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Assessment of *in vitro* synergy of daptomycin or vancomycin plus ceftaroline for daptomycin non-susceptible *Staphylococcus aureus*

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ABSTRACT

The combination of vancomycin or daptomycin plus ceftaroline has showed synergistic results in vitro. This study aimed to investigate in vitro synergy of vancomycin or daptomycin plus ceftaroline for seven patients with daptomycin non-susceptible *Staphylococcus aureus* (SA) bacteremia
Thirteen isolates from seven patients were evaluated: two methicillin-susceptible and five methicillin-resistant SA infections. All patients were treated with daptomycin and became non-susceptible (minimum inhibitory concentration (MIC) >1 µg/mL) with therapy or had resistant strains initially. Time kill experiments were completed with $0.25 \times MIC$, $0.5 \times MIC$, and $0.75 \times MIC$ concentrations. No synergy was seen at $0.25 \times MIC$. Synergy was observed for 4 isolates with vancomycin plus ceftaroline and with daptomycin plus ceftaroline for 2 isolates at $0.5 \times MIC$.
These results are in accordance with literature that supports synergistic combinations of daptomycin or vancomycin with ceftaroline for SA bacteremia. Daptomycin non-susceptible SA bacteremia presents a treatment challenge.
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Staphylococcus aureus (SA) is a virulent organism with the ability to develop resistance against many different antimicrobial classes (Lowy, 2003) for which the Centers for Diseases Control has named a serious antibiotic-resistant threat. For patients with persistent bacteremia, deep-seated infections, or resistant isolates, the management of these infections can be challenging. Current best practice recommendations suggest combination therapy with daptomycin and another anti-staphylococcal agent if methicillin-resistant SA (MRSA) bacteremia persists or vancomycin therapy fails (Liu et al., 2011).

Many antimicrobial combinations utilizing different mechanisms of action have been studied both *in vitro* and *in vivo* in hopes to elucidate a regimen able to effectively treat these patients (Nguyen & Graber, 2010; Steigbigel et al., 1975). The addition of a beta-lactam to daptomycin therapy has been shown to increase daptomycin killing by enhancing daptomycin binding leading to rapid bacteremia clearance (Dhand et al., 2011). Additionally, the combination of vancomycin or daptomycin plus ceftaroline has showed promising *in vitro* synergistic results. Rose and colleagues reported synergistic combinations of daptomycin or vancomycin with ceftaroline that resulted in significantly lower

daptomycin MICs compared to monotherapy (Rose et al., 2012). Furthermore, Rybak and colleagues demonstrated *in vitro* improved killing with the combination of these antimicrobials versus either agent alone, although it is important to note that synergy was seen in sixty-seven percent of strains tested (Werth et al., 2014).

However, we lack clear treatment guidelines for treatment of daptomycin non-susceptible SA bacteremias. More *in vitro* and *in vivo* studies need to be completed to evaluate the synergistic relationship of commonly used anti-MRSA agents. We present our *in vitro* synergy results with combinations of vancomycin or daptomycin plus ceftaroline for seven patients with daptomycin non-susceptible SA bacteremia.

1. Materials and methods

The study was performed at a five-hospital health system, Henry Ford Health System, in Detroit, Michigan. Blood isolates initial identification and sensitivities were performed in the clinical microbiology laboratory as part of routine patient care. Additional MIC and synergy testing were completed within the infectious diseases research laboratory. We included all of the daptomycin non-susceptible SA strains isolated from 2015–2017. Daptomycin non-susceptibility was defined as a MIC >1 µg/ml. MICs were determined by manual broth microdilution (BMD) using Clinical and Laboratory Standards Institute standards (CLSI, 2015).

