Journal of Hospital Infection 132 (2023) 8–19

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevier.com/locate/jhin

An emerging Panton–Valentine leukocidin-positive CC5-meticillin-resistant *Staphylococcus aureus*-IVc clone recovered from hospital and community settings over a 17-year period from 12 countries investigated by whole-genome sequencing

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ARTICLE INFO

Article history:
Received 9 November 2022
Accepted 26 November 2022
Available online 5 December 2022

Keywords:
CC5-MRSA-IVc
Dissemination

SUMMARY

Background: A novel Panton–Valentine leukocidin (PVL)-positive meticillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC)5-MRSA-IVc ('Sri Lankan' clone) was recently described from Sri Lanka. Similar isolates caused a recent Irish hospital outbreak.
Aim: To investigate the international dissemination and diversity of PVL-positive CC5-MRSA-IVc isolates from hospital and community settings using whole-genome sequencing (WGS).
Methods: Core-genome single nucleotide polymorphism (cgSNP) analysis, core-genome multi-locus sequence typing (cgMLST) and microarray-based detection of antimicrobial-resistance and virulence genes were used to investigate PVL-positive CC5-MRSA-IVc (*N* =

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https://doi.org/10.1016/j.jhin.2022.11.015
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Epidemiology
Phylogenomics
PVL
Sri Lankan clone

214 including 46 'Sri Lankan' clone) from hospital and community settings in 12 countries over 17 years. Comparators included 29 PVL-positive and 23 PVL-negative CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V.

Results: Maximum-likelihood cgSNP analysis grouped 209/214 (97.7%) CC5-MRSA-IVc into Clade I; average of 110 cgSNPs between isolates. Clade III contained the five remaining CC5-MRSA-IVc; average of 92 cgSNPs between isolates. Clade II contained seven PVL-positive CC5-MRSA-IVa comparators, whereas the remaining 45 comparators formed an outlier group. Minimum-spanning cgMLST analysis revealed a comparably low average of 57 allelic differences between all CC5/ST5-MRSA-IVc. All 214 CC5/ST5-MRSA-IVc were identified as 'Sri Lankan' clone, predominantly *spa* type t002 (186/214) with low population diversity and harboured a similar range of virulence genes and variable antimicrobial-resistance genes. All 214 Sri Lankan clone isolates and Clade II comparators harboured a 9616-bp chromosomal PVL-encoding phage remnant, suggesting both arose from a PVL-positive meticillin-susceptible ancestor. Over half of Sri Lankan clone isolates were from infections (142/214), and where detailed metadata were available (168/214), most were community associated (85/168).

Conclusions: Stable chromosomal retention of *pvl* may facilitate Sri-Lankan clone dissemination.

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Introduction

Meticillin-resistant *Staphylococcus aureus* (MRSA) contributes significantly to the prevalence of infectious diseases worldwide. Expression of Panton—Valentine leukocidin (PVL), a bicomponent beta-barrel toxin that causes leukocyte lysis or apoptosis via pore formation [1], has been associated with increased MRSA virulence and transmissibility [2—4]. PVL is encoded by the *lukF-PV* and *lukS-PV* genes (known as *pvl*) harboured by lysogenic converting bacteriophages [5]. Carriage of *pvl* was traditionally associated with community-associated (CA)-MRSA, frequently responsible for skin and soft tissue infections (SSTIs), however CA-MRSA lineages are increasingly associated with hospital infection and outbreaks [6—9]. In some cases, highly virulent CA-MRSA with increased transmissibility and greater clonal diversity have surpassed healthcare-associated (HA)-MRSA as the dominant hospital MRSA lineages [7,10,11]. Over the last decade, PVL-positive MRSA clones causing mainly superficial SSTIs have emerged in Irish healthcare and community settings [12,13]. In 2020, the Irish National MRSA Reference Laboratory (NMRSARL) identified *pvl* in 25% of all non-bloodstream infection MRSA submitted for investigation, up from 20% in 2017 and attributed this to an increase in HA-outbreaks [14,15].

McTavish *et al.* recently described a dominant PVL-positive clonal complex (CC) 5 MRSA lineage harbouring a type IVc staphylococcal cassette chromosome *mec* (SCC*mec*) element in Sri Lanka and also identified it in the UK and Australia, referred to hereafter as the 'Sri-Lankan clone' [16]. This lineage was also recently reported in the United Arab Emirates [17]. The emergence of a PVL-positive CC5-MRSA-IVc lineage in Irish hospitals was reported in 2021 [13]. Multiple suspected PVL-positive CA-MRSA outbreaks were investigated and a PVL-positive CC5-MRSA-IVc lineage was identified as being responsible for a 15-month maternity unit outbreak involving 13 patients. The widespread dissemination of novel MRSA lineages with subsequent replacement of predominant clonal types is not uncommon and has been described previously in Ireland and internationally [18—21].

To date, only localized investigations focused on country-specific genomic characterization of PVL-positive ST5-MRSA-IVc isolates, such as the Sri-Lankan clone, have been reported [13,16,17]. This study sought to further investigate the PVL-positive CC5-MRSA-IVc population from Irish hospital and community settings in comparison with a comprehensive collection of similar isolates from 12 countries spanning 17 years using whole-genome sequencing (WGS). WGS provides unrivalled sensitivity and precision for comparing and monitoring the development and spread of historical, current, and emerging clones, as well as tracing infection outbreaks with extremely high resolution.

Methods

MRSA isolates

MRSA isolates ($N = 266$) recovered between 2003 and 2022 were investigated: (1) 214 PVL-positive CC5/sequence type (ST)5-MRSA-IVc isolates (2005—2022) from 12 countries similar to and including 46 previously described Sri-Lankan clone isolates [16] and (2) 52 comparator CC5/ST5-MRSA—SCC—I/II/IVa/IVc/IVg/V isolates (29 PVL-positive and 23 PVL-negative) recovered between 2003 and 2021. Isolates were cryogenically stored at -80 °C. Detailed isolate information and available metadata are provided in Table I and in Supplementary Table S1.

