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CASE REPORT

Recurrent Methicillin-Resistant *Staphylococcus aureus* Cutaneous Abscesses and Selection of Reduced Chlorhexidine Susceptibility during Chlorhexidine Use

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We describe the selection of reduced chlorhexidine susceptibility during chlorhexidine use in a patient with two episodes of cutaneous USA300 methicillin-resistant *Staphylococcus aureus* abscess. The second clinical isolate harbors a novel plasmid that encodes the QacA efflux pump. Greater use of chlorhexidine for disease prevention warrants surveillance for resistance.

CASE REPORT

An 18-year-old man undergoing infantry basic training at Fort Benning, GA, presented to the outpatient clinic in July complaining of a painful skin lesion on his left knee. On physical examination, the patient was afebrile (37°C) and examination was remarkable only for a prepatellar nodule that was erythematous, warm, indurated, tender, and fluctuant. The patient had no other skin lesions and no lymphadenopathy. The remainder of his examination was normal, and the patient did not report a history of cutaneous abscesses. The patient was diagnosed with a cutaneous abscess without joint involvement and underwent incision and drainage. The purulent material underwent standard wound culture and yielded methicillin-resistant *Staphylococcus aureus* (MRSA) with the BD Phoenix automated microbiology system (Becton, Dickinson, Sparks, MD). The isolate was resistant to oxacillin and erythromycin and had inducible clindamycin resistance, as determined by double-disk diffusion (1), but was susceptible to trimethoprim-sulfamethoxazole (TMP-SMX), doxycycline, levofloxacin, linezolid, daptomycin, and vancomycin (Table 1). With no known medication allergies, the patient was treated with a 10-day course of twice-daily double-strength TMP-SMX and underwent serial follow-up examinations and wound care with complete resolution of his abscess within 14 days.

Nine weeks after his initial presentation, the patient returned to the clinic. This time, he complained of a similar painful lesion on his left foot. On physical examination, the patient was again afebrile (37°C) and examination was remarkable only for an inflamed and fluctuant nodule on the dorsum of his left foot. The patient was again diagnosed with a cutaneous abscess and underwent incision and drainage, with standard wound cultures yielding MRSA. This second clinical isolate had the same antibiotic susceptibility pattern as the first MRSA isolate; however, it was resistant to levofloxacin (Table 1). The patient was treated in a similar fashion as for the first episode and recovered without additional recurrences.

The patient was a soldier participating in a prospective cluster-randomized trial aimed at preventing skin and soft tissue infections (SSTIs), which are common in this population (2). The patient was in a study group that received chlorhexidine for weekly showering (4% chlorhexidine gluconate, Hibiclens; Mölnlycke Health Care, Norcross, GA). As part of the protocol, the patient completed a questionnaire at the time of his second episode that queried his chlorhexidine use; he reported using the agent every other week throughout his training. The patient would have used chlorhexidine once or twice before his first episode and four or five times prior to the second episode.

The two MRSA isolates underwent molecular analysis, including typing by pulsed-field gel electrophoresis (PFGE) (3), multilocus sequence typing (MLST) (4), and PCR assays for toxin (5) and resistance genes (6, 7). PFGE findings were resolved and analyzed with BioNumerics (Applied Math, Austin, TX). Both MRSA isolates were sequence type 8 (ST8), USA300, staphylococcal cassette chromosome *mec* type IV, positive for Panton-Valentine leukocidin (PVL, encoded by *lukS-PV*), and negative for high-level mupirocin resistance (*mupA*). The first clinical isolate (C01) was negative for the chlorhexidine resistance genes (*qacA/B*), but the second clinical isolate (C02) was positive for *qacA/B*. As part of the research protocol (2), sampling of the anterior nares at the second episode revealed that the patient was colonized with a separate, unrelated, methicillin-susceptible *S. aureus* strain (ST30).

Both clinical isolates underwent chlorhexidine susceptibility testing. Briefly, about 4×10^4 CFU from an overnight culture were inoculated into 1 ml of cation-adjusted Mueller-Hinton II broth (BD BBL) containing purified chlorhexidine (Sigma) at concentrations ranging from 0 to 1 μg/ml. The cultures were grown overnight with shaking (220 rpm). Upon visual analysis (Fig. 1), the MIC of chlorhexidine for the C01 isolate was 0.3 μg/ml, while that for the C02 isolate was 0.8 μg/ml, an approximate 2.7-fold increase over that for C01.

