roary version 3.13.0 [17]. Phylogenetic trees were constructed using the neighbour-joining algorithm on MEGA version X [18] and visualised using iTOL version 6.3 [19].

2.4. MLST profiling and SCCmec typing

Multilocus sequence typing (MLST) profiles (sequence type [ST] and clonal complex [CC]) were determined by submitting each genome sequence to the PubMLST *S. aureus* website [20,21]. Previously undescribed MLST alleles were deposited to the PubMLST database where the alleles were curated. For MRSA genomes, the staphylococcal cassette chromosome *mec* (SCC*mec*) type was determined using the SCC*mec*Finder 1.2 online database [22].

2.5. Analysis of virulence factors and antimicrobial resistance genes

Resistance and virulence genes of interest were selected following a literature review, examining association with clinical outcomes [23] and phenotypic susceptibility [24], respectively. Reference genes were mapped to all reconstructed genomes using BLAST [25].

2.6. Epidemiological analyses

WGS data were linked to the ISAIAH dataset [2]. The dataset comprised clinical information on demographics, comorbidities, focus of infection, investigations, disease severity, treatment and patient outcomes from the hospital, laboratory and radiology records [2].

Community-onset SAB was defined as a positive blood culture(s) collected ≤48 h after hospital presentation. A healthcare-associated SAB definition was adapted from the National Healthcare Safety Network Centres for Disease Control and Prevention definitions (Supplementary Appendix S2) and included SAB associated with a device or surgical site focus or complicating neutropenia [26]. Relapse was defined as repeat *S. aureus* sterile site culture(s) or hospital representation deemed by site investigators to relate to the initial SAB occurring 15–90 days post primary bacteraemia. Ninety-day all-cause mortality was collected for in-hospital deaths.

2.7. Statistical analyses

Statistical analyses were performed using R version 3.6.3 (R Core Team, 2020). χ^2 or Fisher's exact tests were used to compare categorical variables, and Student's *t* test or Mann-Whitney U test were used to compare continuous variables. Potentially significant covariates were considered a priori (age, sex, country and MRSA status), and those with P value <0.1 on univariate analysis were included in the multivariable regression model. Pairwise correlation coefficients were examined between variables before inclusion in the multivariable model to avoid collinearity. Stepwise backward elimination was performed, and *P* values <0.05 were considered statistically significant. Model performance was assessed using the C-statistic for discrimination and the Hosmer-Lemeshow test for calibration. Ethics approval was obtained from each laboratory hospital site.

3. Results

Overall, 353 *S. aureus* isolates from unique patients for whom there was linked clinical data available were included in the study, collected from eight paediatric hospitals located across Australia (77%, 273/353) and New Zealand (23%, 80/353). The 353 isolates sequenced (Fig. 1) were from a total of 552 SAB episodes in the ISAIAH cohort [2]. The median age of children with SAB was six years (interquartile range 1–11), 61% (217/353) were male, 16% (35/273) of Australian children identified as Aboriginal or Torres Strait Islander, and 45% (36/80) of New Zealand children identified