Irish MRSA

All 47 Irish MRSA isolates investigated were submitted to the NMRSARL between 2013 and 2022. These included 14 previously described PVL-positive ST5/t002-MRSA-IVc maternity unit outbreak isolates recovered between 2018 and 2020 that were similar to the Sri-Lankan clone and had a median of three (average:

Table I
Antimicrobial resistance and virulence-associated gene profiles of 214 Panton–Valentine leukocidin (PVL)-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional PVL-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates investigated

Country	Isolates (*N*)	Year(s) of isolation	*spa*-ST-SCC*mec* (*N*)	Antimicrobial resistance genes (*N*)	PVL (+/−)	IEC type (*N*)	Reference
Algeria	Comparator (1)	2003	t450-ST5-IVa	*aadD*, *erm*(C), *fosB*, *lmrP*, *mprF*, *tet*(M), *vga*(A), *sdrM*	+	B	This study
Australia	Sri-Lankan clone (1)	2015	t002-ST5-IVc	*blaZ*, *fosB*, *lmrP*, *sdrM*	+	G	McTavish *et al.* [16]
Czech Republic	Sri-Lankan clone (6)	2018–2021	t002-ST5-IVc	*blaZ* (4), *fosB* (6), *lmrP* (5), *sdrM* (6), *mprF* (6), *erm*(C) (1)	+	G (4) E (1) Novel type 3 (*sak*, *sep*) (1)	This study
	Comparators (2)	2019–2021	t002-ST5-IVa (1) t002-ST5-II (1)	*aadD* (1), *blaZ* (2), *erm*(A) (1), *fosB* (2), *kdpA/B/C/D/E* (1), *lmrP* (2), *mprF* (2), *sdrM* (2), *xylR* (1)	−	G (1) B (1)	
Denmark	Sri-Lankan clone (66)	2007–2021	t002-ST5-IVc	*blaZ* (61), *fosB* (65), *lmrP* (65), *mprF* (65), *sdrM* (66), *erm*(C) (31), *tet*(K) (3), *mupA* (3), *qacA* (3), *qacC* (1), *cat* (1)	+	G (59) F (7)	This study
	Comparators (10)	2013–2015	t002-ST5-IVa (2) t002-ST5-V (8)	*blaZ* (9), *fosB* (10), *lmrP* (10), *mprF* (10), *sdrM* (10), *aacA-aphD* (7), *erm*(C) (2), *tet*(K) (1)	+ (7) − (3)	G (8) F (1) B (1)	
Germany	Sri-Lankan clone (20)	2011–2019	t002-ST5-IVc (15) t535-ST5-IVc (2) t579-ST5-IVc (1) ND-ST5-IVc (2)	*blaZ* (18), *erm*(C) (11), *fosB* (20), *lmrP* (20), *mprF* (20), *sdrM* (19), *tet*(K) (1), *qacA* (1), *msr*(A) (1)	+	G (16) E (1) Novel type 1 (*sep* only) (1) Novel type 2 (*sak*, *scn*, *sea*, *sep*) (1)	This study
	Comparators (2)	2014–2017	t105-ST5-IVc	*blaZ* (1), *fosB* (1), *lmrP* (1), *mprF* (2), *sdrM* (1)	+	B	
Ireland	Sri-Lankan clone (30)	2013–2022	t002-ST5-IVc	*blaZ* (29), *fosB* (30), *lmrP* (30), *mprF* (30), *sdrM* (30), *erm*(C) (5)	+	G (29) None (1)	This study, McManus *et al.* [13]
	Comparator (17)	2013–2019	t002-ST5-I (1) t002-ST5-IVa (2) t002-ST5-IVc (3) t002-ST5-IVg (2) t311-ST5-V (9)	*blaZ* (13), *erm*(C) (10), *fosB* (17), *lmrP* (17), *mprF* (17), *sdrM* (17), *fusC* (10), *fexA* (1), *aadD* (1), *qacA* (1), *merA* (1)	+ (2) − (15)	G (2) F (2) B (3) E (9) Novel type 1 (*sep* only) (1)	
Kuwait	Sri-Lankan clone (1)	2013	t002-ST5-IVc	*blaZ*, *erm*(C), *fosB*, *lmrP*, *mprF*, *sdrM*	+	G	This study
	Comparator (1)	2013	t002-ST5-IVa	*blaZ*, *erm*(C), *fosB*, *lmrP*, *mprF*, *sdrM*	+	G	
Norway	Sri-Lankan clone (24)	2007–2021	t002-ST5-IVc (23) t1062-ST5-IVc (1)	*blaZ* (23), *fosB* (24), *lmrP* (24), *mprF* (24), *sdrM* (24), *erm*(C) (9), *tet*(K) (2), *vga*(A) (1)	+	G (23) Novel Type 2 (*sak*, *scn*, *sea*, *sep*) (1)	This study

	Comparators (12)	2003–2020	t311-ST5-IVa (4) t311-ST5-IVc (2) t002-ST5-IVa (3) t105-ST5-IVc (1) t3089-ST5-IVa (1) t442-ST5-V (1)	*blaZ* (12), *fosB* (12), *lmrP* (12), *mprF* (12), *sdrM* (12), *erm*(C) (1), *tet*(K) (1), *aacA-aphD* (2), *dfrA* (2), *tet*(M) (2), *aphA3* (3), *mph*(C) (1), *msr*(A) (1), *sat* (3), *qacC* (1)	+ (11) − (1)	G (3) B (5) A (4)	
Saudi Arabia	Sri-Lankan clone (4)	2010–2017	t002-ST5-IVc	*blaZ* (3), *fosB* (4), *lmrP* (4), *mprF* (4), *sdrM* (4), *erm*(C) (2), *aphA3* (1), *sat* (1)	+	G (4)	This study
	Comparator (1)	2010	t311-ST5-IVa	*blaZ, fosB, lmrP, mprF, sdrM*	−	B	
Senegal	Comparators (2)	2007	t311-ST5-IVa	*aacA-aphD* (1), *aadD* (1), *blaZ* (1), *dfrA* (2), *fosB* (2), *lmrP* (2), *mprF* (2), *qacC* (1), *tet*(M) (2), *sdrM* (2)	+	B (2)	This study
Slovakia	Comparator (1)	2020	t002-ST5-IVc	*blaZ, fosB, lmrP, mprF, sdrM*	−	B	This study
Sri Lanka	Sri-Lankan clone (33)	2014	t002-ST5-IVc (21) t010-ST5-IVc (1) t045-ST5-IVc (2) t062-ST5-IVc (4) t1062-ST5-IVc (1) ND-ST5-IVc (4)	*blaZ* (33), *fosB* (33), *lmrP* (33), *mprF* (33), *sdrM* (33), *erm*(C) (14), *tet*(K) (4)	+	G (31) Novel type 1 (*sep* only) (1) Novel Type 2 *(sak, scn, sea, sep)* (1)	McTavish *et al.* [16]
Sweden	Sri-Lankan clone (2)	2005–2009	t002-ST5-IVc	*blaZ* (2), *erm*(C) (1), *fosB* (2), *lmrP* (2), *mprF* (2), *sdrM* (2), *tet*(K) (1)	+	G (2)	This study
United Arab Emirates	Sri-Lankan clone (15)	2017–2019	t002-ST5-IVc (12) t010-ST5-IVc (1) t045-ST5-IVc (1) t306-ST5-IVc (1)	*blaZ* (15), *erm*(C) (9), *fosB* (15), *lmrP* (17), *mprF* (17), *sdrM* (17)	+	G (15)	This study
	Comparator (3)	2018	t105-ST5-IVc (2) t002-ST5-IVa (1)	*blaZ* (1), *erm*(C) (1), *fosB* (3), *lmrP* (3), *mprF* (3), *sdrM* (3)	+	G (1) B (2)	
UK	Sri-Lankan clone (12)	2005–2015	t002-ST5-IVc (5) t062-ST5-IVc (2) ND-ST5-IVc (5)	*blaZ* (12), *fosB* (12), *lmrP* (12), *mprF* (12), *sdrM* (12), *erm*(C) (4), *tet*(K) (2), *sat* (1)	+	G (11) D (1)	McTavish *et al.* [16]

