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Original Article

Investigating a possible link between antiseptic treatment and the increased occurrence of daptomycin-resistant *Staphylococcus aureus*

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ABSTRACT

Objectives: Because of a steady increase in the detection of daptomycin-resistant (DAP-R) *Staphylococcus aureus* at three medical centres in Cologne, Germany, molecular surveillance was established from June 2016 to June 2018 to investigate the causes of the emergence and spread of respective isolates. Seventy-five *S. aureus* isolates, both DAP-R and DAP-susceptible, were collected from 42 patients for further analysis.

Methods: Broth microdilution was used to determine the MICs for DAP and polyhexamethylene biguanide/polyhexanide (PHMB). To investigate the effect of PHMB on the development of DAP resistance, we performed selection experiments with PHMB. All isolates studied were subjected to whole-genome sequencing. Epidemiological, clinical, microbiological and molecular data were analysed comparatively.

Results: Acquisition of DAP resistance was mainly observed in patients with acute and chronic wounds (40/42, 96.2%) treated with antiseptic (32/42, 76.2%) rather than systemic antibiotic therapy using DAP or vancomycin (7/42, 16.7%). DAP-R *S. aureus* had a diverse genetic background; however, within individual patients, isolates were closely related. At least three potential transmission events were detected. Most DAP-R isolates had concomitant elevated MICs for PHMB (50/54, 92.6%), and *in vitro* selection experiments confirmed that PHMB treatment is capable of generating DAP resistance. DAP resistance could be linked to 12 different polymorphisms in the *mprF* gene in the majority of clinical isolates (52/54, 96.3%) as well as in all *in vitro* selected strains.

Discussion: DAP resistance in *S. aureus* can occur independently of prior antibiotic therapy and can be selected by PHMB. Therefore, wound treatment with PHMB may trigger individual resistance development associated with gain-of-function mutations in the *mprF* gene. **Andreas F. Wendel, Clin Microbiol Infect 2023;29:1334.e1–1334.e6**

Introduction

Staphylococcus aureus is one of the most prevalent community- and hospital-acquired pathogens and causes a wide range of local and systemic infections [1]. The rise of methicillin-resistant (MRSA) and even multidrug-resistant *S. aureus* is a public health concern. The cyclic lipopeptide antibiotic daptomycin (DAP) is one of the antibiotics of last resort for MRSA infections [2]. Therefore, emergence of DAP resistance in *S. aureus* is a growing concern.

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Development of DAP resistance (European Committee on Antimicrobial Susceptibility Testing, EUCAST / Clinical and Laboratory Standards Institute, CLSI breakpoint: S $\leq$ 1 mg/L, R > 1 mg/L) in *S. aureus* usually relates to an alteration in cell membrane charge in which the *mprF* gene is consistently implicated. *MprF* encodes a bifunctional enzyme, the multiple peptide resistance factor (MprF). MprF is involved in the production of phospholipid lysophosphatidylglycerol (LysPG) and the translocation of LysPG from the inner to the outer leaflet of the cytoplasmic membrane [3]. Certain mutations in *mprF* lead to a gain-of-function phenotype resulting in a decrease of negative surface charge associated with cross-resistance to various cationic antimicrobials, including DAP [4].

Treatment of wound infections may include both, systemic antibiotic treatment and local use of antiseptics, such as polyhexamethylene biguanide (PHMB), chlorhexidine (both biguanides) or octenidine [5]. The cationic PHMB is a synthetic polymer with structural similarity to antimicrobial peptides, which—similar to DAP—interacts with the negatively charged bacterial cell membrane, resulting in collapse of membrane potential and finally in bacterial death [6]. However, recent data show different target structures within the bacterial cell [7]. PHMB is used for antiseptic bathing, decolonization of MRSA and as wound antiseptic especially for burns [8].

At the same time, *in vitro* studies suggest that exposure to biocides might result in reduced susceptibility to biocides and antibiotics [9].

Given this knowledge, in this study we aimed to analyse the putative causes and molecular mechanisms underlying the increasing occurrence of daptomycin-resistant (DAP-R) *S. aureus* isolates at three medical centres in Cologne, Germany.