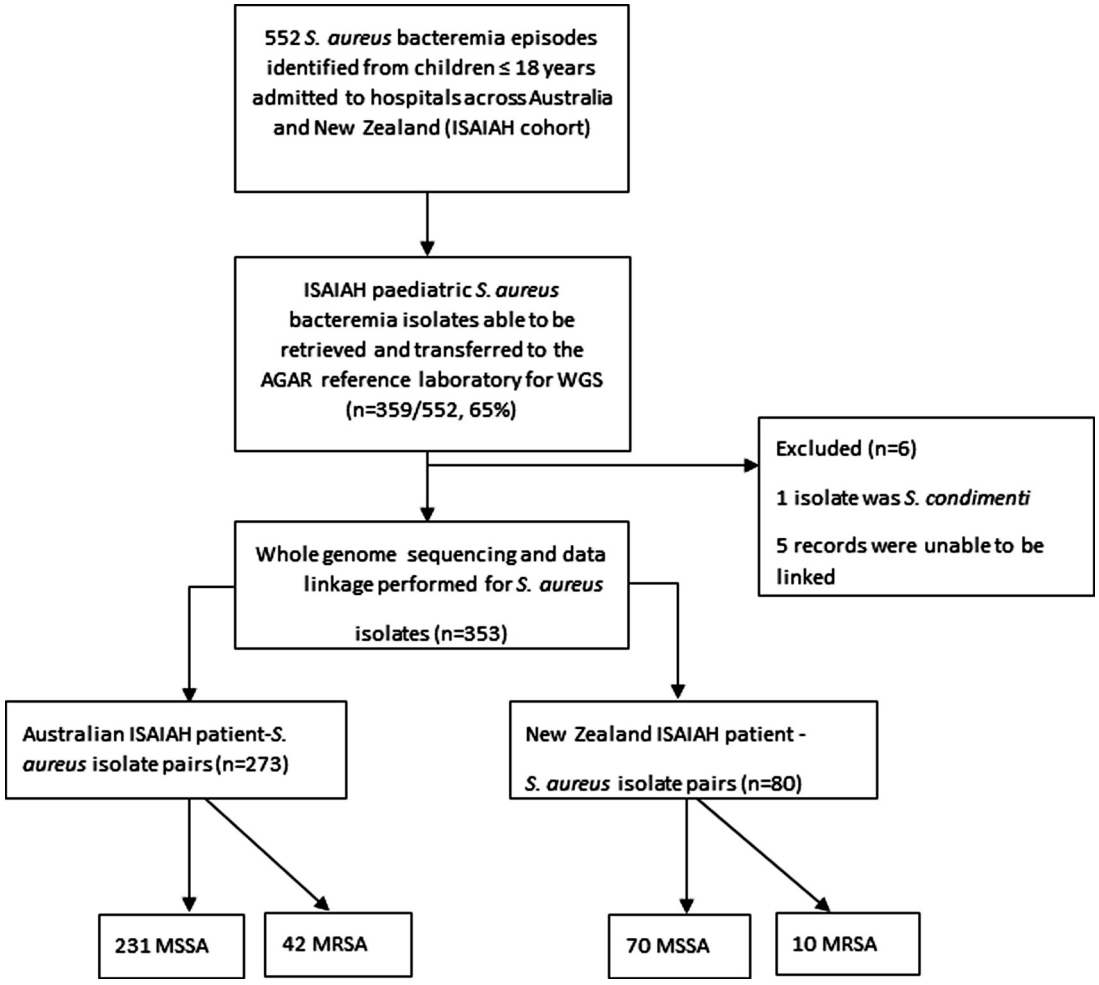

Fig. 1. Study flow diagram of *S. aureus* isolates included in the study (n=353) from unique patients for whom there was linked clinical data available. MSSA, methicillin susceptible *S. aureus*; MRSA, methicillin resistant *S. aureus*; ISAIAH, Invasive *S. aureus* infections and hospitalization study; AGAR, Australian Group on Antimicrobial resistance; WGS, whole genome sequencing.

as Māori or Pacific ethnicity (Table 1). Community-onset SAB comprised 75% (264/353) of the cohort overall, and almost one-third of paediatric SAB was healthcare-associated (35%, 125/353).

The most frequent foci of SAB in children were osteoarticular (47%, 166/353), device-related (27%, 97/353) and SSTI (13%, 45/353). Approximately two-thirds of cases (69%, 245/353) had a single focus of *S. aureus* infection, and one in seven children (14%, 51/353) had multifocal disease (pulmonary and bone most commonly [41%, 21/51]). Clinical outcomes forming the composite endpoint (40%,141/353) included ICU admission (21%, 74/353), LOS >30 days (28%, 99/353), 90-day relapse (4%, 14/353) and 90-day mortality (2%, 6/353).

3.1. Molecular epidemiology

Ninety-two different sequence types (STs) and 23 clonal complexes (CCs) were identified in Australia and New Zealand. Nine CCs were identified in Australia only (Fig. 2). The most frequently identified STs were ST5 (18%, 62/353), ST30 (8%, 29/353) and ST93 (7%, 25/353; Supplementary Appendix S3). CC5 predominated, comprising 21% (74/353) of isolates, followed by CC30 (12%,44/353) and CC1 (12%, 41/353) (Fig. 2). CC5-ST5 was the principal molecular phenotype for each of the main SAB foci of infection including osteoarticular, device-related and SSTI (Supplementary Appendix S4).

3.2. Antimicrobial resistance in SAB

MSSA was the responsible pathogen for 85% (301/353) of SAB episodes (community-onset SAB [84%, 221/264]; healthcare-associated SAB [87%, 109/125; Table 1]). One isolate, ST2250, identified by the MALDI-TOF as MSSA, was subsequently identified by WGS as *Staphylococcus argenteus.* All MRSA isolates harboured *mecA* (15%, 52/353). Non-multi-resistant MRSA comprised the majority of MRSA bacteraemia cases (98%, 51/52), with only one isolate (0.2%) identified as a mrMRSA (ST22-MRSA-IV [CC22]) that was healthcare-associated.

Penicillin-susceptible *S. aureus* (PSSA) bacteraemia occurred in 13% (38/301) of MSSA isolates; however, four PSSA isolates (11%) encoded a penicillinase (*blaZ* positive). Overall, there was an 86% (280/326) concordance between phenotypic and genotypic penicillin susceptibility testing (Supplementary Appendix S5). Cotrimoxazole resistance was present in 5% (18/353) of isolates overall, predominantly in MSSA (78%, 14/18) compared with MRSA bacteraemia (22% 4/18). Clindamycin inducible and constitutive resistance was detected in 9% (31/353) and 0.6% (2/353) of isolates, respectively. Inducible clindamycin resistance was mainly in MSSA bacteraemia (90%, 28/31) compared with MRSA (10%, 3/31), correlating with 84% (27/32) concordance with genotypic (*erm* gene) testing. Further antimicrobial susceptibility testing results are presented in Supplementary Appendix S5.