Blood isolates from 7 patients with SA bacteremia were evaluated: two with methicillin-susceptible and five with MRSA infections. All the patients initially had non-susceptible strains or were treated with

daptomycin and became non-susceptible during therapy. Thirteen isolates were obtained; six patients had first and last isolates evaluated, and one patient had one isolate obtained, as he expired before another isolate could be acquired. Two of the first isolates and all of the last isolates were daptomycin non-susceptible with MICs greater than 1 μg/mL. We also performed synergies using quality control strains, *Staphylococcus aureus* ATCC 29213 and *Klebsiella pneumoniae* ATCC 700603. Cation-adjusted Mueller-Hinton II broth (CA-MHB; BBL, Sparks, MD) was used for all BMD and synergy time kill experiments (TKE) and supplemented with $CaCl_2$ for a final concentration of 50 mg/L for all daptomycin testing. Combinations of daptomycin plus ceftaroline and vancomycin plus ceftaroline were tested for synergy, and all TKE were completed in duplicate using sub-inhibitory $0.25 \times$ MIC, $0.5 \times$ MIC, and $0.75 \times$ MIC concentrations. If an isolate showed synergy at $0.5 \times$ MIC, $0.75 \times$ MIC TKE were not completed. Methods of synergy testing were completed as previously described (NCCLS, 1999). The original bacterial inoculum was approximately 5×10^5 CFU/mL. Testing was performed in four tubes (A-D) with 10ml total volumes; Tubes A and B as vancomycin/daptomycin and ceftaroline alone, tube C as the combination of vancomycin/ceftaroline or daptomycin/ceftaroline, and tube D as a growth control. Synergy was defined as ≥2 log_{10} decrease in CFU/mL between the combination and its most active constituent after 24 h (Eliopoulos & Moellering, 1991).

Pulsed-field gel electrophoresis (PFGE) was performed on each isolate to evaluate strain typing. Genomic DNA was prepared using a modified version of a previously described method (McDougal et al., 2003) and was digested with *Sma*I restriction endonuclease. A CHEF-DR III system (Bio-Rad, Hercules, CA) was used to perform all PFGE. Data for all SA isolates were entered into a Gel Doc XR+ Gel Documentation System (Bio-Rad, Hercules, CA) database and PFGE patterns were compared using BioNumerics software (Applied Maths, St-Martens-Latem, Belgium). Isolates were placed in the same PFGE group if *Sma*I restriction patterns were ≥80% similarity using the Dice coefficient. Isolates were classified into PFGE groups according to the US Centers for Disease Control and Prevention (CDC) classification system (McDougal et al., 2003). Whole genome sequencing (WGS) was performed for DNA from 13 isolates and two quality control strains; methicillin-sensitive *Staphylococcus aureus* ATCC 29213 and methicillin-resistant control strain FoxR was extracted and purified using the DNeasy® Blood and Tissue Kit protocol (Qiagen). WGS was performed by the Applied Genomics Technology Center in Detroit, MI using Nextera XT DNA Library Prep Kitgenerated libraries run as 300 bp paired-end reads on a MiSeq. The following genetic loci previously implicated in daptomycin non-susceptibility were investigated using WGS: vraR (Iwata et al., 2017), vraS (Iwata et al., 2017), mprF (Tran et al., 2015), dltA (Tran et al., 2015), cls2 (Tran et al., 2015), and gdpD (Arias et al., 2011).

2. Results

In the quality control strains, synergy was observed using SA 29213 in both vancomycin/ceftaroline and daptomycin/ceftaroline combinations, while, as predicted, none were seen with KP700603 in both combinations. No synergy was noted for any isolate with either vancomycin or daptomycin plus ceftaroline at $0.25 \times$ MIC. At $0.5 \times$ MIC, synergy of vancomycin and ceftaroline was observed in 4 patients. For the 3 patients that synergy was not observed, $0.75 \times$ MIC became synergistic, but only in the last isolates obtained from the patient. Only two patients had synergy with daptomycin and ceftaroline at $0.5 \times$ MIC. Again, this was only observed in the last isolates obtained from the patients. By increasing the concentration to $0.75 \times$ MIC, all but one patient had synergy observed with daptomycin and ceftaroline. See Table 1 for first and last isolate MICs. See Fig. 1a–g for 24-h TKE for each patient. Please note 4-hour TKE were not able to be completed in the $0.75 \times$ MIC daptomycin and ceftaroline studies for patients 2 and 3.