Methods

Setting

The study was performed in three hospitals in Cologne, Germany (tertiary and secondary care centre and children's hospital; 700, 400 and 260 beds, respectively).

Patients and strain collection

Forty-two patients colonized or infected with a DAP-R *S. aureus* were enrolled in this study from June 2016 to June 2018. All available *S. aureus* isolates of these patients were included in further analyses.

Epidemiological and transmission analysis

Epidemiological and clinical data were collected from patients' clinical records. Transmission events were considered confirmed, if genetically related isolates were isolated from patients who stayed at the same ward or were cared for by the same healthcare worker at the same time.

Identification and susceptibility testing

Species identification and antimicrobial susceptibility testing were performed with the VITEK 2 system (GP-ID and AST-P632, Biomérieux, France) according to the manufacturer's recommendations. DAP resistance was confirmed by gradient test (Etest, Biomérieux, France). Confirmation of species and antibiotic susceptibility was done at the National Reference Centre for Staphylococci and Enterococci, as previously described [10]. MICs were determined by broth microdilution according to EUCAST criteria [11] (see Table S1). *In vitro* activity of DAP was measured in cation-adjusted Mueller-Hinton broth (MHB) supplemented with 50 mg Ca^{2+}/L. The MICs of PHMB were determined by broth microdilution in a twofold serial dilution in MHB with PHMB (Fagron, Glinde, Germany) concentrations ranging from 32 to 0.063 mg/L. *Enterococcus faecalis* American Type Culture Collection (ATCC) 29212 (MIC 2 mg/L) [12] was used as control. Wells were inoculated with ~5×10^5 CFU/mL and MICs were read after 24 hours at 37°C. MICs were determined in biological triplicates and a mean MIC was calculated.

In vitro selection of polyhexanide non-susceptible isolates

Two DAP-S clinical isolates (19-00151, 19-00152; Table S1, Fig. S2) were placed under increasing selection pressure by PHMB. Approximately 5×10^4 CFU were incubated in 220 µL MHB with or without 2 mg/L PHMB at 37°C in a microtiter plate and shaken at 100 rpm intermittently. Turbidity was measured every hour at 600 nm. After 24 hours, 20 µL of the PHMB-containing culture was over-inoculated in 200 µL of MHB with or without 4 mg/L PHMB and 8 mg/L, respectively. If growth was delayed after 24 hours with PHMB, the culture was over-inoculated into MHB of the same PHMB concentration. After each selection step (4 and 8 mg/L PHMB), cultures were plated on MH-agar and single colonies were picked and cultured on MH-agar with 0, 1 and 2 mg/L PHMB; afterwards DAP susceptibility testing was performed. In total, 18 isolates, exhibiting a DAP MIC of 4 mg/L, were subjected to whole-genome sequencing.

Whole-genome sequencing, molecular typing and phylogenetic analyses

Genomic DNA was extracted from overnight cultures in tryptic soy broth using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Karlsruhe, Germany) according to the manufacturers' instructions. Sequencing libraries were generated with the Nextera XT DNA Library Preparation Kit and paired-end sequencing was performed either on a MiSeq or a NextSeq with the 2 $\times$ 250 MiSeq version 3 and the 2 $\times$ 150 NextSeq Rapid SBS version 2 reagent kit (Illumina, San Diego, CA, United States), respectively.

Quality control using FastQC version 01.11.7 (Babraham Bioinformatics, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and *de novo* assembly via SPAdes version 3.11.1 or version 3.13.1 [13] were performed within SeqSphere$^+$ version 7.1.0 or version 8.0.1 (data analysis for the selection experiments was conducted with later versions; Ridom, Münster, Germany) [14]. SeqSphere$^+$ was also used to extract *spa*-type [15], multi-locus sequence-type (MLST, including the *S. aureus* loci *arcC*, *aroE*, *glp*, *gmk*, *pta*, *tpi* and *yqiL*) [16] and core-genome MLST (cgMLST) complex type [17] from *de novo* assembled contigs. On the basis of cgMLST, isolates were grouped into clusters using a threshold of a maximum of ten differing loci within a cluster.