Table 1
Baseline clinical characteristic of Australian and New Zealand children with *S. aureus* bacteraemia

	Total, no. (%) (n=353)	MSSA, no. (%) (n=301)	MRSA, no. (%) (n=52)	P value (MSSA v MRSA)
Clinical phenotype				
Age in years, median (IQR)	6 (1-11)	6 (1-11)	5 (2-10)	0.007[a]
Male, n (%)	217 (61)	184 (61)	33 (63)	0.785
Location				
Australia	273 (77)	231 (77)	42 (81)	0.524
New Zealand	80 (23)	70 (23)	10 (19)	0.524
Ethnicity				
Aboriginal children in Australia SAB	35 (16)	16 (5)	19 (37)	<0.001[b]
Māori and Pacific children in New Zealand SAB	36 (45)	29 (10)	7 (13)	0.514
Classification				
Community-onset	264 (75)	221 (73)	43 (83)	0.127
Healthcare-associated	125 (35)	109 (36)	16 (31)	0.486
Source of infection				
Osteoarticular	166 (47)	140 (47)	26 (50)	0.689
Device-related	97 (27)	83 (28)	14 (27)	0.882
Skin and soft tissue	45 (13)	36 (12)	9 (17)	0.319
Endovascular	44 (12)	25 (8)	19 (37)	<0.001[b]
Pleuropulmonary	40 (11)	25 (8)	15 (29)	<0.001[b]
Foci of infection				
No focus	57 (16)	53 (18)	4 (8)	0.073
Multifocal disease	51 (14)	32 (11)	19 (37)	<0.001[b]
Severity and outcome				
ICU admission	74 (21)	56 (19)	18 (35)	0.009[c]
LOS >30 days	99 (28)	80 (27)	19 (37)	0.141
90-day relapse	14 (4)	13 (4)	1 (2)	0.481
90-day mortality	6 (2)	5 (2)	1 (2)	1.000
Composite outcome	141 (40)	114 (38)	27 (52)	0.0575

SAB, *S. aureus* bacteremia; PSSA, penicillin susceptible *S. aureus*; MRSA, methicillin resistant *S. aureus*; ST, sequence type; CC, clonal complex; IQR, interquartile range, ICU, intensive care unit; LOS, length of stay.
[a] $p < 0.001$
[b] $p < 0.01$
[c] $p < 0.05$