PFGE was used to assess if there were intrinsic changes, such as the introduction of a new strain or modification of the existing strain, contributing to the evolution of daptomycin non-susceptibility in 13 SA isolates obtained from seven patients (see Fig. 2). Four of these isolates were methicillin-susceptible and the remaining 9 were methicillin-resistant. Table 2 demonstrates comparison of isolate strains with established CDC SA strains (McDougal et al., 2003). Among methicillin-resistant isolates, 4 were classified as USA100 (92325, 92450, 94246, 94654), 3 as USA300 (95689, 97635, 98833), and 2 as USA1000 (100547, 100553). Among methicillin-susceptible isolates, 2 were classified as USA900 (92965, 98482) and the other 2 were determined to be non-USA100-1100 strains. Variability of strain type was not observed between first and last isolate for all 7 patients. Each patient's isolates were one strain type that was distinguishable from the strain types identified in the other patients.

WGS was used to characterize genetic patterns of daptomycin resistance in the first and last isolates of each patient, as shown in Table 2. In all 7 patients, there was no change in genetic composition between the first and last isolates. vraR, vraS, mprF, dltA, and cls2 were present in all isolates independent of methicillin or daptomycin susceptibilities. However, the presence of gdpD was more selective. gdpD was not seen in isolates that were methicillin-susceptible (4/13) and among the 8 isolates classified as daptomycin non-susceptible, gdpD was present in 6 of 8 isolates.

3. Discussion

The results of this study are in accordance with recent literature supporting synergistic combinations of daptomycin or vancomycin with ceftaroline for SA bacteremia. Werth and colleagues reported synergy with the use of vancomycin and ceftaroline combination in methicillin-resistant vancomycin-intermediate SA isolates. They compared this combination to vancomycin and oxacillin and found the ceftaroline combination to be much more active, noting synergy in all but one strain (Werth et al., 2013). Likewise, Barber and colleagues reported a similar phenomenon seen in a single patient with daptomycin-non-susceptible MRSA bacteremia. *In vitro* TKE revealed synergy with the use of ceftaroline in combination with daptomycin or vancomycin compared to any agent alone. Of note, the vancomycin and ceftaroline combination showed greater killing compared to the combination of daptomycin and ceftaroline. The team also reported a successful clinical outcome of the patient with clearance of blood cultures after switching to the vancomycin and ceftaroline combination (Barber et al., 2015).

Table 1
Staphylococcus aureus minimum inhibitory concentrations (μg/mL) of first and last isolates tested.