Previous reports have found that mutations in the promoter of

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TABLE 1 Molecular characteristics and antimicrobial susceptibilities of clinical MRSA isolates

Characteristic	C01	C02
MLST result	ST8	ST8
PFGE type	USA300	USA300
Susceptibility to:		
Oxacillin	Resistant	Resistant
Erythromycin	Resistant	Resistant
Clindamycin	Inducible resistance	Inducible resistance
TMP-SMX	Susceptible	Susceptible
Doxycycline	Susceptible	Susceptible
Daptomycin	Susceptible	Susceptible
Vancomycin	Susceptible	Susceptible
Levofloxacin	Susceptible	Resistant
qacA	Negative	Positive
mecA	Positive	Positive
SCC*mec* type	IV	IV
PVL	Positive	Positive
mupA	Negative	Negative
norA	Positive	Positive
Chlorhexidine MIC (μg/ml)	0.3	0.8

the *norA* efflux pump can lead to increases in *norA* transcription, which result in increased resistance to antiseptic agents such as chlorhexidine (8, 9). The promoter region of *norA* was PCR amplified and sequenced with the 5′-GTCTTGGTCATCTGCAAAG GTTG-3′ and 5′-GACTGGTATTACTAAACCGATACC-3′ primers. Additionally, the 5′-GGTGGTATGAGTGCTGGTATGG-3′ and 5′-GCATACGATGTGAAACTTCTGCC-3′ primers were used to assess *norA* transcription via reverse transcription (RT)-PCR. Total RNA was extracted from the C01 and C02 isolates with the Qiagen EasyRNA kit. Prior to RNA extraction, *S. aureus* was lysed by incubation for 1 h in lysis buffer containing Tris-EDTA buffer, lysostaphin (20 μg/ml), and proteinase K (200 μg/ml). cDNA was synthesized from total RNA with the QuantiTect RT kit (Qiagen). All PCR and sequencing reactions were performed as previously described (10). RT-PCR was performed with 10-μl reaction mixtures containing 1× SYBR green (Qiagen) and 1.5 μM each primer. Reaction mixtures were incubated for 5 min at 95°C, followed by 35 cycles of 95°C for 10 s and then 50°C for 10 s. Fluorescence readings were acquired at the end of each cycle with the Qiagen Rotor-Gene Q RT-PCR machine. Sequencing data analysis revealed that the C01 and C02 *norA* promoters were identical in nucleotide composition. Not surprisingly, *norA* expression in the C02 isolate was indistinguishable from that in the C01 isolate.

Plasmid extraction with the Qiagen Plasmid Purification kit and subsequent PCR amplification (5′-GCTGCATTTATGACAA TGTTTG-3′ and 5′-AATCCCACCTACTAAAGCAG-3′) (11) and visualization on a 1% agarose gel revealed that the *qacA* gene was detectable only in the C02 isolate. Given the high level of nucleotide sequence similarity between the *qacA* and *qacB* genes, we determined the *qacA* DNA sequence from the C02 plasmid and then compared it to the canonical *qacA* (accession no. GU565967.1) and *qacB* (accession no. AF053772.1) sequences. Of the seven amino acid differences described by Paulsen et al. that distinguish *qacA* from *qacB*, six of the *qacA* residues were observed in the *qacA* gene from C02; this includes a key aspartic acid residue at amino acid position 323 (12). The one amino acid residue that differed from the *qacA* consensus occurred at the first position, where an alternative lysine start codon was found. In total, the data suggest that C02 harbors the *qacA* gene. Finally, to ensure that the C02 isolate was the only strain that expressed the *qacA* efflux pump, we utilized cDNA as a template for *qacA* PCR amplification with the same *qacA* detection/sequencing primers as mentioned before. Amplicon detection on a 1% agarose gel confirmed that the C02 isolate actively expressed the *qacA* gene while the C01 isolate did not.

Previous reports have found that *qacA* is typically carried on the pSK1 family of plasmids (13, 14). We therefore sequenced approximately 550 bp upstream of the *qacA* gene with the 5′-CT CCAATCCTTATAGACCGTGC-3′ primer and found a high level of nucleotide sequence similarity to the pSK1 DNA sequence (GenBank accession no. NC_014369) (>99% nucleotide sequence similarity). To determine if the plasmid was indeed pSK1, we next used the pSK1 plasmid sequence to design a series of 10 PCR primer pairs that span the entire plasmid (Table 2). We found that only the primer pair that encompassed the *qacA* gene (primer pair 9) yielded a PCR amplicon of the correct size in the C02 isolate. This led us to conclude that the *qacA* gene found in the C02 isolate may be carried on a pSK1-like plasmid but not on pSK1 itself.