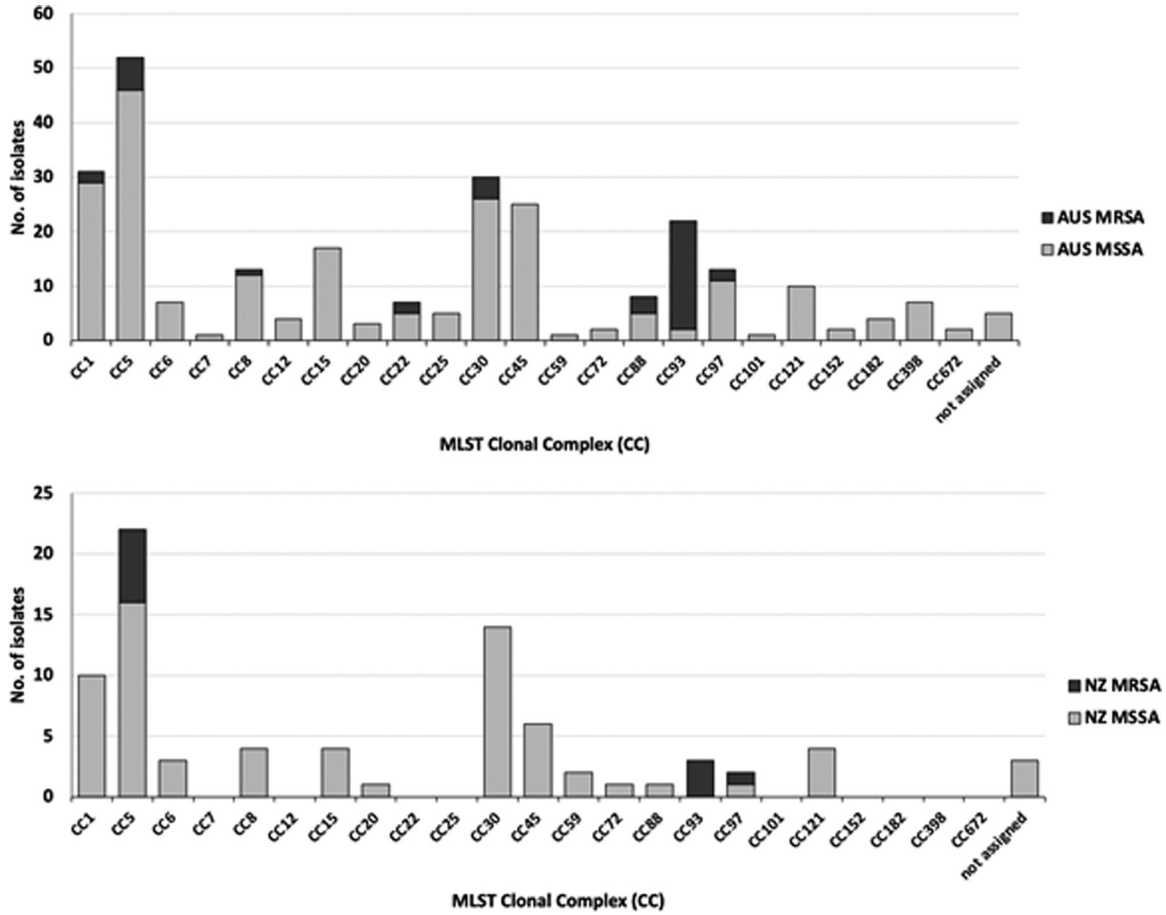

Fig. 2. Clonal complexes (CCs) for methicillin-resistant and methicillin-susceptible paediatric *S. aureus* bacteraemia isolates from Australia and New Zealand. MRSA, methicillin resistant *S. aureus*; MSSA, methicillin susceptible *S. aureus*, MLST, multilocation sequence type; CC, clonal complex; Aus, Australia; NZ, New Zealand.

Table 2
Baseline molecular characteristics of Australian and New Zealand children with *S. aureus* bacteraemia

	Total, no. (%) (n=353)	MSSA, no. (%) (n=301)	MRSA, no. (%) (n=52)	P value (MSSA v MRSA)
Number of different sequence types (ST)	92	88/92 (96)	13/92 (14)	<0.001[a]
ST5	62 (18)	50 (17)	12 (23)	0.298
ST30	29 (8)	25 (8)	4 (8)	1.000
ST93	25 (7)	2 (.01)	23 (44)	<0.001[a]
ST1	21 (6)	19 (6)	2 (4)	0.566
ST45	20 (6)	20 (7)	0 (0)	0.049[c]
Number of different clonal complexes (CC)	23	23/23 (100)	8/23 (35)	<0.001[a]
CC5	74 (21)	61 (20)	13 (25)	0.412
CC30	44 (12)	40 (13)	4 (8)	0.311
CC1	41 (12)	38 (13)	1 (2)	0.021[c]
CC45	31 (9)	31 (10)	0 (0)	0.061
CC93	26 (8)	2 (1)	24 (46)	<0.001[a]
CC15	21 (6)	21 (7)	0 (0)	0.049[c]
CC121	14 (4)	14 (5)	0 (0)	0.099
CC not assigned	8 (2)	8 (3)	0 (0)	0.206
Virulence genes				
Panton-Valentine leukocidin (PVL: lukF+lukS)	76 (22)	45 (15)	31 (60)	<0.001[a]
Staphylokinase (sakb)	307 (87)	255 (85)	52 (100)	0.003[b]
Alpha hemolysin (hla)	342 (97)	291 (97)	51 (98)	0.689
Toxin syndrome toxin (tst)	42 (12)	40 (13)	2 (4)	0.062
S. aureus enterotoxin d (sed)	25 (7)	22 (7)	3 (6)	<0.001[a]
S. aureus enterotoxin A (sea)	83 (24)	70 (23)	13 (25)	0.753
S. aureus enterotoxin (selq)	36 (10)	33 (11)	3 (6)	0.273
Enterotoxin like protein (sel)	39 (11)	31 (10)	8 (15)	0.283
Staphylococcal protein A (spa)	220 (62)	192 (64)	28 (54)	0.169
Fibronectin binding protein A (fnbA)	250 (71)	207 (69)	43 (83)	0.040[c]
Clumping factor B precursor (clfB)	240 (68)	208 (69)	32 (62)	0.318
Willebrand factor-binding protein (vWbp)	169 (48)	137 (46)	32 (61)	0.046[c]
Adhesive protein (sdrC)	266 (75)	227 (75)	38 (73)	0.759
Adhesive protein (sdrD)	197 (56)	159 (53)	38 (73)	0.007[b]
Adhesive protein (sdrE)	266 (75)	219 (73)	47 (90)	0.009[b]
Chemotaxis inhibitory protein (chp)	204 (58)	162 (54)	42 (81)	<0.001[a]
S. aureus collagen adhesin (cna)	34 (10)	33 (11)	1 (2)	0.043[c]
S. aureus secrete coagulase (coa)	142 (40)	135 (45)	7 (13)	<0.001[a]

[a] $p < 0.0001$
[b] $p < 0.01$
[c] $p < 0.05$

Greater diversity among MSSA compared with MRSA isolates was observed, with more STs (96%, 89/92) and CCs (100%, 23/23) compared to MRSA ([ST; 14%, 13/92]; [CCs; 35%, 8/23]) (Table 2). Genetic relatedness was evident between MSSA and MRSA isolates in Australia and New Zealand, with no country-specific cluster identified (Fig. 3). CCs associated with only MSSA bacteraemia included CC45, CC15 and CC121 (Table 2). Common MSSA healthcare-associated clones included CC15 (48%, 11/21) and CC8 (33%, 7/21). There were eight MSSA STs that could not be classified into a CC (ST6508, ST5491, ST207, ST6497, ST5111, ST6887, ST2250, ST6494). Although there were no MRSA-only CCs, 92% (24/26) of CC93 were MRSA (Table 2).