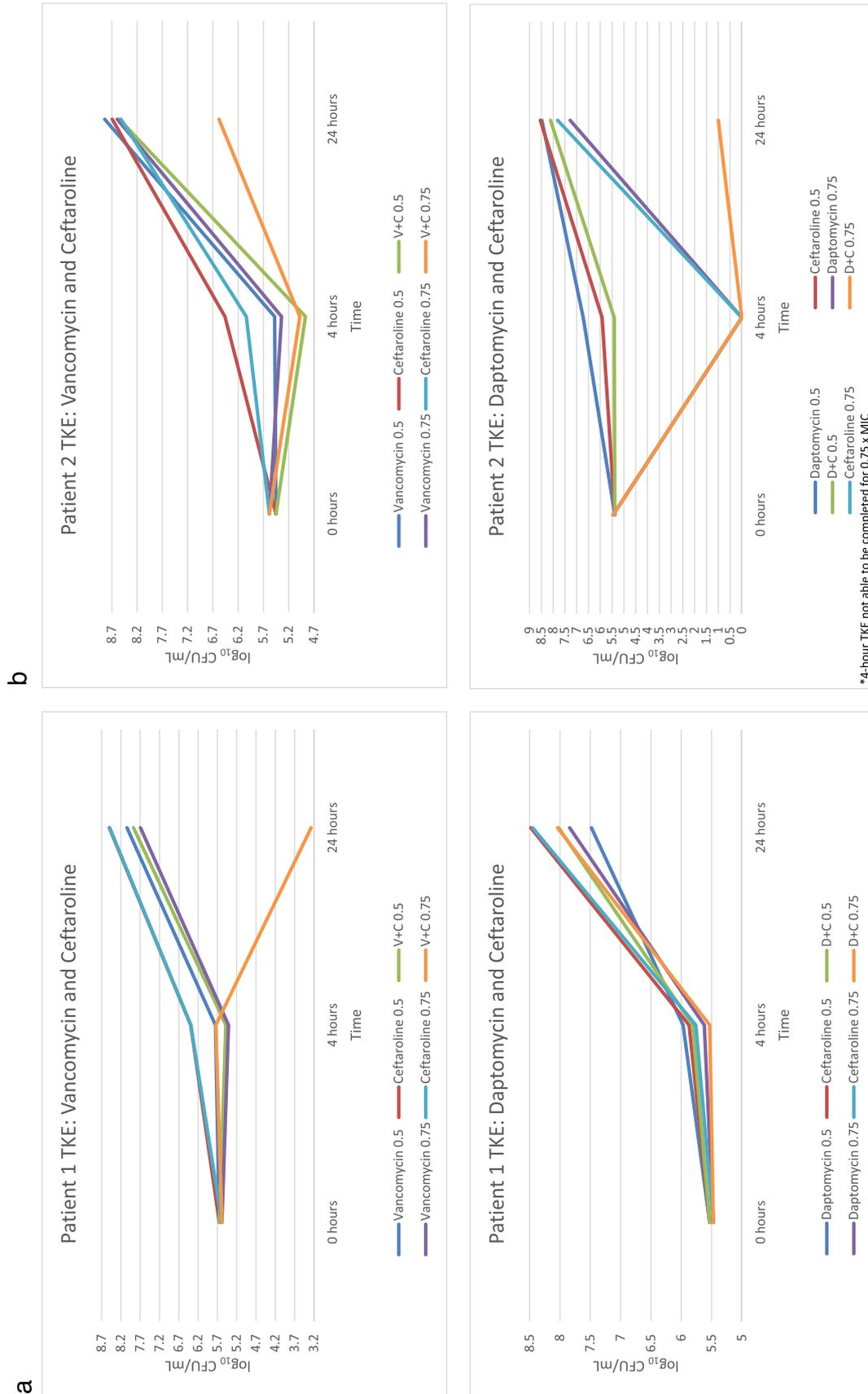|	Isolate	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Vancomycin MIC	First	1	1	1	1	2	1	4
	Last	2	2	2	2	2	1	-
Daptomycin MIC	First	1	1	1	0.5	2	1	8
	Last	16	8	2	16	4	2	-
Ceftaroline MIC	First	0.25	0.5	0.25	0.5	0.25	0.25	0.25
	Last	0.06	0.5	0.25	0.12	0.25	0.25	-

