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Chlorhexidine bigluconate resistance in Methicillin Resistant *Staphylococcus aureus*

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Disclaimer: The primary dataset used in this project was collected by the Georgia Emerging Infections Program (GAEIP). The GAEIP was not involved in the analyses presented in this thesis.

**Comments**

**Degree:** Master of Public Health

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# Chlorhexidine bigluconate resistance in Methicillin Resistant *Staphylococcus aureus*

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Abstract

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By Eric Evans

Background: With increased chlorhexidine use in infection control practices, concerns of bacterial tolerance has grown. Methicillin-resistant *Staphylococcus aureus* (MRSA) are likely frequently exposed to chlorhexidine. Reduced chlorhexidine susceptibility in staphylococci is associated with quaternary ammonium compound (qac*)* efflux proteins. Mechanisms of tolerance to chlorhexidine beyond the qac proteins are also not well understood. Surveillance conducted by the Georgia EIP provided data to test patient factors contributing to increased chlorhexidine resistance. We serially exposed MRSA to increasing concentrations of chlorhexidine in a *qac*-negative MRSA strain to induce chlorhexidine tolerance.

Methods: 90 MRSA strains collected by the Georgia EIP were tested for MIC of chlorhexidine using standardized test provided by the CDC. Patient information was used to analyze whether any specific variable contributed to increased resistance. Additional laboratory experimentation, seeking to induce chlorhexidine tolerance, was completed. Using both liquid and plate cultures, colonies were selected and exposed to serial passaging in BHI broth with increasing concentrations (0.1 -10 µg/mL) of chlorhexidine for 24-72 hours. After each passage to a higher concentration of chlorhexidine, colonies were examined and confirmed to be SA, tested by broth microdilution to confirm the minimum inhibitory concentration (MIC) to chlorhexidine and frozen.

Results: No significant relationships between any of the patient variables and chlorhexidine tolerance were identified. Notably, samples collected before and after widespread chlorhexidine usage did not show increased chlorhexidine tolerance. All in vitro experiments trying to induce chlorhexidine tolerance failed to produce a tolerant mutant.

Discussion: Despite growing concerns about chlorhexidine tolerance in MRSA, this study does not show an increased chlorhexidine tolerance in MRSA despite widespread use in their respective healthcare facilities. In vitro evolution of increased tolerance to chlorhexidine susceptibility was not successful. These combined experiments suggest development of chlorhexidine resistance in MRSA is likely not as simple as previously assumed.

Introduction

First cultured in the 1880s, *S. aureus* is a major human pathogen and the leading cause of skin and soft tissue infections such as boils, wound abscesses, and cellulitis to more serious, life-threatening infections such as pneumonia osteomyelitis, infective endocarditis, bacteremia and sepsis (Kourtis, 2019; Dale, 1995). In addition, *S. aureus* is also a commensal bacterium with which approximately 30% of the human population is transiently colonized and therefore the leading cause of device-related infections (Wertheim, 2005). S. *aureus* can evade many immune responses to cause infections through colonization of mucosal membranes or wound sites. Mortality rates from *S. aureus* infections during the pre-antibiotic era were seen to be as high as 83% (van Hal, 2012). With the development of antibiotics, such as penicillin, common infections like those caused by *S. aureus,* became less of a threat to infected patients, making previously severe infections manageable and recoverable. Antibiotics were deemed "wonder drugs" as they seemed to heal a patient quickly and efficiently (Davies, 2010). Unfortunately, the respite provided by these medicines was short-lived as resistance to sulfonamides and penicillin was found in the 1930s and 1940s respectively (Chambers, 2009).

The introduction of methicillin helped to briefly stem the tide, but resistance to methicillin was observed by 1961 (Chambers, 2009). The observed resistance to methicillin and penicillin lead to *S. aureus* being additionally classified as Methicillin-Resistant (MRSA) or Methicillin-Susceptible (MSSA). Infections from *S. aureus* can be further categorized as healthcare- or hospital-acquired and community-acquired infections (Dantes, 2011). With resistance to antibiotics beginning to develop, the emergence of multi-drug resistant organisms (MDROs) followed closely behind. As their name suggests, MDROs are microorganisms that demonstrate a high level of

resistance to a range of antibiotics usually used to treat them. As was demonstrated with antibiotics used early on in medical history and the resistance that soon followed, there is a strong correlation between antibiotic use and increases in antibiotic resistance (Hardy, 2018).