To assess clonality between the two isolates, total DNA from both isolates was prepared via phenol-chloroform extraction and subjected to Pacific Biosciences RS II SMRT whole-genome sequencing (University of Maryland, School of Medicine, Institute for Genome Sciences). A single closed circular chromosome and plasmid were obtained for each isolate. A comprehensive list of the chromosome and plasmid characteristics is included in Table 3. Genome analysis revealed that the C01 isolate contained 2,770 chromosomal open reading frames (ORFs) and was approximately 2.92 Mbp in length. Conversely, the C02 isolate had a reduced chromosome of 2.86 Mbp encoding 2,704 ORFs. In addition to whole gene changes, we observed >140 single nucleotide polymorphisms (SNPs) between the two chromosomes, which suggests that the two isolates are genetically distinct. Of note, two nonsynonymous SNPs in the C02 *gyrA* and *grlA* genes (C251T and T239A, respectively) were detected. These SNPs result in an S84L amino acid mutation in GyrA and an F80Y mutation in GrlA, which have previously been shown to contribute to quinolone resistance (15–17) and therefore likely explain the levofloxacin resistance of the C02 isolate.

The pC01 and pC02 plasmid sequences were analyzed with

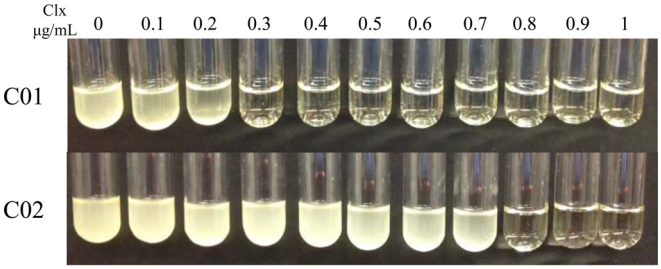

FIG 1 MICs of chlorhexidine (Clx) for *S. aureus* abscess isolates C01 and C02. The chlorhexidine concentrations tested ranged from 0 to 1 μg/ml. All cultures were inoculated with approximately 4×10^4 CFU and grown overnight at 37°C with shaking at 220 rpm. The MIC for the C01 strain was determined to be 0.3 μg/ml, while that for the C02 isolate was 0.8 μg/ml.

TABLE 2 pSK1-like plasmid PCR primer panel

Primer pair	Primer (5′-3′)	
	Forward	Reverse
1	GGAGCACTAGTAGCAACTTTCATC	CCAGAGCCGATGCTACGC
2	GCCTTAAAATTCCAGGCGC	GCTGAAAGTTATAGAGCGGC
3	GAAGCACTTGCATACGATAGTG	GCTCACGCTATACCGACATTC
4	CTAACGTGCGATCAGATGCTTG	GCACCCTCAGAAGCCATTC
5	CGCAGTTGGAGCAAGTGAG	CTTTATCTTCGACTCTATCACGAAC
6	CATCATAGCACCAGTCATCAG	GTGTGCGATCATCGCGTCTATTC
7	CAATTACCTTGGCACTTACCAAATG	GGTTGGAAGAACGCACATATG
8	CTTAGATAGTAGCCAACGGCTAC	CATCGTATCGATCTTGTTGTCC
9	CGATCGCACGGTCTATAAGG	GCTTTGAATCTCTTCGCTTTTCAG
10	CGAAGACGCCTTTCAATATACCG	CCTAGAGCTTGCCATGTATATG

the Basic Local Alignment Search Tool (BLAST; NCBI). While the pC01 sequence was nearly perfectly identical to known *S. aureus* plasmids such as SAP046A (GenBank accession no. NC_013294.1), the pC02 plasmid was significantly larger and less than 40% of the plasmid contained regions similar to those of other sequences in the NCBI database. Annotation of the pC02 plasmid revealed the presence of numerous proteins involved in a range of cellular processes, including DNA replication (Ssb, TopB), transcriptional regulation (QacR), and substrate translocation (CadC). The fully annotated pC02 map is depicted in Fig. 2 (18). The *qacA*-containing pC02 plasmid appears to be novel and may represent an additional class of antimicrobial resistance plasmids in *S. aureus*.