3.3. Virulence genes in paediatric SAB

The frequency of virulence genes varied considerably—for example, 97% of isolates (342/353) harboured *hla* (encoding alpha hemolysin), and yet only 7% (25/353) harboured *sed* (encoding *S. aureus* enterotoxin D; Table 2). Seven virulence genes occurred more frequently among paediatric MRSA vs MSSA bacteraemia, including *sed*, the *luk-F* and *luk-S* PVL-associated genes, and the *chp* gene (encoding chemotaxis inhibitory protein; Table 2). Two virulence genes were more common in paediatric MSSA compared with MRSA bacteraemia infections, including *cna* (encoding collagen adhesin) and *coa* (encoding coagulase; Table 2).

PVL was identified in 22% (76/353) of isolates (Table 2). The majority of PVL$^+$ isolates were MSSA (59%, 45/76), from community-onset (92%, 70/76) infections, and occurred in older children (median 8 years [IQR 3–12] vs PVL-negative SAB; median 5 years [IQR 1–11] $P = 0.007$). The most common PVL$^+$ CCs included CC93 (33%, 25/76), CC1 (16%, 12/76) and CC 121 (14%, 11/76) (Supplementary Appendix S7).

3.4. Associations between phenotype, genotype and outcomes

Using univariate or multivariable analyses, no differences were observed between the frequency of any CC, virulence or antimicrobial resistance gene when examined by the 90-day all-cause mortality endpoint (although the low frequency of this outcome [2%, 6/353] precluded some of the analyses; Table 3). For community-onset SAB, variables predictive of the composite outcome (90-day mortality, relapse, LOS >30 days or ICU admission) on univariate analysis included CC93 (OR 3.3 [95% confidence interval [CI] 1.4–8.3], $P = 0.007$) as well as methicillin resistance (OR 1.9 [95% CI 1.0–3.6], $P = 0.03$) and PVL positivity (OR 1.7 [95% CI 1.0–2.8], $P = 0.04$; Table 3). However, the only microbiological variable on multivariable analysis was PVL positivity (aOR 2.6 [95% CI 1.0–6.2]), $P = 0.04$; Table 3), which was predominantly influenced by LOS >30 days within this composite outcome (Supplementary Appendix S6).

All clinical markers of *S. aureus* disease severity examined on univariate analysis were significantly associated with PVL$^+$ SAB compared to PVL$^-$ SAB, including multifocal disease, endovascular infection, multiorgan dysfunction and surgical source control, as well as the number of surgeries, peak C-reactive protein (CRP), days with SAB and fever, LOS and total duration (intravenous ± oral) of antibiotic therapy (Supplementary Appendix S8). These findings remained significant across different SAB clinical phenotypes including pulmonary, osteoarticular and SSTI foci (Supplementary Appendix S8).

Table 3
Univariate and multivariable logistic regression examining (1) paediatric *S. aureus* bacteraemia 90-day all-cause mortality and (2) a composite outcome for community-onset *S. aureus* bacteraemia comprising 90-day all-cause mortality, 90-day relapse, ICU admission or length of stay >30 days