Genotypic information for 266 study isolates (Sri-Lankan clone and comparator isolates) recovered from 15 different countries between 2003 and 2022. Sri-Lankan clone isolates were recovered from 12 countries across Europe, Asia, Australia, and the Middle East between 2005 and 2022. 214 PVL+ CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional PVL-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates were investigated. The isolates were subjected to whole-genome sequencing and subsequent analyses and profiling to determine antimicrobial resistance gene patterns and virulence gene profiles. Genotypic information was extracted from whole-genome data using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany) genotyping, and *S. aureus* Genotyping Kit 2.0 (Abbott) microarray technology [19,23–25]. The isolates also underwent core-genome multi-locus sequence typing and single nucleotide polymorphic analyses.

IEC, immune evasion cluster; ND, not determined – isolates not available and *spa* types could not be determined using *in-silico* techniques on the available genomic sequence data; *sak*, staphylokinase gene; SCC*mec*, staphylococcal chromosomal cassette harbouring *mecA*; *scn*, staphylococcal complement inhibitor; *sea*, staphylococcal enterotoxin a gene; *sep*, staphylococcal enterotoxin p gene; ST, sequence type.

six; range: 0–27) core-genome multi-locus sequence type (cgMLST) allelic differences between isolates [13]. The emergence of this clonal type in Irish hospitals appeared to be recent but a search of the NMRSARL collection for PVL-positive t002-MRSA-IVc and related *spa* types revealed 16 additional isolates recovered between 2013 and 2022 (Table I and Supplementary Table S1). These included two more recent (2021) patient isolates from the maternity unit and 14 patient isolates from nine other hospitals. Seventeen comparator NMRSARL isolates (two PVL-positive ST5-MRSA-IVa and 15 PVL-negative ST5-MRSA-I/IVc/IVg/V) recovered between 2013 and 2019 were also investigated (Table I and Supplementary Table S1).

International MRSA

Additional PVL-positive ST5-MRSA-IVc isolates or WGS datasets from disparate geographical locations were sought for comparison. Contact with international collaborators, an extensive search of the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA)/GenBank, the European Nucleotide Archive (ENA) databases and a literature search (Supplementary Table S2) yielded 219 international isolates or WGS datasets (80 clinical isolates and 139 WGS sequences; Table I and Supplementary Table S1). Of these, 184 were PVL-positive ST5-MRSA-IVc isolates similar to the Sri-Lankan clone, whereas 35 were PVL-positive ($N = 27$) and PVL-negative ($N = 8$) ST5-MRSA-II/IVa/IVc/V comparator isolates.

Clinical isolates

Fifty-six PVL-positive ST5-MRSA-IVc isolates recovered between 2005–2021 in the Czech Republic ($N = 6$), Germany ($N = 4$), Kuwait ($N = 1$), Norway ($N = 24$), Saudi Arabia ($N = 4$), Sweden ($N = 2$) and the United Arab Emirates (UAE) ($N = 15$) underwent WGS at the Dublin Dental University Hospital Microbiology Research Unit (Ireland) (Table I and Supplementary Table S1). Twenty-four ST5-MRSA-II/IVa/IVc/V comparator isolates (19 PVL-positive and five PVL-negative) recovered in Algeria ($N = 1$), the Czech Republic ($N = 2$), Germany ($N = 1$), Kuwait ($N = 1$), Norway ($N = 12$), Saudi Arabia ($N = 1$), Senegal ($N = 2$), Slovakia ($N = 1$) and the UAE ($N = 3$) between 2003 and 2021 were also sequenced (Table I and Supplementary Table S1).

Whole-genome sequences

WGS datasets for 46 previously described PVL-positive ST5-MRSA-IVc Sri-Lankan clone isolates were downloaded from ENA (accession number PRJEB27049) [16]. These patient isolates were recovered in a Sri-Lankan hospital over four months in 2014 ($N = 33$), the UK between 2005 and 2015 ($N = 12$) and Australia in 2015 ($N = 1$) (Table I). WGS datasets for PVL-positive ST5-MRSA-IVc isolates from Denmark (2007–2021) ($N = 66$) and Germany (2011–2019) ($N = 16$) were received. Comparator STST5-MRSA-IVa/IVc/V WGS datasets from Denmark (seven PVL-positive and three PVL-negative; 2013–2015) and Germany (one PVL-positive; 2017) were also included (Table I and Supplementary Table S1).

Genomic DNA extraction and whole-genome sequencing

For short-read sequencing, genomic DNA was extracted and sequencing libraries prepared using the Illumina® DNA Prep Kit (Illumina, Eindhoven, The Netherlands) as described previously [19]. Libraries were scaled to exhibit $\geq 50\times$ coverage and sequenced using a 600-cycle MiSeq paired-end Reagent Kit v3 (Illumina) on an Illumina MiSeq sequencer according to the manufacturer's instructions. Short- and long-read datasets for isolates sequenced in Dublin were submitted to the NCBI SRA database under BioProject Nos. PRJNA896922 and PRJNA638834). Short-read datasets for Danish isolates were submitted to the NCBI SRA database under BioProject Nos. PRJNA839593, PRJNA865897, PRJNA869909 and PRJNA898141.

For long-read sequencing, genomic DNA extractions and library preparations were performed as described previously [22]. Sequencing was performed on the MinION platform using a R9.4.1 Flow Cell with the MinKNOW software v20.10 (Oxford Nanopore Technologies, UK) as per manufacturer's instructions.

