Ceftaroline-Heteroresistant *Staphylococcus aureus*

Stephanie N. Saravolatz, Hayley Martin, Joan Pawlak, Leonard B. Johnson, Louis D. Saravolatz
St. John Hospital and Medical Center and Wayne State University School of Medicine, Detroit, Michigan, USA

Heteroresistance refers to the presence, within a large population of antimicrobial-susceptible microorganisms, of subpopulations with lesser susceptibilities. Ceftaroline is a novel cephalosporin with activity against methicillin-resistant *Staphylococcus aureus* (MRSA). The aim of this study was to detect the prevalence of ceftaroline heteroresistance *in vitro* in a select group of *S. aureus* strains. There were 57 isolates selected for evaluation, 20 MRSA, 20 vancomycin-intermediate *S. aureus* (VISA), 7 daptomycin-nonsusceptible *S. aureus* (DNSSA), 6 linezolid-nonsusceptible *S. aureus* (LNSSA), and 4 heteroresistant VISA (hVISA) isolates. MICs and minimal bactericidal concentrations were determined using the broth microdilution method according to CLSI guidelines. All of the isolates were analyzed by pulsed-field gel electrophoresis. The staphylococcal cassette chromosome mec (SCCmec) types were determined by a multiplex PCR. Population analysis profiles (PAPs) were performed to determine heteroresistance for all of the isolates using plates made by adding various amounts of ceftaroline to brain heart infusion agar. The frequencies of resistant subpopulations were 1 in 10⁴ to 10⁵ organisms. We determined that 12 of the 57 (21%) isolates were heteroresistant subpopulations with lesser susceptibilities (4). Although the clinical significance is still unclear, heteroresistance has been reported among various antimicrobial agents used against *S. aureus*, including beta-lactams and vancomycin. Typically, the subpopulations with lesser susceptibilities are present at frequencies of 1 subclone in every 10⁴ to 10⁶ colonies. This is why it is difficult to detect these clones in normal broth microdilution MIC testing using an inoculum of 5 × 10⁴ CFU/well. Population analysis profiles (PAPs), which use a larger inoculum size, are considered the most reliable method for detecting heteroresistant subpopulations. The aim of this study was to detect the prevalence of ceftaroline heteroresistance *in vitro* in a select group of *Staphylococcus aureus* strains.

**Materials and Methods**

Isolates. A collection of 57 isolates was selected for evaluation. Methicillin-resistant *S. aureus* (MRSA) (*n* = 20), heteroresistant vancomycin-intermediate *S. aureus* (hVISA) (*n* = 4), and daptomycin-nonsusceptible *S. aureus* (DNSSA) (*n* = 7) isolates were obtained from patients admitted to St. John Hospital and Medical Center (Detroit, MI). VISA (*n* = 20) and four of the linezolid-nonsusceptible *S. aureus* (LNSSA) isolates were obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program (supported under NIAID/NIH contract HHSN272200700055C). Two LNSSA isolates were obtained from Robin Memorial Hospital in Ohio. Either ceftaroline was not given to these patients, or their exposure was unknown.

**Susceptibility testing.** MICs were determined using microdilution tests with cation-adjusted Mueller-Hinton broth. MICs were determined in accordance with CLSI guidelines. MICs were read visually as the lowest drug concentration well with no visible bacterial growth. Minimal bactericidal concentrations (MBC) were determined to be the antibiotic concentration that reduced the number of viable cells by ≥99% as determined by colony counts. We also determined MICs using an Etest strip containing ceftaroline.

**Molecular testing.** Staphylococcal cassette chromosome mec (SCCmec) types were determined by using a multiplex PCR method on all isolates (5). The isolates were analyzed by pulsed-field gel electrophoresis (PFGE) using the restriction enzyme Smal. The PFGE gel patterns were compared with the development of a dendrogram using GelCompar II software (Applied Maths). Percent similarities were derived from the unweighted-pair group method using arithmetic averages (UPGMA) and based on Dice coefficients. The band position tolerance was set at 1.25, and optimization was set at 0.5%. An isolate was determined to belong to a PFGE strain group (USA-100 to USA-1100) if its similarity coefficient was ≥80% (6). The USA-100 to USA-1100 strains used for comparison were obtained from the NARSA.

**Heteroresistance testing procedure.** PAP assays to detect heteroresistance were performed as previously described (7) with the following modifications. Testing plates were prepared by adding ceftaroline to brain heart infusion (BHI) agar (Difco). The BHI agar was prepared according to the manufacturer’s instructions. Ceftaroline (CPT) powder was reconstituted and added to the BHI agar plates at concentrations of 0.25, 0.5,
The isolates to be tested were grown overnight on blood agar plates (BAP). The overnight BAP culture was suspended in saline and used to prepare samples at 10^7 CFU/ml and 10^4 CFU/ml. Aliquots of the samples containing 10^7 CFU/ml were then spiral plated (Whitley automatic spiral plater; Microbiology International) onto a drug-free BHI agar plate and onto BHI agar plates containing ceftaroline at all concentrations to determine the presence of heteroresistance. Aliquots of the samples containing 10^4 CFU/ml were spiral plated onto the drug-free BHI agar plate and onto plates containing 0.25, 0.5, and 0.75 g/ml of ceftaroline. This was done to obtain an accurate determination of the numbers of CFU/ml. The plates were incubated at 35°C for 48 h in ambient air. The colonies were counted after 48 h using a ProtoCOL automated colony counter (Synbiosis, Frederick, MD, USA).