	n (%)	90-day all-cause mortality overall SAB				Composite outcome (community-onset SAB)			
		Univariate analysis		MVL regression		Univariate analysis		MVL regression	
		OR (95% CI)	*P* value	aOR (95% CI)	*P* value	OR (95% CI)	*P* value	aOR (95% CI)	*P* value
Baseline characteristics									
Age in years, median (IQR)[a]	6 (01–11)	0.99 (0.99–1.00)	0.621	1.00 (0.99–1.00)	0.80	0.99 (0.99–0.99)	<0.001[b]	1.56 (0.59–3.95)	0.351
Sex (male) [a]	217 (61)	3.12 (0.50–60.22)	0.301	2.21(0.30–44.37)	0.49	1.25 (0.80–1.96)	0.319	1.45(0.68–3.16)	0.34
Location (country: Australia) [a]	273 (77)	0.58 (0.11–4.24)	0.534	0.63 (0.10–5.63)	5.63	0.57 (0.34–0.94)	0.029[b]	0.60(0.24–1.58)	0.28
Focus of infection									
Osteoarticular focus[c]	166 (47)	-	-			0.45 (0.29–0.71)	0.0005[b]	1.21(0.56–2.64)	0.63
Pleuropulmonary focus[c]	40 (11)	1.58 (0.08–10.14)	0.68			6.01 (2.87–13.84)	<0.001 [b]	5.32 (1.81–16.41)	0.003 [b]
Multifocal SAB[c]	51 (14)	1.18 (0.06–7.52)	0.881			3.11 (1.69–5.89)	<0.001 [b]	2.71(1.00–7.23)	0.046 [b]
Comorbidities									
Congenital heart disease	41 (12)	4.05 (0.55–21.52)	0.113			10.68 (4.66–28.95)	<0.001 [b]	12.95(3.02–68.92)	<0.001 [b]
Prematurity[c]	30 (9)	5.05 (0.68–27.11)	0.068 [b]	5.81 (0.51–64.86)	0.14	11.66 (4.39–40.34)	<0.001 [b]	1.83 (0.19–13.32)	0.566
Severity									
Multiorgan dysfunction[c]	29 (9)	10.96 (1.95–61.89)	0.004 [b]	10.97 (1.5–81.5)	0.015 [b]	47.10 (9.84–845.77)	<0.001[d]		
Frequent *S. aureus* CC									
CC5	74 (21)	0.75 (0.04–4.76)	0.796			1.10 (0.65–1.86)	<0.001 [b]	0.922 (0.28–2.756)	0.887
CC30	44 (12)	-	-			0.82 (0.42–1.58)	0.567		
CC1	41 (12)	1.63 (0.08–10.45)	0.661			1.12 (0.56–2.18)	0.751		
CC45	31(9)	-	-			0.73(0.32–1.59)	0.442		
CC93	26 (7)	2.5 (0.13–16.33)	0.41			3.31(1.43–8.32)	0.007	2.91 (0.51–19.45)	0.244
CC15	21 (6)	3.4 (0.2–22.2)	0.28			0.55 (0.19–1.39)	0.229		
CC8	21 (6)	-	-			1.07 (0.43–2.61)	0.876		
C121	14 (4)	-	-			1.45 (0.49–4.32)	0.498		
S. aureus antimicrobial susceptibility									
MRSA [a]	52 (15)	1.21 (0.06–7.71)	0.864			1.93 (1.05–3.57)	0.035 [b]	0.50(0.10–1.89)	0.346
PSSA	27 (8)	2.40 (0.12–15.63)	0.432			0.83 (0.35–1.83)	0.645		
S. aureus virulence genes									
S. aureus immune evasion genes									
Alpha hemolysin (hla)[e]	342 (97)	-	-			0.69 (0.19–2.54)	0.569		
PVL[e,f]	76 (22)	1.81 (0.25–9.49)	0.496	1.27 (0.09–12.2)	0.84	1.69 (1.01–2.85)	0.047 [b]	2.57(1.04–6.22)	0.038 [b]
Staphylokinase (sak [f])	307 (87)	0.73 (0.11–14.14)	0.775			1.65 (0.86–3.33)	0.142		
Staphylococcal protein A (spa)	220 (62)	1.23 (0.24–8.99)	0.809			1.23(0.79–1.92)	0.365		
Willebrand factor-binding protein (vWbp)	169 (48)	1.10 (0.20–6.01)	0.908			1.53(0.99–2.37)	0.053 [b]	1.13 (0.49–2.66)	0.775
Chemotaxis inhibitory protein (chp)	204 (58)	0.73 (0.13–4.01)	0.707			0.99 (0.64–1.53)	0.961		
S. aureus secrete coagulase (coa)	142 (40)	1.54 (0.28–8.43)	0.6			0.70(0.45–1.09)	0.113		
S. aureus quorum sensing regulators									
Accessory gene regulator subgroup 4 (Agr_subIV [e,f])	21/306 (7)	-	-			1.05 (0.41–2.56)	0.919		
Accessory gene regulator subgroup 3 (Agr_subIII)	97/306 (32)	1.06 (0.15–5.55)	0.943			1.35 (0.82–2.21)	0.236		
Accessory gene regulator subgroup 2 (Agr_subII)	87/306 (28)	1.28 (0.17–6.66)	0.782			0.85 (0.51–1.41)	0.541		
	n (%)	90-day all-cause mortality overall SAB				Composite outcome (community-onset SAB)			
		Univariate analysis		MVL regression		Univariate analysis		MVL regression	
		OR (95% CI)	*P* value	aOR (95% CI)	*P* value	OR (95% CI)	*P* value	aOR (95% CI)	*P* value
Accessory gene regulator subgroup 1 (Agr_subI)	101/306 (33)	1.03 (0.14–5.38)	0.972			0.85(0.52–1.39)	0.523		
S. aureus toxin genes									
Toxin syndrome toxin (tst) [e]	42 (12)	-	-			0.90 (0.45–1.74)	0.760		
S. aureus enterotoxin d (sed) [e]	25 (7)	2.74 (0.14–17.94)	0.367			1.60 (0.70–3.67)	0.259		
S. aureus enterotoxin A (sea) [e]	83 (24)	1.67 (0.23–8.71)	0.56			1.08 (0.65–1.79)	0.759		
S. aureus enterotoxin Q (seq)	36 (10)	-	-			1.39(0.69–2.82)	0.353		
S. aureus enterotoxin L (sel)	39 (11)	-	-			1.32(0.67–2.61)	0.416		
S. aureus adhesin genes									
Collagen adhesin (cna)[e]	34 (10)	1.85 (0.09–11.92)	0.58			0.99 (0.48–2.04)	0.995		
Adhesive protein (sdrC)	266 (75)	1.68 (0.27–32.42)	0.638			0.94(0.57–1.55)	0.808		
Adhesive protein (sdrD)	197 (56)	3.94 (0.63–75.95)	0.213			1.41(0.91–2.20)	0.120		
Adhesive protein (sdrE) [e]	266 (75)	-	-			1.07(0.65–1.77)	0.791		
Fibronectin binding protein A (fnbA) [e]	250 (71)	-	-			1.31(0.82–2.13)	0.264		
Clumping factor B precursor (clfB) [e]	240 (68)	0.90 (0.17–6.57)	0.905			0.97 (0.61–1.55)	0.911		