On the basis of cgMLST, accessory genome and MLST data, 59 709 single-nucleotide polymorphisms (SNPs) in 1752 loci present in all isolates were used to calculate a neighbour-joining tree in Geneious Prime 2020.2.3 (Biomatters) that was further annotated with iTOL version 6 [18].

SNP and indel analysis

Seven genetic loci previously associated with DAP resistance in staphylococci [19,20] were extracted from *de novo* assembled contigs of all sequenced isolates. Corresponding protein sequences were aligned and compared with those of the DAP-S reference strain COL (GenBank acc. no. CP000046.1) within SeqSphere$^+$ version 8.0.1.

Sequence comparisons of isolates obtained from *in vitro* selection experiments were performed by mapping trimmed paired-end reads for the selected isolates to the *de novo* assembled contigs of the corresponding DAP-S isolate using the in-house pipeline batchMap version 2.0.0, as previously described [21]. Resulting alignments were further analysed in Geneious Prime 2020.2.3.

Ethical approval

The study was approved by the Ethics Committee of the Faculty of Health of the Witten/Herdecke University (study number 169/2018).

Results

Clinical and epidemiological results

Patients and epidemiology

From June 2016 to June 2018, DAP-R *S. aureus* isolates were confirmed in 42 patients (31 MRSA and 11 methcillin-sensitive *S. aureus,* MSSA). Most affected patients were hospitalized in the tertiary care centre and stayed in surgical departments. Initial detection was primarily from samples of acute or chronic wounds. About half of the patients were infected with DAP-R *S. aureus* (Table 1), but none developed bactereamia with a DAP-R strain starting from the infected wound. Multiple *S. aureus* isolates were available from 14 patients. Both, DAP-S and DAP-R strains were isolated from 11 patients (Table S1, Fig. S1).

Antibiotic and antiseptic usage

Seven patients were treated systemically with DAP and/or vancomycin (VAN) in the 3 months before detection of DAP-R *S. aureus*. Most patients ($n = 31$, 73.8 %) were exposed to the antiseptics PHMB and/or octenidine and underwent topical wound treatment or antiseptic bathing (Table 2).

Microbiological results

Bacterial isolates and susceptibility testing

A total of 75 (54 DAP-R and 21 DAP-S) *S. aureus* isolates were available for further analysis (Fig. S1). All DAP-R isolates exhibited DAP MICs of 2 to 4 mg/L. No isolate exceeded the susceptibility breakpoint for VAN (EUCAST: S $\leq$ 2 mg/L, R > 2 mg/L; CLSI S $\leq$ 2 mg/L, R > 16 mg/L; Table S1). In general, DAP-R isolates also showed increased MIC values for PHMB (Fig. 1). While mean PHMB MICs in DAP-S *S. aureus* ($n = 21$) ranged between 0.5 and 0.67 mg/L (with only 3 isolates exhibiting MICs >0.5 mg/L in single experiments), PHMB MIC values in DAP-R isolates ($n = 54$) were elevated twofold on average and ranged from 0.5 and 1.33 mg/L (only one isolate showing a MIC of 0.5 mg/L and 10 mean MICs <1 mg/L). Complete resistance patterns of all isolates are summarized in Table S1.

In vitro selection

In one of the two PHMB-exposed strains (19-00152, see Table S1), we were able to generate isolates with elevated DAP and PHMB MICs. All resulting 19-00152-descendants exhibited a DAP MIC of 4 mg/L.

Molecular characterization

Molecular typing and transmission analysis

Isolates examined could be assigned to a broad spectrum of clonal lineages with CC22 and CC5 dominating in both, MRSA and MSSA (Table S1, Fig. S1). On the basis of cgMLST analysis, 14 different clusters were identified comprising 2 to 11 isolates (Fig. 2).