PAPs were generated by plotting the log_{10} CFU/ml against the antibiotic concentrations. The frequency of resistant subpopulations at the highest drug concentration was calculated by dividing the number of colonies grown on an antibiotic-containing plate by the colony count from the same bacterial inoculum plated onto the antibiotic-free plate (8). We considered any sample with a susceptible ceftaroline MIC and growth in the intermediate or resistant range as heteroresistant. All samples were run three separate times, and the results were averaged for a final result.

Colonies that grew on the plates containing the highest concentration of ceftaroline were removed and subcultured daily for 7 days onto antibiotic-free medium. The isolates were subcultured, and MICs were determined daily in order to determine if the resistance was stable (9).

RESULTS

Table 1 provides the data for PAPs for the isolates that did not show growth above 1 g/ml of ceftaroline on PAP plates.
demonstrate heterogeneous growth in the presence of ceftaroline was 8.1%, as reported by Liu and Chambers (10), who reviewed 14 studies. More recently, it was reported at a higher rate of 8.1%, as noted by Khatib et al. (11).

This rate of ceftaroline heteroresistance may be of concern if heteroresistance is a precursor to resistant isolates. If the selective pressure of prolonged exposure to an antimicrobial agent enhances the likelihood of the emergence of organisms resistant to the therapeutic agent administered, we would expect to see an increasingnumber of clinical cases resistant to ceftaroline with more widespread use of this agent. The comparison of ceftaroline heteroresistance in this study to vancomycin heteroresistance in other studies may not be valid with the selection bias used in identifying our organisms as opposed to the random selection of MRSA isolates used in the vancomycin heteroresistance studies.

The mechanism for ceftaroline heteroresistance is unknown. We know that these strains are virulent, as they were isolated from clinical infections, including bacteremia, pneumonia, and wound infections. We do not know if these strains are more or less virulent than other ceftaroline-susceptible strains. To date, clinical cases of failure due to ceftaroline heteroresistance have not been reported.

Vancomycin-heteroresistant strains have demonstrated lower MICs for vancomycin compared to susceptible strains, as shown in Table 1. In this study, we found that 12 (21%) of the 57 tested strains demonstrated heteroresistance to ceftaroline. This rate is lower than previously reported rates of vancomycin heteroresistance, which ranges from 1.1% to 2.2%. The rate of ceftaroline heteroresistance in this study was 8.1%, which is not significantly different from the rates reported in other studies.

The implications of ceftaroline heteroresistance are significant, as this agent is being increasingly used in clinical settings. If heteroresistance is a precursor to resistance, then the selective pressure of ceftaroline may contribute to the development of resistance in clinical isolates. Therefore, it is important to monitor the emergence of heteroresistance and to consider the use of alternative treatment options.

The data from this study suggest that ceftaroline heteroresistance is more widespread than previously reported, and further research is needed to understand the mechanism of heteroresistance and its implications in clinical settings.

**DISCUSSION**

Among the 57 isolates tested, 12 (21%) of the strains demonstrated heteroresistance by the population analysis profiling method, which is considered an acceptable method for determining heteroresistance. Heteroresistance was found in strains which were nonsusceptible to daptomycin, resistant to linezolid, and intermediate in susceptibility to vancomycin. Finally, we did identify one strain heteroresistant to ceftaroline and vancomycin. The rate of ceftaroline heteroresistance in this study was higher than the rate of vancomycin heteroresistance (2.17% among MRSA isolates) reported by Liu and Chambers (10), who reviewed 14 studies. More recently, it was reported at a higher rate of 8.1%, as noted by Khatib et al. (11).

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growth rates and thicker cell walls than vancomycin-susceptible strains (12). In addition, these strains also produce greater quantities of PBP2 and PBP2’ (13). Several isolates with high ceftaroline MICs (4 μg/ml) obtained from an antibiotic resistance surveillance system demonstrated decreased PBP2a binding affinity due to alterations in the PBP2a (14). Although our study did not evaluate strains for PBPs production or cell wall thickness, we did not see a correlation with ceftaroline heteroresistance and vancomycin heteroresistance. Recent in vitro studies of ceftaroline activity against MRSA isolates with reduced vancomycin susceptibility demonstrated increased activity compared with isolates with lower vancomycin MICs (15); however, this observation was not seen among the isolates evaluated in this study.

The clinical significance of ceftaroline heteroresistance is unclear. Heteroresistant strains were mainly SCCmec type II (75%), and no strains were found to be SCCmec type III or IVa. The small sample size of the CA-MRSA isolates in this study limits the generalizability of the results. Studies evaluating the frequency of ceftaroline heteroresistance among a larger set of CA-MRSA isolates should be performed to confirm this finding.

The information in this study should suggest caution by clinicians using ceftaroline in patients with resistance to other anti-MRSA agents, as heteroresistance was seen in isolates demonstrating reduced susceptibilities to daptomycin and linezolid, and increased ceftaroline MICs were noted in four VISA isolates. To date, the occurrence of ceftaroline heteroresistance has not been shown to be a risk factor for the clinical failure of ceftaroline, and the mechanism for the development of these strains is not known. Further work should be done to evaluate the risk factors for and clinical significance of ceftaroline heteroresistance.

ACKNOWLEDGMENT

Funding for this study was provided by Forest Laboratories, Inc.

REFERENCES