Resistance to antibiotics has been observed to develop through many different pathways (Domingues, 2012). The evolutionary nature of microbes allows for random genetic mutations that give the bacteria the ability to survive normally difficult situations. This adaptation can also be induced through the introduction of the bacteria to an environment where adaptation is necessary for survival. Another way that bacteria can develop resistance is horizontal gene transfer. Horizontal gene transfer (HGT) is a method by which bacteria exchange genetic material, such as plasmids or other transposable sections of genetic material, to improve survival. These sections of genetic material often contain new genes (resistance genes) that provide novel ways to survive normally lethal conditions. One example of a gene exchanged through HGT is the *mec* gene, which codes for resistance to penicillin and similar antibiotics. Historically, the major driving factor for resistance acquisition in *S. aureus* has been HGT (Davies, 2010).

Chlorhexidine digluconate (CHG) is a biguanine compound with topical antibacterial activity commonly used since the early 1950s. With proven efficacy in reducing hospital associated infections like *S. aureus*, CHG is used for a variety of purposes in medical facilities, such as pre-procedure skin preparation, handwashing, and whole-body bathing (Climo, 2013; Septimus, 2016). CHG operates through binding to exposed anionic sites in the cell surfaces, effectively destabilizing the cell membrane, causing it to leak potassium ions and protons. CHG is an effective general cleaner and

pre-surgical antiseptic, reducing skin flora better than povidone iodine and has longer residual activity (Horner, 2012). With an increase in how frequently CHG usage is being recommended, increased resistance has also been observed (Hardy, 2018). Resistance to CHG has already been seen in vitro in both Klebsiella and Enterococcus (Wand, 2016; Bhardwaj, 2017). In both studies cited above, organisms that demonstrated an increased resistance to CHG also showed a subsequent increased resistance to additional antibiotics. As CHG is currently the most widely used antiseptic, CHG resistance in bacteria will likely become a significant health threat in the future.

One of the common pathways for resistance to CHG to develop is efflux pumps. Efflux pumps, also previously observed to increase antibiotic resistance, operate by removing the antimicrobial from the cell by "pumping" it out. While eleven different genes are known to encode for efflux-based resistance, the most common gene associated with CHG resistance in MRSA is the *qacA* gene (Horner, 2012). HGT of this gene has been confirmed, increasing the concerns of public health officials. Despite concerns of HGT proliferation of this gene, greater abundance of CHG resistant MRSA has yet to be reported.

CHG resistance is of great concern in the public health world because of the frequency at which CHG is being used. As has previously been demonstrated, increased usage of antimicrobials can lead to increased resistance observed. With this in mind, collaborative research between the CDC and its Emerging Infections Program (EIP) sites in the United States was begun to determine the levels of CHG resistance present before and after widespread usage. The data from the Georgia EIP site on MRSA strains has not been analyzed for additional factors that could contribute to CHG resistance. Though

studies relating to the *qacA* gene and CHG resistance have been completed, experiments seeking to induce resistance through other genes are yet to be published. This study seeks to find, through statistical analysis, patient risk factors and, through experimental methods, additional genetic components that contribute to CHG resistance in *S. aureus*.

## Methods

The data used was collected by the Georgia EIP or by the researchers themselves. The data from the Georgia EIP was collected from 2005 to 2018 and included 90 MRSA samples (see Table 1). These samples were collected from facilities in Health District 3 – Metropolitan Atlanta. Thirty of the samples were collected before CHG was used in the source facility routinely. The remaining sixty were collected after reported routine CHG use began in the source facility. The individuals who had the MRSA infections ranged from 26-79 years old with a mean of 56 years old and a median of 57 years old. The patients included in the study were relatively evenly distributed between men and women. All MRSA isolates included in this data were collected from the blood of the patients. Each sample was epidemiologically classified using the same definitions. Samples that were collected greater than 3 calendar days after acute care hospital admission were designated as Hospital Onset (HO). Those that were collected 3 days or less after acute care hospital admission or in an outpatient setting and at least one of the following: acute care hospitalization within past year, surgery within past year, acute or chronic dialysis within past year, residence in long term care facility within the past year, admission to a long term acute care hospital within the past year, or central venous catheter in place within 2 calendar days prior to culture were designated as Healthcare

Associated Community Onset (HACO). Samples collected three days or less after acute care hospital admission or in an outpatient setting and the patient that didn't have any of the health-care risk factors were classified as Community Associated (CA).