Chlorhexidine is a broad-spectrum topical biguanide cationic antiseptic agent with activity against *S. aureus* (19, 20). Although it has been used for decades in various roles, ranging from hand washing to preoperative skin preparation, chlorhexidine has been increasingly employed for the prevention of both nosocomial (21–24) and community-associated infections (2, 25–27). Evidence from large randomized-control trials points to the importance of chlorhexidine in the prevention of the spread of drug-resistant organisms and hospital-acquired infections (21, 24, 28). Indeed, chlorhexidine has also been an integral component of strategies aimed at the prevention of recurrent MRSA SSTIs in individuals and households (26, 29).

Despite its widespread use, the prevalence of chlorhexidine resistance in the United States is low (approximately 1%) (25, 30, 31); this is in contrast to observations in other countries (11, 32). When used in large trials in both community and hospital settings, chlorhexidine resistance has been only rarely reported (21, 24, 27, 31). Nevertheless, with the widespread and increasing use of this agent, experience has shown that concern about the potential emergence of chlorhexidine resistance is appropriate (32). Additional studies that investigate the frequency of chlorhexidine use and selection of chlorhexidine-resistant strains must be conducted to ensure proper chlorhexidine stewardship.

TABLE 3 Genome and plasmid statistics

Cell component	Size (bp)	% GC	No. of ORFs	Avg gene length (bp)	% Coding
Chromosome					
C01	2,918,599	32.8	2,770	879	84.5
C02	2,864,998	32.8	2,704	882	84.4
Plasmid					
pC01	27,044	30.6	32	592	70.1
pC02	61,537	29.5	71	677	78.2

The plasmid-borne *qacA* gene, in particular, encodes an efflux pump that has been shown in numerous reports to confer resistance to numerous hydrophobic compounds, including cationic biocides such as chlorhexidine (20, 33, 34). While there are no established breakpoints for chlorhexidine resistance, the presence of these genes has been associated both with increased MICs and with untoward clinical outcomes (35–38). Interestingly, multiple reports have identified chlorhexidine-resistant *S. aureus* isolates with MICs of ≥4 μg/ml (30, 31, 38). The isolate described in this report, however, showed a reduced MIC (≤0.8 μg/ml). While the reason for the lower MIC is not clear, this may be due to reduced translation efficiency due to the alternate start codon found in the C02 *qacA* gene (39). The presence of *qacA*/*B* and an increased MIC are sometimes poorly correlated (30); however, in our isolates, an increase in the MIC for the *qacA*-positive C02 strain was clearly observed. We cannot determine the overall clinical impact of this reduced chlorhexidine susceptibility in our patient other than to note that he developed a second USA300 MRSA abscess.

Chlorhexidine has residual antibacterial activity, which may be beneficial in reducing the bacterial burden or preventing the spread of resistant organisms (40); however, this residual activity may also contribute to an environment that ultimately fosters resistance (11). Since our patient utilized chlorhexidine every other week, this may have played a part in the selection of reduced chlorhexidine susceptibility in the patient. Evidence suggests that the *qacA* gene may be able to be horizontally transferred across various staphylococcal species (33, 41). This typically is plasmid mediated since numerous reports have shown that the *qacA* gene is often carried on a plasmid from the pSK1 family of vectors (34, 42). Although we do not know its original source, *qacA* in our identified clinical MRSA isolate was carried on a large, uncharacterized plasmid that shows limited similarity to pSK1. This finding suggests that transmission of *qacA* is not limited to the pSK1-like vectors. Furthermore, the identification of this novel *qacA*-containing plasmid combined with the now ubiquitous use of chlorhexidine, highlights the need for increased surveillance programs that would seek to understand the evolution of *qacA* transmission across MRSA isolates and potentially across other staphylococcal species.