Table 1
Characteristics of the 42 patients colonized/infected with DAP-R *S. aureus*

Patient characteristics ($n = 42$)	Number (%)
Age (y)	
Median	60
Range	0–90
Sex	
Male	20 (47.6)
Medical centres	
Tertiary care	34 (81)
Secondary care	5 (11.9)
Children's hospital	3 (7.1)
Ward type	
ICU	13 (31)
General ward	23 (54.7)
Outpatient clinic	6 (14.3)
Medical departments	
Plastic surgery	23 (54.8)
Trauma surgery	4 (9.5)
Visceral surgery	4 (9.5)
Vascular surgery	3 (7.1)
Surgery (other)	4 (9.5)
Internal medicine	2 (4.8)
General paediatrics	2 (4.8)
First positive specimen (source)	
Acute wound[a]	7 (16.7)
Chronic wound[a]	31 (73.8)
Screening (nose/throat)	3 (7.1)
Eye	1 (2.4)
Type of wound	
Thermal/chemical burn	11 (26.2)
Surgical site	9 (21.4)
Venous ulcer	7 (16.7)
Decubitus ulcer	6 (14.3)
Skin necrosis	3 (7.1)
Other	4 (9.5)
No wounds	2 (4.8)
Mode of acquisition of DAP-R *S. aureus*[b]	
Healthcare-associated	38 (90.5)
Community-associated	4 (9.5)
Infection with DAP-R *S. aureus*[c]	
Deep incisional SSI	6 (14.3)
Osteomyelitis	4 (9.5)
Soft tissue infection	3 (7.1)
Decubitus ulcer infection	2 (4.8)
Superficial incisional SSI	2 (4.8)
Burn wound infection	1 (2.4)
Skin infection	1 (2.4)
Colonization	23 (54.7)

DAP-R, daptomycin-resistant; ICU, intensive care unit; SSI, surgical site infection.

[a] Acute wounds ≤42 d, chronic wounds >42 d [30].

[b] Healthcare-associated, detection >48 h after hospital admission or contact to the healthcare system (e.g. hospital stay, outpatient clinic, professional wound care) within the last 30 d.

[c] Healthcare-associated infections based on CDC/NHSN criteria [available from: https://www.cdc.gov/nhsn/pdfs/pscmanual/17pscnosinfdef_current.pdf].

Four clusters contained isolates from more than on patient (Cluster [C]1: 3 patients; C7, C13, C14: 2 patients). Seven clusters included both, DAP-R and DAP-S isolates and six of those contained isolates from a single patient only. In patient P37, 2 isolates (DAP-R and DAP-S) differed in 20 cgMLST loci and can therefore also be considered at least closely related. From 4 patients, genetically distinct isolates were obtained and at least one DAP-S strain differed genetically from DAP-R strain(s) (patients 1, 9, 13 and 39). With the inclusion of epidemiologic data, 3 patient-to-patient transmissions could be confirmed (cluster 13, P10 → P12, MSSA transmission; cluster 1, P38 → P39 → P41, MRSA transmission). Two other putative transmission events (P40 → P14; P21 → P23) could not be verified.

Table 2
Antibiotic and antiseptic treatment within 3 months before isolation of DAP-R *S. aureus*

Treatment	Agent	No. of patients treated (%)
Antiseptic wound treatment[a]	PHMB only	23 (54.8)
	OCT only	5 (11.9)
	Povidone-iodine only	2 (4.8)
	PHMB and OCT	1 (2.4)
	PHMB and povidone-iodine	1 (2.4)
Antiseptic bathing (universal)	PHMB only	12 (28.6)
	OCT only	3 (7.1)
Topical antibiotic treatment	Mafenide acetate	2 (4.8)
Relevant systemic antibiotic treatment	DAP only	1 (2.4)
	VAN only	4 (9.5)
	VAN and DAP	2 (4.8)

DAP-R, daptomycin-resistant; OCT, octenidine; PHMB, polyhexamethylene biguanide; VAN, vancomycin.
[a] Antiseptic therapy with chlorhexidine was not established [30].