**Determination of MIC to CHG**

All cultures to be tested were grown up on Trypticase Soy Agar (TSA) with 5% Sheep's Blood (BAP) for 18-24 hours. MIC panels were previously made in-house by the CDC and had a range in CHG concentration of 0-64 µg/mL. Using a sterile swab, inoculum was prepared by suspending growth from the overnight plate into a tube of 5 mL of sterile saline. The tube was vortexed and adjusted to match a 0.5 McFarland standard or approximately $1x10^8$ cells. The suspended cells were then diluted by adding 0.25 mL of the inoculum into a tube of 4.75 mL of saline. The new tube was mixed gently and poured into a plastic reservoir. A 12-channel pipettor, loaded only on 11 channels, was used to deliver 10 µL to 11 of the wells of the CDC-prepared 96-well plate. The inoculum was pipetted up and down 4-5 times to mix and 10 µL was aspirated back into the tips before being placed in a biological waste container. This process was repeated, placing a new organism to be tested on each row of the plate. The completed 96-well plate was placed in an 8 in. x 8 in. resealable plastic bag and excess air from the bag was removed to avoid moisture condensation. A 1 µL sterile loop of the inoculum was plated onto a quadrant of a BAP for a purity check. Both the 96-well plate and the purity BAP plates were incubated at 35°C for 16-20 hours. The protocol encouraged use of incubators that did not have carbon-dioxide enhanced atmospheres. Plates were analyzed within the required timeframe of 16-20 hours. The MIC was read as the lowest

concentration of CHG that completely inhibited visible growth of the organism. All results were compared to the growth control well. Purity BAP plates were also confirmed to contain only a single organism.

**Statistical Methods**

All data was imported into SAS 9.4, parsed, cleaned, and combined into a single dataset. Analysis of the relationship between each of the variables and the CHG MIC value for the organisms was then completed based off of the type of variable, i.e. binomial, categorical (see Table 2). For each variable, the mean CHG MIC values for the different categories were compared for significant differences. For binomial variables, pooled or Satterthwaite t-tests comparing the mean CHG MIC were completed, using significant differences in variance as a guide as to which to use. For categorical variables, one-way ANOVA was used to analyze significant differences in means across all groups included. Additionally, all variables were analyzed to see if any additional relationships were present, i.e. interaction, confounding. The results found in Table 2 show the test used, the t- or F-value of the test, and the p-value found for each test.

**Serial Passage Experiments**

For liquid cultures, the organism was streaked out for colonies on a BAP. Individual colonies were selected and placed in 1-5mL of BHI Broth with initial concentrations believed to be below the antimicrobial MIC of the organism. These were grown overnight at 37°C in a shaking incubator. If growth was observed, 100µL of the growth was stored in 1mL of 20% glycerol in Tryptic Soy Broth (TSB) and 100µL was

transferred to the next concentration. If growth was not observed, an additional 1-2 days of growth was allowed to confirm no growth at that concentration. The concentration was increased gradually to ensure that adaptation to that concentration had been achieved. A typical incremental concentration increase was 0.1 to 0.25 µg/mL per step. The organism was confirmed using plating and staining methods. If any culture was found to be contaminated it was terminated. This pattern of growth and confirmation were continued until all colonies are terminated or observed to have significant resistance, i.e. two to four times the epidemiologic cut-off found in the CDC/EIP study. For organisms observed to grow at higher concentrations, broth microdilution plates were used to confirm an actual CHG MIC.

For plate cultures, the organism was streaked for colonies on a BAP. Individual colonies were selected and followed one of two different techniques. The first technique involved direct streaking for colonies on the next concentration and overnight growth at 37°C in an incubator. Each step, the concentration was increased by 0.5µg/mL. The organism was confirmed through Catalase, plating, and staining techniques. The second technique was to suspend the colony in 1mL of BHI Broth and then create a lawn on the next concentration using 100µL of the previous growth. These were grown in an incubator overnight at 37°C. Each step, the concentration was increased by 0.5µg/mL. For both techniques, samples were collected and placed in 20% glycerol in TSB and growth continued until all colonies were terminated or observed to have significant resistance, i.e. two to four times the epidemiologic cut-off. For the second plate technique, mupirocin plates were also run as positive controls to have an accurate comparison of the timeline in which resistance should be developing. For organisms

observed to grow at higher concentrations, broth microdilution plates were used to confirm an actual CHG MIC. Organisms seen to grow at higher concentrations of mupirocin had their resistance confirmed through repeated growth on plates of that concentration.