SAB, *S. aureus* bacteraemia; ICU, intensive care unit; MVL, multivariable logistic regression; OR, odds ratio; aOR, adjusted odds ratio; CI, confidence interval; IQR, interquartile range; CC, clonal complex; MRSA, methicillin resistant *S. aureus*; PSSA, penicillin susceptible *S. aureus*; PVL, Panton-Valentine leukocidin.

[a] Multivariable logistic regression model adjusted for by age, sex, location (by country) and methicillin susceptibility.

[b] $P < 0.05$.

[c] Variables identified from the ISAIAH cohort demonstrated to have impacted outcome in children with SAB. - Unable to perform univariate analysis as one of the variables contained zero events.

[d] Excluded as similar variable comprises composite outcome (ICU admission).

[e] Virulence genes that have been previously implicated in complicated adult and paediatric invasive *S. aureus* disease.

[f] Virulence genes that have previously been implicated in adult invasive *S. aureus* mortality.

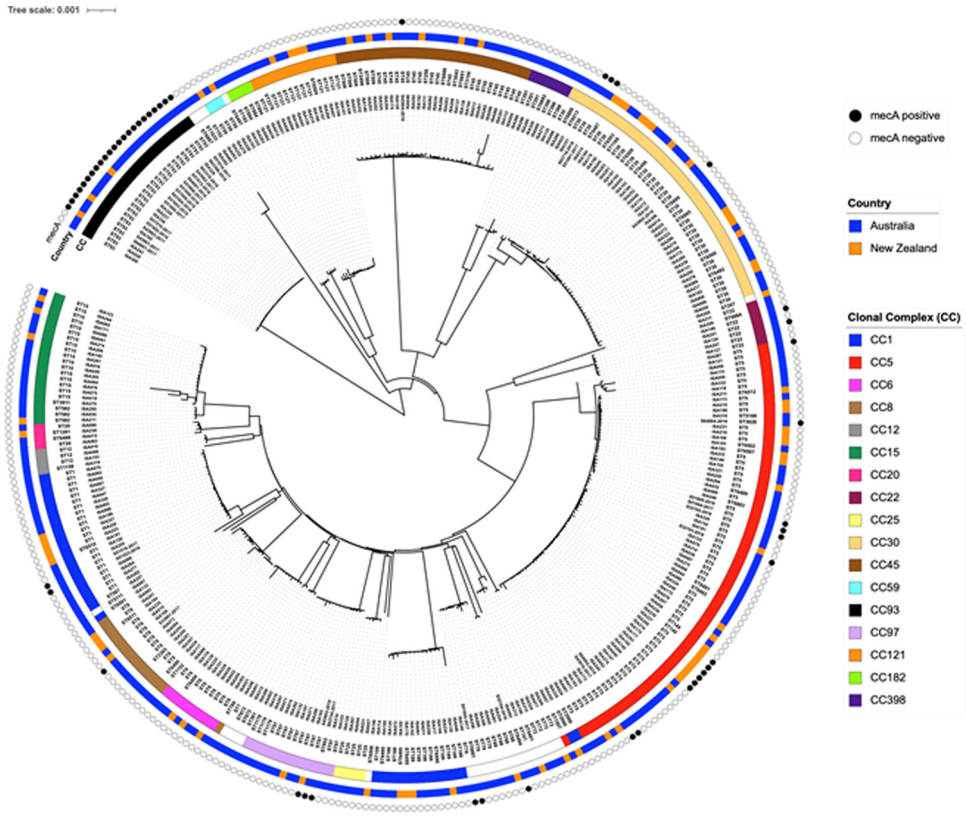

Fig. 3. Phylogenetic tree for methicillin-resistant and methicillin-susceptible paediatric *S. aureus* bacteraemia isolates from Australia and New Zealand. CC, clonal complex. [a]MecA positive refers to the MecA genetic element found in MRSA strains. [b]MecA negative refers to the MecA genetic element not found in MSSA strains.

4. Discussion

We present this analysis from a large repository of WGS data inclusive of both MSSA and MRSA paediatric bacteraemia isolates from the multicentre prospective ISAIAH cohort [2]. Genomic analyses revealed key insights including the virulence of MSSA in the setting of paediatric bacteraemia, the important contribution of MSSA clones to healthcare-associated SAB in children and the predictive association of PVL$^+$ SAB on clinical outcomes in children.