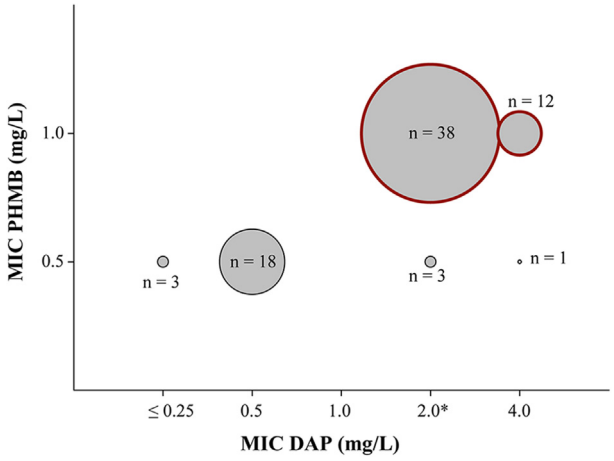

Fig. 1. Minimum inhibitory concentration for DAP (x-axis) and PHMB (y-axis) in mg/L. Mean PHMB MICs were rounded to the next MIC. EUCAST breakpoint for DAP is indicated by an asterisk. For each DAP-PHMB MIC combination, the number of strains is indicated. DAP-resistant isolates with concomitant elevated PHMB MIC are indicated in red. DAP, daptomycin; PHMB, polyhexamethylene biguanide.

SNP analyses

All DAP-S isolates contained a wildtype *mprF* gene. In DAP-R isolates 12 different mutations in *mpr*F were identified. The most common amino acid changes were L826F ($n = 14$), S337L ($n = 13$), T345A ($n = 6$), L341S ($n = 5$) and S295L ($n = 4$). In two DAP-R isolates, no *mpr*F mutation previously associated with DAP resistance was detected (Table S1). DAP-R isolates from the same patient assigned to one cluster carried the same mutation in ten clusters. However, within cluster C1, DAP-R isolates from P38 ($n = 3$) and P41 ($n = 1$) carried the mutation L341S, whereas 3 isolates from P39 carry L826F. Similarly, DAP-R isolates from 2 patients in C7 carried S337L and L291S, respectively.

All 19-00152 progenies generated under PHMB selection showed 3 different amino acid changes in MprF: S295P, S295L, and L341V (Table S2). Amino acid substitutions predicted for six additional loci previously associated with DAP resistance are summarized in Table S3.

Discussion

After an increase of DAP-R *S. aureus* on different surgical wards, an outbreak was suspected. However, whole-genome sequencing confirmed a polyclonal setting and the existence of 14 unrelated clusters of which only 4 contained isolates from several patients. Furthermore, spatiotemporal analysis confirmed transmission events in only one cluster.

The isolation of DAP-S isolates after initial detection of a DAP-R strain in several patients (e.g. P15) suggested the coexistence of susceptible and resistant strains (without or with mutation in *mpr*F, respectively) within the *S. aureus* population colonizing a single patient. By selecting individual colonies in the isolation process, it is random which variant is sampled. We also observed this heterogeneous resistance development in our selection experiments (data not shown).

The observation that DAP-R and DAP-S isolates from the same patient form 'patient-specific' clusters in cgMLST analysis suggests that the majority of affected patients independently acquired DAP resistance. This is further supported by the closely related isolates within 'outbreak cluster' C1, which originate from 3 different patients, but carry 2 different mutations in *mpr*F. The most likely scenario for the development of C1 is that a DAP-S strain was transmitted from P38 to P39, where it developed DAP resistance (L826F) thereafter independently (Fig. 2). Concomitantly, DAP resistance occurred in P38 and the DAP-R strain that developed here was transferred to P41 (L341S). Interestingly, P39 in an initial screening carried a DAP-S t032 MRSA strain, which was outcompeted by an unrelated DAP-R (L826F) t032 MRSA that was repeatedly isolated from the wound.

Although DAP resistance was often suggested to be multifactorial [22], *mpr*F mutations are the most cited polymorphisms associated. In this study, 52 of 54 DAP-R isolates exhibited mutations in *mpr*F, all of which were previously described [3]. In the 2 remaining isolates, no mutations were detected, neither in *mpr*F nor in 6 other investigated loci previously associated with DAP resistance [19,20] (Table S3). This indicates the occurrence of rare and so far unknown mechanisms.