## Results

Analysis of data provided by the combined CDC and GA EIP experiment showed no significant differences in CHG MIC based off any of the variables tested (see Table 2). The most important negative result observed in this data is the lack of a significant difference in MIC values for isolates obtained pre-CHG use when compared to post-CHG use isolates. It would be expected that a significant difference would be observed in CHG MIC between these two periods, but a difference in pre- vs post-CHG use was not observed in the data (p-value: 0.684). Additionally, greater resistance based off the epidemiological classification might also be expected as the infections obtained in a healthcare setting are more likely to have frequent CHG exposure. The relationship observed in the data was not significant (p-value: 0.421). Significant differences in CHG MIC based off the type of insurance of the patient were observed but are likely due to 64 of the samples having missing values for this variable. This data suggests that none of these variables are significant contributors to CHG resistance development in MRSA.

In vitro evolution of reduced CHG susceptibility in MRSA proved to be significantly more difficult than previously believed. Despite repeated attempts with different techniques and organisms, no significant CHG resistance was observed (Table 3). The positive control of mupirocin resistance was observed in the same time frame

and seemed to develop with relative ease. All organisms seen to grow to a significant concentration of CHG resistance were either falsely resistant or contaminated cultures.

## Discussion

The results of this study help to alleviate some anxiety relating to CHG resistance in MRSA. Previous studies, like the one conducted by Hardy et al., have compared MRSA samples from pre- and post-chlorhexidine usage and observed a 3-fold increase in CHG resistance. With such a significant increase observed, it is no wonder that there are concerns about CHG resistance beginning to develop. Within this study we saw no such increase in CHG resistance, despite samples from Health District 3 – Metropolitan Atlanta that were collected over a 13-year time period. Further inquiry showed that all post-2002 cultures that were included in Hardy et al.'s study were collected from a single hospital where extensive use of CHG was documented. After analyzing the results of the Georgia EIP surveillance study, the researcher surmises that it is likely that the observed increased resistance was due to the practices of that facility and were not representative of resistance seen in MRSA as a whole. Not only was there no increased resistance observed over the 13-year time period in which this study's samples were collected, but resistance did not seem to come easily to MRSA. All in vitro experiments trying to create less susceptible mutants failed, despite seeing mupirocin resistance develop under the same conditions in the same strains and timeframe. While not entirely comprehensive, this study shows that evolutionary pressures believed to contribute to CHG resistance in MRSA are likely not as urgent as previously suggested. Despite these

findings, continued surveillance and stewardship are necessary in continuing to prevent further development of CHG resistance

**Table 1.**[1]
Summary statistics of samples collected by the Georgia Emerging Infections Program in Health District 3 – Metropolitan Atlanta from 2005 - 2018