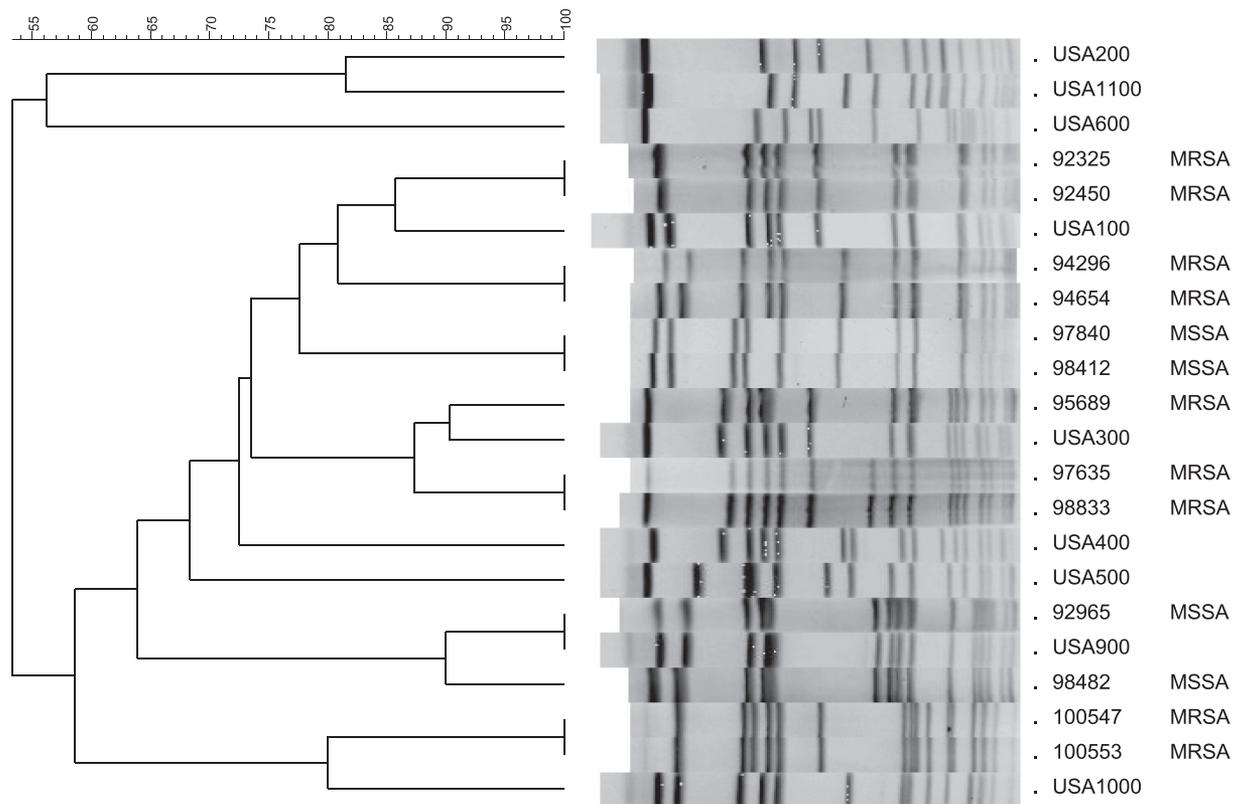Fig. 1. a–g: 24-hour TKE for patients 1–7.

Fig. 2. PFGE dendrogram comparing first and last isolates obtained from seven patients and digested with *SmaI* against established CDC MRSA strains 100-1100. Brackets distinguish each set of patient isolates.