Paediatric molecular analyses incorporating MSSA are currently limited and confined predominantly to SSTI [8,27,28] and to colonisation cohorts [29]. Data presented here highlight the importance of MSSA as a major cause of community-onset infection (84%, 221/264), healthcare-associated SAB (87%, 109/125) and specific MSSA associated virulence genes (*cna* and *coa*). Four published studies include the molecular epidemiology of childhood MSSA bacteraemia, of which only one used WGS and was limited to a Dutch NICU setting (n=112 MSSA bacteraemia) [12]. The other three studies involved children from Poland (n=107; 51 MSSA bacteraemia) [13], the United States (n=222;182 MSSA bacteraemia) [30] and Tunisia (n= 27; 25 MSSA bacteraemia) [31]. Congruent with our study, these four studies demonstrated the predominance of MSSA bacteraemia that accounted for the greatest disease burden, CC diversity and virulence genes [12,13]. Our data also highlight that CC15 and CC8 were MSSA healthcare-associated clones. Locally and internationally MSSA is an under-recognized, leading cause of healthcare-associated infections in children [32,33]. The inclusion of MSSA in molecular epidemiology research to further inform infection prevention policy is critical to advance SAB prevention and treatment.

Using WGS, one MSSA isolate was identified as *S. argenteus,* a coagulase-positive species within the *S. aureus* complex [34], previously thought to be a divergent *S. aureus* clonal lineage. The *S. argenteus* isolate identified in this study was from an eight-year-old with community-onset single focus osteoarticular infection from regional Australia with bacteraemia for a longer duration (4 days) than the overall cohort median of 1 day (IQR 1–3). The patient responded to five weeks total duration of antibiotic therapy with no relapse or death identified.

S. argenteus has been identified in human clinical infection, and from nonhuman primates and bats in Africa [35]. Knowledge regarding *S. argenteus* epidemiology, clinical significance and transmission risk is limited. Emerging evidence suggests that the pathogenicity of *S. argenteus* is similar to that of *S. aureus*. To our knowledge, only one other case of paediatric *S. argenteus* has previously been reported in a child from Japan with lymphadenitis [36]. Our case provides an important addition to the literature regarding the pathogenicity of *S. argenteus* bacteraemia in a paediatric context. We also highlight the presence of a low proportion of *S. argenteus* (0.3%, 1/353) among Australian and New Zealand children with SAB.

The most widely studied *S. aureus* toxin, PVL, has been reported to have a varied and, at times, conflicting impact on *S. aureus* clinical outcomes [11]. From paediatric SAB WGS data here, we demonstrated PVL$^+$ community-onset *S. aureus* infections were associated with a distinct clinical phenotype (e.g., multifocal disease, deep vein thrombosis, necrotizing pneumonia), MSSA bacteraemia, severe disease and poor clinical outcomes. These observations remained true after adjusting for methicillin susceptibility, CC, age and focus of infection. Most of the previous literature reporting on the virulence of PVL$^+$ *S. aureus* infections was in the context of the U.S. 300 MRSA clone [8,37]. Our data are an important addition to the global literature, suggesting that PVL and not its associated clones is a key determinant of poor outcomes in SAB in children. Despite these findings, we did not demonstrate an association between PVL and mortality (acknowledging the small numbers of

deaths limited the ability to detect a difference). The variation in previous reports of the contribution of PVL positivity to disease phenotype was mostly in adult cohorts with a focus on MRSA [38]. Potential confounders in the literature include age (peak incidence of PVL^+ infections are in children and young adults [39]), a focus on hospital-onset infections (most PVL^+ infections are community-onset) [40], or other complex host-pathogen-antimicrobial factors that remain incompletely understood. Further research is required to understand this variation and the potential implications that PVL-producing strains may have on approaches to *S. aureus* clinical management.

Our study has several limitations. The study reports on SAB molecular epidemiology from one region of the world, and this may not be translatable to other regions. Similarly, we did not compare SAB isolates to those causing colonisation or mild infection in children, and these findings may not apply to those settings. Most children in the study were cared for in tertiary paediatric hospitals, which may have introduced selection bias. This study did not examine all *S. aureus* virulence and antimicrobial resistance genes, nor did we examine gene expression, and therefore we may have missed other molecular variables impacting paediatric SAB outcomes. There were some missing clinical data, as it was captured from multiple sources including medical records; however, the mortality endpoint, albeit infrequent, was well documented. Relapse and death may be underestimated, with community, out-of-hospital or private provider presentations or deaths not recorded, although this was estimated to be low. Despite the limitations, our comprehensive genomic analysis of 353 paediatric SAB episodes provides an in-depth description of the molecular phenotype of severe disease and poor outcomes.

5. Conclusion

From this large repository of WGS paediatric *S. aureus* bacteraemia data, we demonstrated the previously under-recognized role MSSA has in harbouring genetic virulence factors and causing healthcare-associated paediatric infections. In addition, despite the relationship some antimicrobial resistance genes, CC type and PVL positivity have on clinical phenotype and disease severity, the only independent molecular predictor of poor clinical outcomes in children with community-onset SAB was PVL positivity. These findings add to the molecular understanding of this common childhood infection and underscore the need for further research to define the potential implications PVL-producing strains may have on approaches to *S. aureus* clinical management.

Declaration of Competing Interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.03.012.

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