| Characteristics | | Overall | | Pre-Chlorhexidine Use | | Post-Chlorhexidine Use | |
|---|---|---|---|---|---|---|---|
| | | N | % | N | % | N | % |
| Chlorhexidine MIC (ug/mL)[2] | | 3.0 (1.3) | | 2.9 (1.1) | | 3.0 (1.4) | |
| Sex | Female | 44 | 48.9 | 14 | 46.7 | 30 | 50 |
| | Male | 46 | 51.1 | 16 | 53.3 | 30 | 50 |
| Age (years)[2] | | 56.04 (13.8) | | 58 (12.4) | | 55.0 (14.4) | |
| Age Range (years) | 20-29 | 4 | 4.4 | 0 | 0.0 | 4 | 6.7 |
| | 30-39 | 9 | 10.0 | 2 | 6.7 | 7 | 11.7 |
| | 40-49 | 9 | 10.0 | 5 | 16.7 | 4 | 6.7 |
| | 50-59 | 26 | 28.9 | 10 | 33.3 | 16 | 26.7 |
| | 60-69 | 19 | 21.1 | 4 | 13.3 | 15 | 25 |
| | 70-79 | 15 | 16.7 | 7 | 23.3 | 8 | 13.3 |
| Hospitalized | Yes | 87 | 96.7 | 29 | 96.7 | 58 | 96.7 |
| | No | 3 | 3.3 | 1 | 3.33 | 2 | 3.33 |
| Epidemiological Classification | CA | 6 | 6.7 | 5 | 16.7 | 1 | 1.67 |
| | HACO | 73 | 81.1 | 17 | 56.7 | 56 | 93.3 |
| | CO | 11 | 12.2 | 8 | 26.7 | 3 | 5.0 |
| Ethnicity | Hispanic | 3 | 3.3 | 0 | 0.0 | 3 | 5.0 |
| | Non-Hispanic | 86 | 95.6 | 30 | 100 | 56 | 93.3 |
| | Unknown | 1 | 1.1 | 0 | 0.0 | 1 | 1.7 |
| Race | Black | 65 | 72.2 | 23 | 76.7 | 42 | 70 |
| | White | 20 | 22.2 | 6 | 20.0 | 14 | 23.3 |
| | Native American or Pacific Islander | 1 | 1.1 | 0 | 0.0 | 1 | 1.7 |
| | Asian | 1 | 1.1 | 1 | 3.3 | 0 | 0.0 |
| | Unknown | 3 | 3.3 | 0 | 0.0 | 3 | 5.0 |
| Insurance | Medicaid/State Program | 2 | 2.2 | 2 | 6.7 | 0 | 0.0 |
| | Medicare | 20 | 22.22 | 15 | 50 | 5 | 8.3 |
| | Private | 2 | 2.2 | 1 | 3.3 | 1 | 3.3 |
| | Prison | 1 | 1.1 | 1 | 3.3 | 1 | 3.3 |
| | No Coverage | 1 | 1.1 | 1 | 3.3 | 0 | 0.0 |
| | Unknown | 64 | 71.1 | 9 | 33.33 | 54 | 90.0 |

[1] Some of the totals do not add up to the full sample due to missing values.

[2] These variables were continuous, so the values provided are Mean (Standard Deviation).

**Table 2.**
Statistical tests comparing mean Chlorhexidine MIC values for significant differences within groups contained in each variable.

| Variable | Statistical Test | t, F-value | p-value (P>t, F) |
|---|---|---|---|
| Pre- or Post-Chlorhexidine Use | Pooled t-test | 0.41 | 0.684 |
| Sex | Satterthwaite t-test | 0.00 | 0.997 |
| Age Range | One-way ANOVA | 1.68 | 0.149 |
| Hospitalization (Y/N) | Pooled t-test | -0.49 | 0.625 |
| Epidemiological Classification | One-way ANOVA | 0.87 | 0.421 |
| Ethnicity | One-way ANOVA | 0.41 | 0.667 |
| Race | One-way ANOVA | 0.35 | 0.843 |
| Insurance[1] | One-way ANOVA | 2.81 | 0.043 |

[1]The significant value observed here was likely due to the high number of missing values for Insurance (64 patients).

**Table 3.**
List of experiments and methods, by strain number and result: MRSA - USA 300 JE2 and MSSA - ATCC 25923. Completed 2019 and 2020.

| Experiment | Medium Used | Organism Number | Observed MIC Level (ug/mL) | Outcome |
|---|---|---|---|---|
| 1 | Overnight Growth in Broth | MRSA - 1 | CHG 0.5 | Contaminated |
| | | MRSA - 2 | CHG 2.0 | Contaminated |
| | | MRSA - 3 | CHG 0.4 | No Growth |
| | | MRSA - 4 | CHG 2.0 | No Growth |
| | | MRSA - 5 | CHG 1.0 | No Growth |
| 2 | Overnight Growth in Broth | MRSA - A1 | CHG 2.0 | Contaminated |
| | | MRSA - A2 | CHG 0.5 | No Growth |
| | | MRSA - A3 | CHG 1.5 | No Growth |
| | | MRSA - A4 | CHG 1.0 | No Growth |
| | | MRSA - B | CHG 1.0 | No Growth |
| | | MRSA - C | CHG 0 (2.0) | No Growth in Initial Concentration |
| 3 | Streaked Plates | MSSA - 1 | CHG 4.0 | No Growth |
| | | MSSA - 2 | CHG 2.0 | No Growth |
| | | MSSA - 3 | CHG 3.0 | No Growth |
| | | MSSA - 4 | CHG 4.0 | No Growth |
| 4 | Lawn Plates | MRSA - 1 | CHG 6.5 | Contaminated |
| | | MRSA - 1 | MUP 1.5 | Contaminated |
| | | MRSA - 2 | CHG 3.0 | No Growth |
| | | MRSA - 2 | MUP 16 | Growth |
| | | MSSA - 1 | CHG 3.5 | No Growth |
| | | MSSA - 1 | MUP 16 | Growth |
| | | MSSA - 2 | CHG 4 | No Growth |
| | | MSSA - 2 | MUP 16 | Growth |