Within our study cohort, patients 4-7 demonstrated synergy with vancomycin plus ceftaroline at $0.5 \times$ MIC, while patients 4 and 6 isolates also had synergy to daptomycin plus ceftaroline at $0.5 \times$ MIC. Patient four received six weeks of vancomycin plus ceftaroline, had recurrence of bacteremia and was retreated with ceftaroline for 6 weeks followed by doxycycline suppression as new hardware had been placed. Patient five was treated with daptomycin 6 mg/kg daily alone, prior to dapto MICs being reported, and expired. Patient 6 was treated initially with daptomycin plus ceftaroline for 2 weeks followed by an 8 week course of vancomycin and survived. Patient 7 was at an outside facility for four weeks prior to transfer had had persistently positive blood cultures in the setting of prosthetic aortic valve endocarditis complicated by aortic root abscess. The patient received combinations of vancomycin with gentamicin followed by daptomycin with gentamicin. He expired at our facility after two days of therapy with daptomycin, gentamicin, and rifampin. Patients 1–3 did not demonstrate synergy at $0.5 \times$ MIC to either combination of antibiotics but did at $0.75 \times$ MIC with vancomycin plus ceftaroline (all patients) and daptomycin plus ceftaroline (patients 2 and 3). Patients 1 and 3 expired. Patient 1 was treated with daptomycin 10mg/kg plus ceftaroline and had clearance of the bacteremia. It recurred several months later in which the isolate was susceptible to daptomycin. The patient enrolled in hospice due to worsening heart and renal failure and expired. Patient 3 was initially treated with vancomycin in the setting of bacteremia and lumbar osteomyelitis. The patient had a recurrence and was briefly treated with high dose daptomycin plus linezolid followed by vancomycin plus linezolid. His infection recurred several years later. Patient 2 survived and was treated with a course of ceftaroline.

Table 2
Daptomycin resistance genes in first and last *S. aureus* isolates as determined by WGS. The exception is Patient 7, who had only one isolate collected to patient death before the collection of a second isolate. Determination of daptomycin susceptibility was based on the CLSI breakpoint of >1 μg/mL (S = susceptible, NS = non-susceptible).

Patient	Isolates	Methicillin resistance status	Daptomycin susceptibility status	vraR	vraS	mprF	dltA	cls2	gdpD
1	92965	MSSA	S	+	+	+	+	+	
	98482	MSSA	NS	+	+	+	+	+	
2	97635	MRSA	S	+	+	+	+	+	+
	98833	MRSA	NS	+	+	+	+	+	+
3	97840	MSSA	S	+	+	+	+	+	
	98412	MSSA	NS	+	+	+	+	+	
4	100547	MRSA	NS	+	+	+	+	+	+
	100553	MRSA	NS	+	+	+	+	+	+
5	92325	MRSA	S	+	+	+	+	+	+
	92450	MRSA	NS	+	+	+	+	+	+
6	94296	MRSA	S	+	+	+	+	+	+
	94654	MRSA	NS	+	+	+	+	+	+
7	95689	MRSA	NS	+	+	+	+	+	+
Control	ATCC29213	MSSA	-	+	+	+	+	+	
Control	FoxR	MRSA	-	+	+	+	+	+	+

Our study population was unique as all patients had at least one daptomycin non-susceptible SA isolate obtained during inpatient treatment, and 2 patients also had methicillin-susceptible strains. While our findings agree with recent synergy literature, some interesting trends were also noted in our study that warrant further investigation.

No isolate had synergy at $0.25 \times$ MIC for either combination but increasing concentrations to $0.5 \times$ MIC led to synergy in the majority of the vancomycin and ceftaroline studies, which was not seen with daptomycin plus ceftaroline. Further increasing the MIC concentration to $0.75 \times$ MIC led to synergy in all patients with vancomycin and ceftaroline and all but one patient in the daptomycin and ceftaroline studies. Since the majority of the daptomycin and ceftaroline studies did not have synergy below $0.75 \times$ MIC, higher daptomycin doses may be required in clinical practice to eradicate SA infections. In recent literature, higher daptomycin doses have been associated with improved mortality for SA bacteremia (Timbrook et al., 2018). Furthermore, in a review of salvage therapy for persistent SA bacteremia by Sakoulas and colleagues, the combination of ceftaroline and daptomycin, most commonly dosed at 8–10 mg/kg, was successful for 25 out of 26 patients receiving that combination (Sakoulas et al., 2014).