Hybrid assemblies were performed by genome scaffolding using paired-end short-read and long-read sequences using the Unicycler v0.5.0 pipeline (https://github.com/rrwick/Unicycler). Assembled genomes were annotated using the web-based RAST v2.0 server (https://rast.nmpdr.org) and visualized using Bandage v0.8.1 (https://rrwick.github.io/Bandage/) and SnapGene v6.0.6 (GSL Biotech LLC; https://www.snapgene.com).

Whole-genome sequence analysis

Short-read FASTQ files were assembled, quality assessed and analysed using BioNumerics software (BioNumerics v8.0; Applied Maths, Sint-Martens-Latem, Belgium), Ridom SeqSphere+ software v7.0.4 (Ridom GmbH, Münster, Germany) and web-based SCC*mec*Finder tool (https://cge.cbs.dtu.dk/services/SCCmecFinder/) as described previously [13].

Molecular characterization

DNA microarray profiling was undertaken using the *S. aureus* Genotyping Kit 2.0 (Abbott (Alere Technologies GmbH), Jena, Germany) or WGS analysis. The DNA microarray chip harbours 333 target sequences for approximately 170 antimicrobial-resistance and virulence-associated genes and other genes and sequences that can assign *S. aureus* to CCs and/or STs as described previously [23,24]. WGS-based DNA microarray profiling was undertaken using *in silico* probes of the *S. aureus* Genotyping Kit 2.0. Probe sequences map on to assembled genomes to predict DNA array hybridization patterns [25] and these patterns were compared with *in vitro* array results. Additional investigations into alleles of interest were performed using the Clustal Omega multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/), and NCBI BLAST search engine (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *S. aureus* immune evasion cluster (IEC) types were assigned using microarray profiling. β-haemolysin converting bacteriophages encode combinations of the IEC genes (*sea*/*sak*/*scn*/*sep*/*chp*), permitting isolates to be clustered into one of eight IEC types based on the combination of genes carried [26].

Phylogenetic analysis

Relatedness between MRSA recovered over an extended time period (2003–2022) was investigated using cgMLST. A