Works Cited

Bhardwaj P, Hans A, Ruikar K, Guan Z, Palmer KL. Reduced Chlorhexidine and Daptomycin Susceptibility in Vancomycin-Resistant Enterococcus faecium after Serial Chlorhexidine Exposure. *Antimicrob Agents Chemother*. 2017;62(1):e01235-17. Published 2017 Dec 21. doi:10.1128/AAC.01235-17

Chambers HF, Deleo FR. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat Rev Microbiol.* 2009;7(9):629–641. doi:10.1038/nrmicro2200

Climo MW, Yokoe DS, Warren DK, et al. Effect of daily chlorhexidine bathing on hospital-acquired infection [published correction appears in N Engl J Med. 2013 Jun 13;368(24):2341]. N Engl J Med. 2013;368(6):533–542. doi:10.1056/NEJMoa1113849

Dale GE, Broger C, Hartman PG, et al. Characterization of the gene for the chromosomal dihydrofolate reductase (DHFR) of Staphylococcus epidermidis ATCC 14990: the origin of the trimethoprim-resistant S1 DHFR from Staphylococcus aureus?. *J Bacteriol.* 1995;177(11):2965–2970. doi:10.1128/jb.177.11.2965-2970.1995

Dantes R, Mu Y, Belflower R, et al. National Burden of Invasive Methicillin-Resistant Staphylococcus aureus Infections, United States, 2011. *JAMA Intern Med*. 2013;173(21):1970–1978. doi:10.1001/jamainternmed.2013.10423

Davies J, Davies D. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev*. 2010;74(3):417–433. doi:10.1128/MMBR.00016-10

Domingues S, Nielsen KM, da Silva GJ. Various pathways leading to the acquisition of antibiotic resistance by natural transformation. Mob Genet Elements. 2012;2(6):257–260. doi:10.4161/mge.23089

Hardy K, Sunnucks K, Gil H, et al. Increased Usage of Antiseptics Is Associated with Reduced Susceptibility in Clinical Isolates of Staphylococcus aureus. *mBio*. 2018;9(3). doi:10.1128/mbio.00894-18.

Horner C, Mawer D, Wilcox M. Reduced susceptibility to chlorhexidine in staphylococci: is it increasing and does it matter?, *J Antimicrob Chemother.* 2012; 67(11): 2547-2559 https://doi.org/10.1093/jac/dks284

Kourtis AP, Hatfield K, Baggs J, et al. Vital Signs: Epidemiology and Recent Trends in Methicillin-Resistant and in Methicillin-Susceptible Staphylococcus aureus Bloodstream Infections — United States. *MMWR Morb Mortal Wkly Rep* 2019;68:214–219. DOI: http://dx.doi.org/10.15585/mmwr.mm6809e1

Septimus EJ, Schweizer ML. Decolonization in Prevention of Health Care-Associated Infections. Clin Microbiol Rev. 2016;29(2):201–222. doi:10.1128/CMR.00049-15

van Hal SJ, Jensen SO, Vaska VL, Espedido BA, Paterson DL, Gosbell IB. Predictors of mortality in Staphylococcus aureus Bacteremia. *Clin Microbiol Rev*. 2012;25(2):362–386. doi:10.1128/CMR.05022-11

Wand ME, Bock LJ, Bonney LC, Sutton JM. Mechanisms of Increased Resistance to Chlorhexidine and Cross-Resistance to Colistin following Exposure of Klebsiella pneumoniae Clinical Isolates to Chlorhexidine. *Antimicrob Agents Chemother*. 2016;61(1):e01162-16. Published 2016 Dec 27. doi:10.1128/AAC.01162-16