Another interesting phenomenon observed in our study was that synergy was more likely to be observed at lower MIC concentrations if the vancomycin or daptomycin MIC was elevated. For example, patient four's first staphylococcal isolate with a daptomycin MIC of 0.5 μg/mL did not have synergy observed at any MIC concentration for daptomycin and ceftaroline, but the patient's last isolate with a daptomycin MIC of 16 μg/mL had daptomycin and ceftaroline synergy observed at $0.5 \times$ MIC. This was also seen in patients one through three for the vancomycin plus ceftaroline combination and patients 5 and 6 for the daptomycin plus ceftaroline combination. The reasoning behind this phenomenon may be explained by one of the resistance mechanisms of SA. These bacteria have developed many different mechanisms of resistance, including synthesis of PBP2a which replaces other PBPs and limits all beta-lactam binding, as seen in MRSA. It can also alter peptidoglycan synthesis that leads to thickened cell walls and resistance to vancomycin (Lowy, 2003). Daptomycin resistance may result from cell membrane changes, including modification of a negative charge to a more positive charge, increased rigidity or fluidity of the membrane, or reduction of the phospholipid head groups phosphotidylglycerol and cardiolipin (Bayer et al., 2014). Any combination of these resistance mechanisms can produce resistance to an antimicrobial, but leave other antimicrobials susceptible or more susceptible depending on the mechanism of action of the antimicrobial. Mutations leading to changes in the cell wall which raise vancomycin and/or daptomycin MICs could lead the organism to be more susceptible to synergy when these antimicrobials are combined with ceftaroline or any other beta-lactam, also known as the "seesaw effect".

There are limitations to the current study. Our study was completed in a small sample of 13 isolates of seven patients that were all treated at the same institution, which cannot be generalized to all daptomycin non-susceptible isolates. These synergy tests were only completed in daptomycin non-susceptible strains, and the results cannot be applied to daptomycin susceptible strains. Synergy testing was completed with ceftaroline plus vancomycin or daptomycin, but no other antimicrobials, which leads to further questions about other agents to use in combination that may have synergistic relationships. Furthermore, our in vitro synergy data does not necessarily translate to in vivo synergy outcomes, which must be evaluated in larger patient populations to determine the most appropriate therapy for these patients. In vitro experiments depend on laboratory technician judgement and technique, which may lead to errors in interpretation, although our duplicate experiments did not differ in synergy results.

Our study highlights the importance of further investigation into differences in SA strain types, as genomic sequencing may reveal genes associated with antimicrobial combination therapy success or failure. Our laboratory investigated these patients' SA isolates using pulse field gel electrophoresis and found that changes in daptomycin susceptibility were within one strain type without modification or introduction of additional strain types.

In the absence of additional strain types or modification of strain type, as determined by PFGE, it can be ascertained that for this group of isolates, the development of daptomycin non-susceptibility is due to intrinsic changes. The presence of cell envelope modification genes vraS, vraR, dltA, mprF and cls2 in both daptomycin-susceptible and daptomycin-non-susceptible isolates indicates the possibility of gene upregulation in the setting of prolonged antibiotic treatment. This has been previously demonstrated with vraSR and mprF (Mehta et al., 2012), as well as, dltA (Cafiso et al., 2014). Further investigation is needed to pinpoint mutations in these loci between first and last isolates that could have contributed to the development of daptomycin resistance.

Additionally, gdpD was only seen in MRSA isolates and was isolated in 6 of 8 daptomycin non-susceptible strains, suggesting an association of gene expression with methicillin status and its contribution to the development of daptomycin resistance (Sundaram et al., 2018).

This study also highlights the need for animal models to evaluate the effect of daptomycin dosing on synergy with ceftaroline for SA bacteremia. Investigating further into SA resistance and treatment options will allow us to better understand the most appropriate management of these difficult to treat infections.

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Author statement

Mary A. Hutton: Investigation, formal analysis, writing – original draft; **Ayesha Sundaram**: writing – original draft; **Mary B. Perri**: Methodology, validation, formal analysis, investigation; **Marcus J. Zervos**: Conceptualization, supervision **Erica S. Herc**: Writing – Reviewing & Editing, visualization, supervision.

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