The National Reference Centre for Staphylococci in Germany receives a continuously increasing number of DAP-R isolates; however, DAP resistance is still rare worldwide [23]. It usually develops during DAP or VAN therapy [24], which is typically administered in patients with MRSA infections. The isolates analysed showed only moderately increased MICs for DAP and almost no MIC elevation for glycopeptides (5 DAP-R isolates presented a VAN MIC of 2 mg/L), although a putative link between the development of DAP resistance and the *S. aureus* VAN heteroresistance phenotype has been suggested previously [25]. However, according to the patient records in this study, only a minority of the patients had been exposed to DAP or glycopeptides before isolation of DAP-R *S. aureus*, indicating an alternative selection mode. Searching for the place and mode of selection of DAP resistance, we encountered an epidemiological link of DAP-R isolates with a hospital stay at different surgical departments, where PHMB treatment is integral part of the wound management standard. In fact, a significant proportion of study patients received antiseptic wound care. Because this is not always recorded individually, the proportion treated may be even higher than documented. It is well known that the widespread application of antiseptics can lead to the selection of bacteria with reduced susceptibility for antibiotics [26] and *in vitro* selection of DAP-R *S. aureus* has recently been demonstrated for PHMB [27]. In this context, it was also noticed that a large

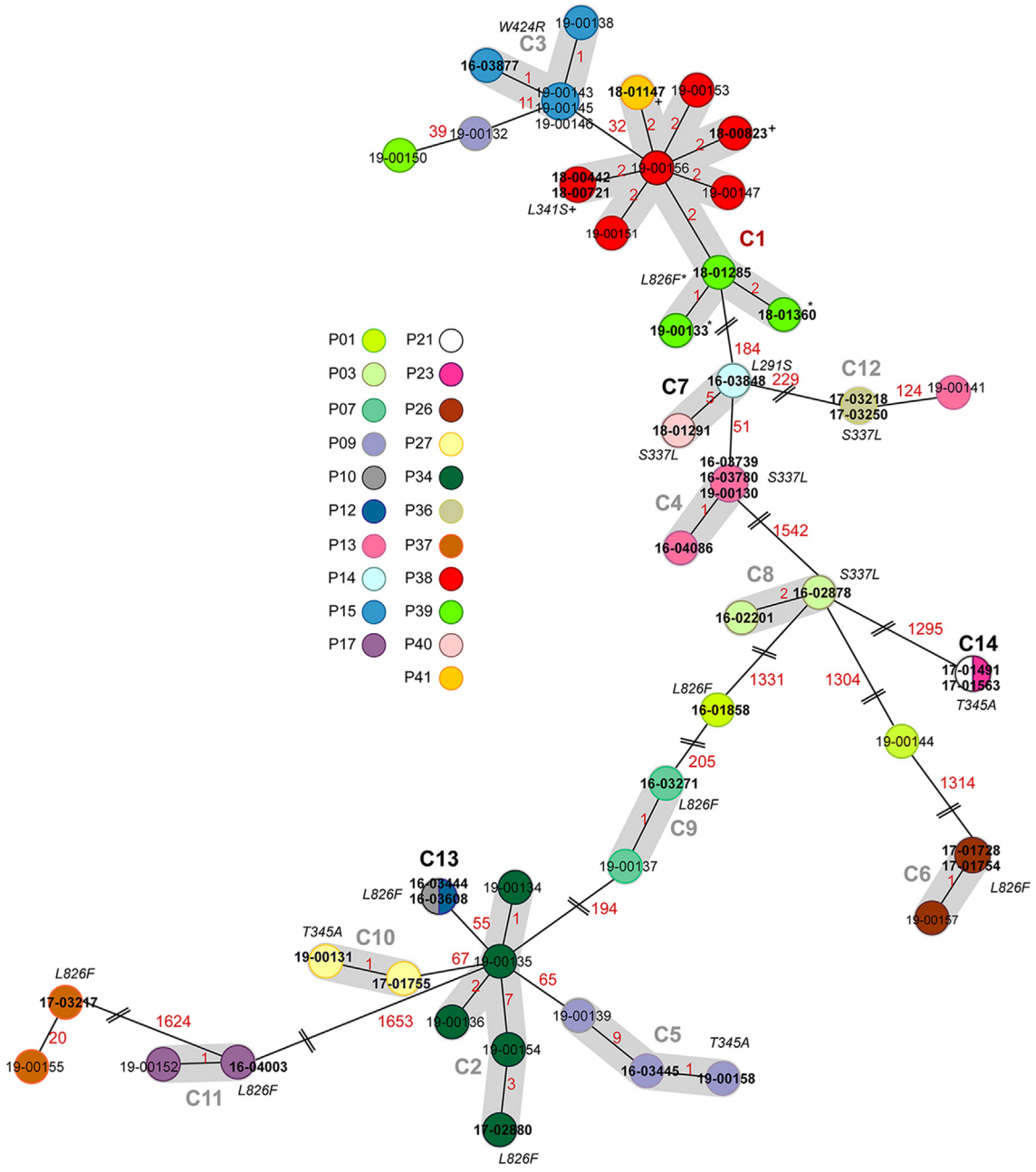

Fig. 2. Minimum spanning tree for 54 isolates. Included are isolates from 14 patients with more than 1 isolate recovered and 7 single patients isolates that belonged to a cluster in the cgMLST analysis of all isolates within the study ($n = 75$ isolates, Table S1). The tree is based on a cgMLST analysis comprising 1752 loci of the *S. aureus* genome. Circles represent up to three isolates each and are coloured according to patients. Isolates indicated in bold are DAP resistant. Grey shadows indicate clusters. Isolates of a cluster differ from the nearest related isolate in a maximum of 10 core-genome loci (MST cluster threshold: 10). Clusters are labelled C1–C14. Clusters containing isolates from several patients are labelled in black, those in which an outbreak could be verified epidemiologically are marked in red, all others in grey. MprF AA substitutions in DAP-resistant isolates are given in italics. Generally, a single MprF AA substitution was found in each cluster with exception of C1 and C7. In C1 different substitutions are indicated with * and $^{+}$, respectively. cgMLST, core-genome multi-locus sequence-type.