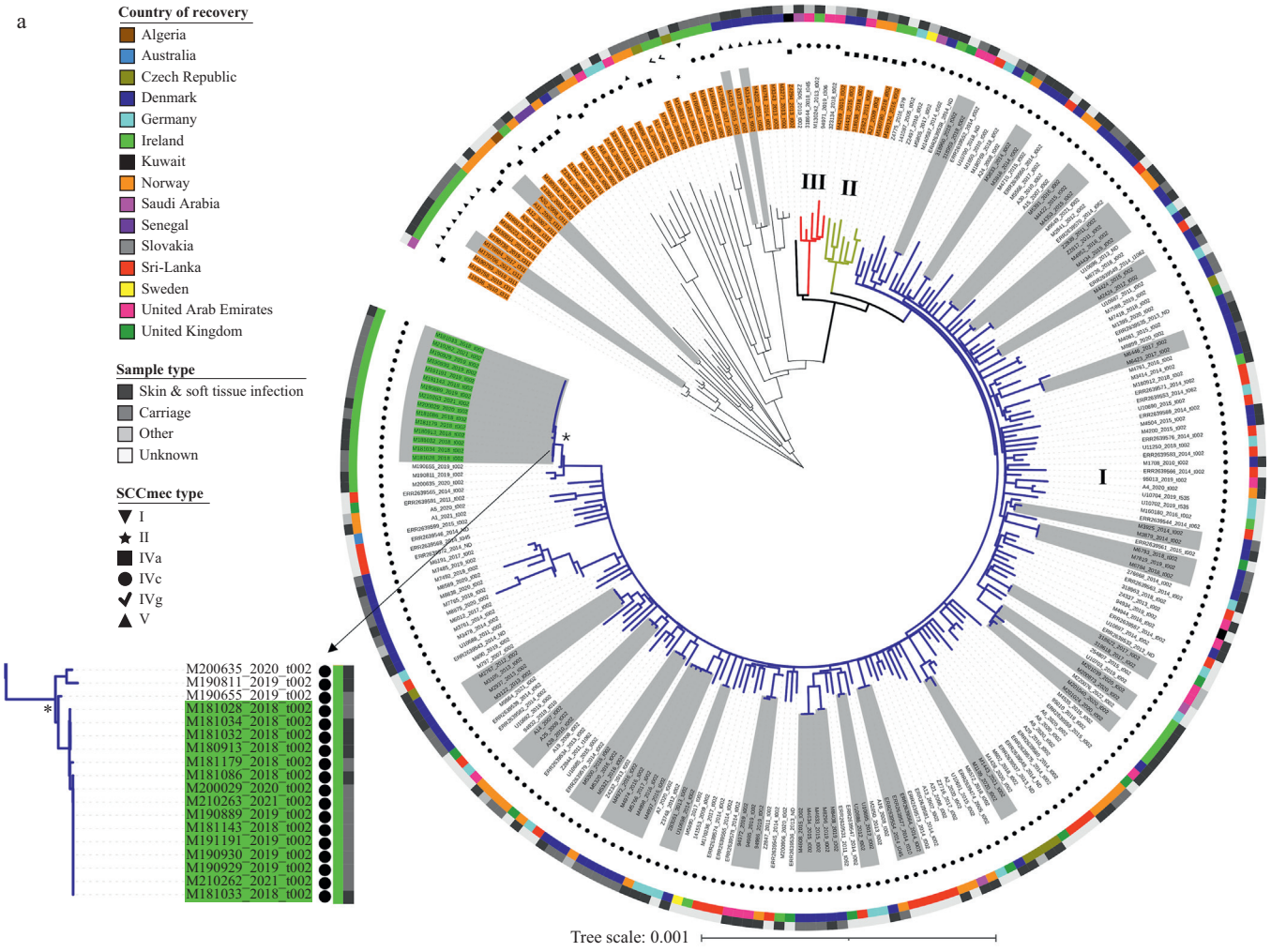

Figure 1. (a) Maximum likelihood tree (MLT) based on phylogenetic analysis of 12,245 core-genome single nucleotide polymorphisms (cgSNPs) for 214 PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional Panton–Valentine leukocidin (PVL)-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates. Sri-Lankan clone isolates were recovered from 12 countries across Europe, Asia, Australia and the Middle East between 2005–2022. Separate node colours/shapes represent country of recovery, sample types and SCC*mec* types as indicated in the legend. Blue branches represent PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates ($N = 209/214$) forming Clade I. Green branches represent PVL-positive CC5/ST5-MRSA-IVa comparator isolates ($N = 7/52$) forming Clade II. Red branches represent the remaining PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates ($N = 5/214$) forming Clade III. The thick black branch represents a PVL-positive CC5/ST5-MRSA-IVa comparator isolate ($n = 1$) branching out next to Clade III. The thin black branches represent the comparator outgroup isolates ($N = 44$) which separate away from Clades I–III. Labels for the 52 comparator isolates are highlighted in orange. Isolate names, year of recovery and *spa* types are all indicated in the branch labelling. Country-specific isolate pairs or clusters containing closely related isolates that differed by ≤10 cgSNPs are shaded in grey. The divergent subgroup of 15 Irish isolates (lacking the *bbp* gene) within the large Sri-Lankan clade (Clade I) is indicated by an asterisk and the isolate names are highlighted in green. The epidemiological and genotypic information for each isolate investigated is provided in Table I and Supplementary Table S1. Corresponding SNP distance matrix data is provided in Supplementary Table S3. (b) Minimum spanning tree (MST) based on core genome multi-locus sequence type (cgMLST) analysis of 1861 target genes for 214 PVL-positive CC5/ST5-MRSA-IVc Sri-Lankan clone isolates and 52 additional PVL-positive ($N = 29$) and PVL-negative ($N = 23$) CC5/ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates. Separate node colours represent country of isolation as indicated in the legend. Partitions within nodes represent the presence of ≥2 isolates per node. Comparator isolates are indicated in by red squares. Closely related clusters of isolates (≤20 allelic differences to the closest neighbouring isolate within the RIG) are outlined within grey shadowing. Branch numbers indicate the number of allelic differences between neighbouring isolates. Node numbers indicate the 36 related isolate groups (RIGs) in the population (Supplementary Table S1). The subgroup of closely related Irish isolates consisting of 15 isolates with a distinct genotypic profile to other Sri-Lankan clone isolates is outlined in green. The corresponding cgMLST pairwise isolate distance matrix is provided in Supplementary Table S4. The cgMLST-based MST was constructed using Ridom SeqSphere+ v7.0.4 (Ridom GmbH, Münster, Germany).

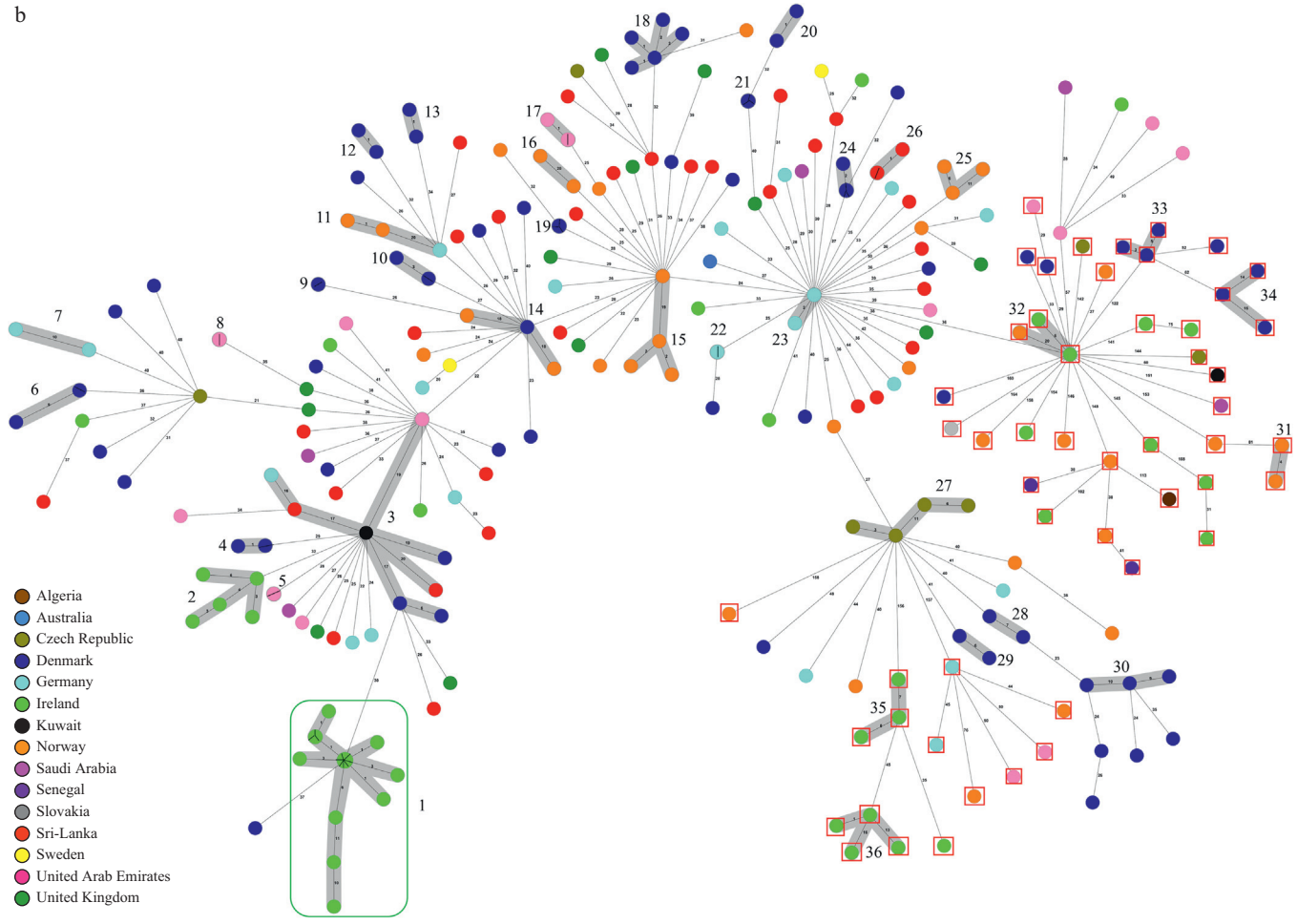

Figure 1. (continued)

minimum spanning tree (MST) based on 1861 core-gene loci was generated for all isolates using the SeqSphere+ (Ridom) cgMLST scheme as previously described [27,28]. Core-genome alignment and variant calling based on single-nucleotide polymorphisms (SNPs) was performed on all isolates mapped against a study-specific reference genome from MRSA isolate 141087 (2005; earliest year of recovery for Sri-Lankan clone isolates investigated), using Snippy v4.6.0 (https://github.com/tseemann/snippy). Recombinant SNPs were removed using Gubbins v3.2.1 (https://github.com/nickjcroucher/gubbins) and a pairwise cgSNP distance matrix generated using snp-dists v3 (https://github.com/tseemann/snp-dists). A cgSNP-based maximum-likelihood tree (MLT) was constructed through IQ-TREE v2.2.0 (http://www.iqtree.org) using recommended IQ-TREE guidelines. The phylogenetic tree was visualized and annotated through Interactive Tree of Life v6.5.8 (https://itol.embl.de).