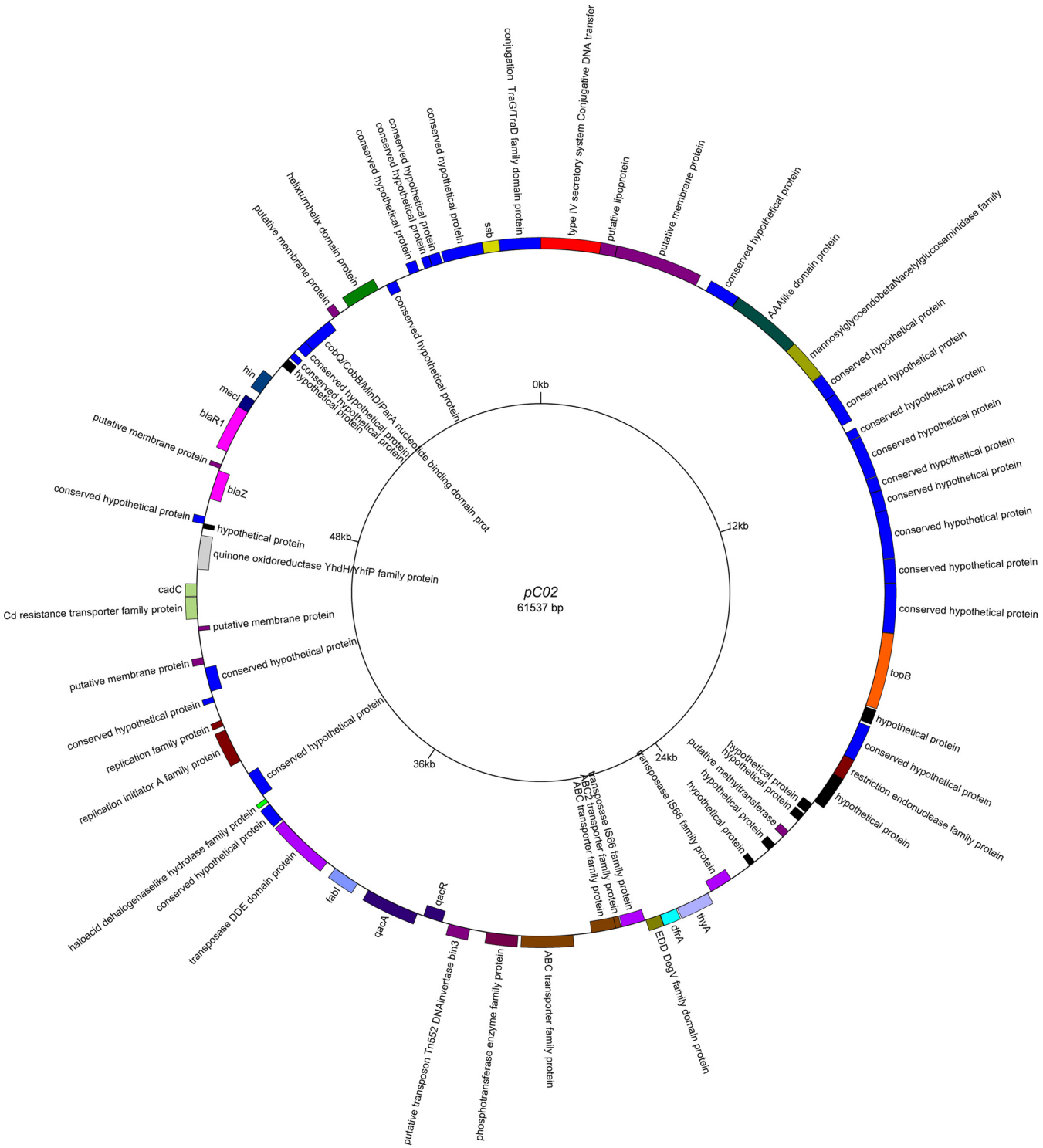

FIG 2 Annotated map of the pC02 plasmid. Sequencing and annotation were performed at the University of Maryland Institute for Genome Sciences and visualized with GenomeVX.

In summary, to our knowledge, this is the first report of selection for increased chlorhexidine MICs while using chlorhexidine in a community-based patient with recurrent USA300 MRSA SSTIs. In light of recent clinical trials that show the benefit of chlorhexidine in the prevention of drug-resistant infections, the medical community should anticipate greater use of this agent and consequently increased resistance. Further study and surveillance for the emergence of chlorhexidine resistance should be considered in health care and community settings that use chlorhexidine for disease prevention.

Nucleotide sequence accession numbers. The C01 and C02 genomes and plasmids were submitted to NCBI and given accession no. CP012118, CP012119, CP012120, and CP012121.

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