proportion of the DAP-R isolates had elevated MIC values for PHMB, although these were below the epidemiological cut-off value of 4 mg/L proposed by Fabry et al. [28]. To date, there are few interpretative criteria for determining biocide susceptibility, and biocide resistance is thought to be rare [29]. However, our results and the knowledge about the similarities in structure and mode of action between PHMB and DAP prompted us to conduct PHMB selection experiments, which finally generated isolates with both, elevated PHMB and DAP MICs. These results led us to hypothesize that PHMB treatment might have induced individual resistance development, which was mainly associated with mutations in *mprF*.

One important limitation of the present study is the biased strain collection enriched by DAP-R isolates and where DAP-S strains were often not available for further analyses. Thus, the strain collection is biased towards DAP-R isolates, which may reduce the reliability of certain conclusions.

Our results demonstrate the potential impact of antiseptic treatment on the selection of antibiotic resistance, which finally

may even lead to unexpected treatment failure. Therefore, the use of antiseptics should be recorded in more detail to prevent inappropriate use and to better assess, understand and, where possible, counteract parallel developments in the emergence of resistance.

Author contributions

AFW, RO, RS, FM, FL-N and BS conceptualized the study. AFW, RO, CJT-C, HO, FL-N and BS acquired and analysed the data. AFW, FL-N, FM and BS interpreted the results. AFW and BS visualized the data and drafted the manuscript. RO, FL-N, FM, RS and GW reviewed and edited the manuscript. All authors gave final approval of the version to be submitted.

Transparency declaration

The authors declare that they have no conflicts of interest.

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Data availability

Sequence reads of all strains have been deposited as a project at the European Nucleotide Archive (ENA) under the accession number PRJEB59334.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2023.06.007.

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