Results

MRSA

MRSA isolates ($N = 266$) recovered between 2003 and 2022 were investigated. These included 214 PVL-positive CC5/ST5-MRSA-IVc isolates from 12 countries similar to and including 46 'Sri-Lankan clone' isolates [16] and 29 PVL-positive and 23 PVL-negative ST5-MRSA-I/II/IVa/IVc/IVg/V comparator isolates (Table I and Supplementary Table S1). The PVL-positive ST5-MRSA-IVc isolates belonged to eight closely related *spa* types, with t002 predominating (186/214; 86.9%) (Table I and Supplementary Table S1). Six closely related *spa* types were identified among the comparator isolates, half of which were t002 (26/52; 50%) (Table I and Supplementary Table S1).

Phylogenetic analysis of Sri-Lankan clone isolates

To investigate the population structure of Irish PVL-positive CC5/ST5-MRSA-IVc relative to international isolates, all isolates and comparators were subjected to WGS-based phylogenetic analyses. The construction of SNP-based MLT and cgMLST-based MST trees yielded comparable findings regarding isolate relatedness and clustering (Figure 1a, b).

Core-genome SNP analysis

CgSNP analysis based on 12,245 SNPs showed that all 214 PVL-positive CC5/ST5-MRSA-IVc isolates exhibited a pairwise SNP-distance median of 107 (average: 116; range: 0—410) (Supplementary Table S3). The SNP-based MLT grouped the vast majority of isolates (209/214, 97.7%) including all 46 'Sri-

Lankan clone' isolates [16] into one clade, termed Clade I (Figure 1a). Clade I isolates had a median of 106 (average: 110; range: 0–287) SNPs between isolates (Supplementary Table S3). The remaining five PVL-positive ST5-MRSA-IVc grouped into Clade III exhibiting a median of 86 (average: 92; range: 69–127) SNPs (Figure 1a). Clade III differed from Clade I by a median of 232 (average: 237; range: 159–410) SNPs. Most comparator isolates (44/52) formed an outgroup at the base of the MLT (Figure 1a). A single PVL-positive CC5/ST5-MRSA-IVa comparator (isolate Z4294) branched out next to Clade III (thick black branch in Figure 1a). The remaining seven comparators (all PVL-positive ST5/t002-MRSA-IVa) grouped into Clade II, forming the closest neighbour to Clade I (Figure 1a). Clade II differed from Clades I and III by a median of 176 (average: 178; range: 123–331) and 230 (average: 234; range: 207–277) SNPs, respectively (Supplementary Table S3). This tree topology confirmed the identity of all 214 CC5/ST5-MRSA-IVc as 'Sri-Lankan clone'. Hereafter Clades I and III isolates are referred to as 'Sri-Lankan clone'. In general, Sri-Lankan clone isolates did not group according to their country of origin or year of recovery; however, 24 small country-specific clusters of closely related isolates that differed by ≤10 cgSNPs were evident (Figure 1a).

Core-genome MLST analysis

As cgSNP analysis revealed low genotypic diversity among Sri-Lankan clone isolates recovered over 17 years, the previously recommended threshold of ≤24 cgMLST allelic differences for defining closely related isolates [28] was lowered to ≤20. The 214 Sri-Lankan clone isolates exhibited a median of 55 allelic differences from one another (average: 59; range: 0–200) (Supplementary Table S4). The 209 Clade I and five Clade III Sri-Lankan clone isolates exhibited a median of 54 (average: 57; range: 0–153) and 42 (average: 43; range: 28–56) allelic differences, respectively. Clade III isolates differed from Clade I by a median of 116 (average: 117; range: 70–200) allelic differences. Clade II comparator isolates differed from Sri-Lankan clone Clade I and Clade III by a median of 87 (average: 86; range: 36–174) and 106 (average: 93; range: 28–134) allelic differences, respectively (Supplementary Table S4). These findings confirmed limited diversity within the Sri-Lankan clone population.

Thirty-six related isolate groups (RIGs) comprising 123/266 study isolates were evident in the cgMLST-based MST (Figure 1b, Supplementary Table S5). Isolates within each RIG exhibited ≤20 allelic differences to the closest neighbouring isolate in the RIG. Most RIGs included Sri-Lankan clone isolates only (30/36 RIGs) and the remaining six (RIGs 31–36) included comparator isolates only (Figure 1b and Supplementary Table S5). There were 32 country-specific RIGs (27 Sri-Lankan clone isolates only (RIGs 1–2, 4–10, 12–13, 15–30) and five comparator isolates only (RIGs 31, 33–36)) as follows: Denmark ($N = 16$), Norway ($N = 4$), Ireland ($N = 4$), Germany ($N = 3$), UAE ($N = 3$), Sri Lanka ($N = 1$) and Czech Republic ($N = 1$). The remaining four RIGs comprised isolates from two or more countries. RIG-3 comprised eight Sri-Lankan clone isolates from Denmark ($N = 3$), Sri Lanka ($N = 2$), Germany ($N = 1$), Kuwait ($N = 1$) and the UAE ($N = 1$), with an allelic difference range of 6–20 between neighbouring isolates in the RIG and a range of 6–36 allelic differences for the entire RIG (Figure 1b and Supplementary Table S5). Sri-Lankan clone isolates from Norway ($N = 2$) formed two separate RIGs (RIG-11 and RIG-14) with Sri-Lankan clone isolates from Germany ($N = 1$) and Denmark ($N = 1$) with allelic difference ranges of 1–21 and 18–20, respectively. One comparator isolate from Norway formed a third RIG (RIG-32) with two comparator isolates from Ireland.

Irish sub-clade

Potential sub-clades (RIGs 1–36) observed on the cgSNP-based MLT and cgMLST-based MST phylogenetic trees were further investigated using *in silico* DNA microarray profiling and WGS data to identify possible RIG/country-specific characteristics. A genotypic difference was observed between the overall Sri-Lankan clone population and 15/18 Irish Sri-Lankan clone isolates in RIG-1. These 15 isolates formed a distinct Irish sub-clade within the cgSNP-based MLT (Figure 1a) and lacked the *bbp* gene (also known as *sdrE*) encoding a surface-associated, bone sialoprotein-binding protein. The absence of *bbp* in these 15 isolates was confirmed by analysing hybrid assembled genomes (Supplementary Figure S1a).

Genotypic profiling of the Sri-Lankan clone

Strain assignment and antimicrobial resistance genes

DNA microarray profiling, SCC*mec*Finder and Ridom Seqsphere+ template tools for detection of antimicrobial-resistance and virulence-associated genes revealed that most genes in Sri-Lankan clone isolates ($N = 214$) were homogenously distributed (Supplementary Table S1). Microarray analysis grouped Sri-Lankan clone isolates into two categories including 'CC5-MRSA-IVc (*sed*/*sej*/*ser*+)' (200/214; 93.5%) and 'CC5-MRSA-IVc (*sed*/*sej*/*ser*-)' (14/214; 6.5%). The two groups differed by the presence/absence of the *sed*/*sej*/*ser* enterotoxin genes, which were located on a plasmid of approximately 27 kb. Of the five Sri-Lankan clone Clade III isolates (Figure 1a), four isolates were "CC5-MRSA-IVc (*sed*/*sej*/*ser*-)" (Supplementary Table S1). The β-lactamase gene *blaZ* and the multi-drug transporter encoding gene *lmrP* mediating resistance to macrolides, lincosamides, streptogramins and tetracycline was harboured by the majority of Sri-Lankan clone isolates (202/214; 94.4% and 212/214; 99.1%, respectively). Antimicrobial genes detected are shown in Table I and Supplementary Table S1.

IEC types

IEC-type G was predominant amongst Sri-Lankan clone isolates (196/214; 91.6%). The remaining isolates harboured IEC-type F (7/214; 3.3%), IEC-type E (2/214; 0.9%), IEC-type D (1/214; 0.5%) or were non-typeable IEC variants harbouring *sep* only (2/214; 0.9%), *sak* and *sep* (1/214; 0.5%) or *sak*, *scn* and *sea-sep* (4/214; 1.9%). One Sri-Lankan clone isolate (M130242; Supplementary Table S1) lacked lysogenic β-haemolysin converting bacteriophages and carried no IEC genes.

Epidemiological data

Where detailed metadata were available (168/214; 78.5%), just over half of Sri-Lankan clone isolates were CA-MRSA (85/168; 50.6%), while the remainder (50/168; 29.8%) were HA-MRSA or were from hospitalized patients (33/168; 19.6%) (Supplementary Table S1). Most isolates were from infection sites (142/214; 66.3%), with the remainder from carriage (50/214; 23.4%) or unknown sites (22/214; 10.3%). The majority of

infection isolates were from SSTIs (83/142, 58.5%), other infection types (9/142) or were unknown (50/142).

Epidemiological information available for some Sri-Lankan clone isolates from Denmark ($N = 9$), Ireland ($N = 2$) and the UAE ($N = 1$) revealed that these isolates were from patients with international links. The Irish isolates were recovered from patients with a history of travel to Sri Lanka and Turkey, respectively. Within the Danish subset, one patient had been hospitalized in Vietnam, four were from Sri-Lanka and four had travelled to Sri-Lanka. The isolate from the UAE was from a patient from Bangladesh (Supplementary Table S1).

pvl-encoding bacteriophage regions

Clade I and III Sri-Lankan clone ($N = 214$) and Clade II ($N = 7$) comparator isolate short-read assembled genomes were investigated for *pvl*-associated bacteriophage DNA. All isolates harboured the *lukF/S*-PV genes, the phage lysis genes encoding amidase and holin and remnants of phage structural genes encoding the tail fiber and major teichoic acid biosynthesis protein. Genes associated with lysogeny, DNA replication/transcriptional regulation and packaging/structure were not detected [29].

Twenty-six representative Sri-Lankan clone isolates (24 MLT Clade I and two MLT Clade III isolates) available for long- and short-read sequencing underwent hybrid-assembly to further investigate chromosomal regions surrounding *pvl*. These 26/214 isolates (2005–2021) were from Czech Republic ($N = 1$), Denmark ($N = 6$), Germany ($N = 2$), Ireland ($N = 8$), Kuwait ($N = 1$), Norway ($N = 3$), Saudi Arabia ($N = 2$), Sweden ($N = 1$) and the UAE ($N = 2$). Additionally, eight comparator isolates underwent hybrid-assembly (five outgroup and three MLT Clade II comparators, Supplementary Table S1). All 26 Sri-Lankan clone isolates lacked an intact lysogenized *pvl*-encoding phage genome, but harboured a chromosomal remnant encoding the *lukF/S-PV* genes as well as remnants of phage structural and lysis genes (Supplementary Figure S2). In each case, the phage remnant was 9,616 bp, with an intact upstream attachment site (*attL*), but no downstream attachment site (*attR*) (Supplementary Figure S2b). An identical phage remnant was observed in the three Clade II comparator isolates and the single PVL-positive ST5-MRSA-IVa comparator isolate (Z4294) next to Clade III (Figure 1a). The four remaining outgroup comparator isolates all harboured a complete bacteriophage genome of ~45,000 bp which shared 99.99% sequence homology with the well-characterized PVL-encoding phage phiSa2wa (accession no. ON989481.1) (Supplementary Figure S2a) [29]. The phage remnant exhibited 100% sequence homology with the 3′ junction of phage phiSa2wa (Supplementary Figure S2b). Chromosomal sequences adjacent the *pvl*-phage remnant were identical in all isolates investigated by hybrid assembly.

Discussion

The emergence of PVL-positive MRSA is a public health concern globally. These organisms were originally associated with community-onset infections, especially SSTIs but also including necrotizing pneumonia, necrotizing fasciitis, and sepsis [2–4,30,31]. Patients with community onset SSTIs often seek treatment in hospital emergency departments, providing entry routes for CA-MRSA clones into hospitals [32,33]. The spread of PVL-positive CA-MRSA clones into hospitals and resistance to a wide range of antimicrobials is well documented [8,12,34,35].

The increasing prevalence of PVL-positive MRSA isolates from non-bloodstream infections and hospital outbreaks both in Ireland and internationally is concerning [8,13,34,35]. In 2019, McTavish *et al.* characterized a dominant PVL-positive CC5-MRSA-IVc lineage in a Sri Lankan hospital and also identified it in the UK and Australia [16]. In 2021, similar isolates from 13 patients during a protracted Irish maternity unit hospital outbreak were described [13]. Consequently, our investigation sought to compare Irish PVL-positive CC5-MRSA-IVc with the previously reported Sri-Lankan clone and similar international isolates to determine the clone's global distribution, diversity and population structure for the first time.

Phylogenetic analysis of 214 Sri-Lankan clone and 52 comparator isolates revealed that the Sri-Lankan clone is relatively homogenous compared with other PVL-positive CA-MRSA clones that have diverged more significantly over time [35]. Greater diversity maybe revealed in future studies with more disparately recovered isolates. The vast majority of Sri-Lankan clone isolates (209/214, 97.7%) recovered over 17 years grouped into Clade I (Figure 1a) by cgSNP analysis with an average of 110 cgSNPs (57 cgMLST allelic differences) between isolates (Supplementary Tables S3 and S4, respectively). The five remaining Sri-Lankan clone isolates formed Clade III that differed from Clade I by an average of 237 SNPs (117 cgMLST allelic differences). Seven PVL-positive ST5/t002-MRSA-IVa comparator isolates in Clade II formed the closest neighbour to Sri-Lankan Clade I. Segregation of Sri-Lankan clone isolates into country-specific RIGs (27/30 RIGs) by cgMLST probably reflects local transmission and clonal evolution (Supplementary Table S5). Some Danish isolates ($N = 27$) in country-specific RIGs also formed household-specific clusters (RIGs 4, 9–10, 13, 18–21, 24 and 29) (Supplementary Table S1). In some cases, different members of the same household presented with either carriage or infection. Additionally, two isolates recovered from separate patients in a Danish hospital clustered in RIG-30, with 16 cgSNPs (9 cgMLST allelic differences) between isolates (Supplementary Table S1). These findings highlight the significance of CA-MRSA transmission in both community and hospital settings. Only limited inter-country dissemination of closely related Sri-Lankan clone isolates was detected (RIG-3, RIG-11 and RIG-14), although this possibly reflects the limited collection of isolates available for investigation.

Sri-Lankan clone isolates investigated were ST5, predominantly *spa* type t002 or closely related *spa* types and harboured a relatively small number of antimicrobial-resistance genes (Table I and Supplementary Table S1). DNA microarray and WGS data analyses revealed variable IEC gene cluster (*sea/sak/scn/sep/chp*) and plasmid-encoded enterotoxin genes (*sed/sej/ser*) detection, while the majority of other molecular characteristics were highly conserved (Supplementary Table S1). Although IEC-type G (91.6%) was predominant among Sri-Lankan clone isolates, six other IEC types were detected (Supplementary Table S1). Additionally, *sed/sej/ser* enterotoxin genes were absent in only a small number of isolates (6.5%). The absence of the *bbp* gene within Irish maternity unit hospital outbreak-associated isolates (Figure S1a) very likely reflects local loss of the gene as other Irish isolates harboured the gene. Variation in IEC types and enterotoxin

genes probably reflects loss/gain of converting bacteriophages encoding IEC genes and *sed*/*sej*/*ser*-encoding plasmids [36,37]. The prevalence of the multi-drug-resistant PVL-negative European CC1-MRSA-IV clone in Ireland [18,19] exemplifies the importance of mobile genetic elements in the successful dissemination of emerging MRSA clones [31,35,38]. Earls *et al.* described the emergence of European CC1-MRSA-IV from a South-Eastern European meticillin-susceptible *S. aureus* (MSSA) CC1 lineage, and its subsequent rapid expansion across Europe in the late 1990s [18,19]. European CC1-MRSA-IV is now the predominant endemic CC1-MRSA clone in Ireland, associated with community transmissions and multi-hospital outbreaks [19]. Periodic replacement of predominant MRSA clones in Irish hospitals is well-documented [39], thus the recovery of the Sri-Lankan clone in 10 Irish hospitals over a nine-year period (2013—2022) is concerning (Table I and Supplementary Table S1).

The Sri-Lankan clone chromosomally integrated defective 9.6 kb *pvl*-encoding phage remnant (Supplementary Figure S2b) may be a useful genetic marker, as the earliest Sri-Lankan clone study isolate (141087, 2005) harboured this remnant. The remnant probably arose by imprecise excision of a lysogenized *pvl*-phage genome, possibly as a result of a fitness cost imposed on the bacteria through carriage of the entire prophage genome [40]. Stable chromosomal *pvl*-retention without phage mobility-associated genes may provide a survival advantage. Defective *pvl*-encoding bacteriophages with truncated tail formation genes have been described in MRSA [41,42]. Furthermore, a defective *pvl*-phage has been reported in the successful CA-MRSA clone USA300 [43]. In the Sri-Lankan clone, approximately 80% of the phage genome has been deleted leaving the *pvl*-encoding remnant.

The seven PVL-positive ST5/t002-MRSA-IVa comparator isolates in Clade II (the closest neighbour to Sri-Lankan Clade I) and the single ST5-MRSA-IVa comparator isolate Z4294 located adjacent to Clade III in the cgSNP MLT also harboured the 9.6 kb phage remnant. These findings suggest that Clade II isolates, isolate Z4294 and Sri-Lankan clone Clade I and III isolates emerged from a PVL-positive common ancestor harbouring the 9.6 kb phage remnant, very likely a PVL-positive ST5-MSSA [19]. Sri-Lankan clone Clade I then went on to disseminate widely. Interestingly, a PVL-positive CC5-MSSA isolate identified in the puBMLST database by *in silico* PCR that also harboured the 9.6 kb phage remnant clustered beside comparator isolate Z4294 and adjacent to Sri Lankan Clade III (Figure 1 and Supplementary Figure S2).

This study had some limitations. Limited Sri-Lankan clone isolates/WGS datasets were recovered following comprehensive literature and WGS database searches making it difficult to assess its true prevalence (Supplementary Table S2). Historical and contemporary data on MSSA progenitor populations is limited in most MRSA lineages [44], including the Sri-Lankan clone. Future investigations require a more comprehensive isolate collection with good quality metadata, including potential progenitor MSSAs from more numerous and disparate regions.

In conclusion, international and local surveillance of emerging MRSA clones is important for monitoring transmission. The association of Sri-Lankan clone isolates with SSTIs in both community and hospital settings in 12 countries spanning 17 years reflects its emergence internationally. The stable chromosomal integration of *pvl* in the Sri-Lankan clone potentially contributes to its dissemination.

Acknowledgements

The authors wish to acknowledge the support of the staff of the Irish National MRSA Reference Laboratory (NMRSARL), the staff of the referring hospitals who submitted isolates for investigation as well as Elke Müller and Annett Reissig (Jena) for performing array experiments identifying suspect isolates. We thank Edet Udo (Kuwait), Bo Söderquist (Sweden) Anne Tristran (Lyon) for providing some isolates for investigation.

Conflict of interest statement
None of the authors have any conflicts of interest to declare.

Funding sources
This work was primarily supported by Dublin Dental University Hospital Microbiology Research Unit. This work was primarily supported by Dublin Dental University Hospital Microbiology Research Unit grant 9512 (D. Coleman). Jan Tkadlec (Prague) was supported by the National Institute for Virology and Bacteriology project no. LX22NPO5103 (Program Exceles, NextGenerationEU).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2022.11.015.

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