Multistep Resistance Development Studies of Ceftaroline in Gram-Positive and -Negative Bacteria[▽]

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Ceftaroline, the active component of the prodrug ceftaroline fosamil, is a novel broad-spectrum cephalosporin with bactericidal activity against Gram-positive and -negative isolates. This study evaluated the potential for ceftaroline and comparator antibiotics to select for clones of *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis* with elevated MICs. *S. pneumoniae* and *S. pyogenes* isolates in the present study were highly susceptible to ceftaroline (MIC range, 0.004 to 0.25 μg/ml). No streptococcal strains yielded ceftaroline clones with increased MICs (defined as an increase in MIC of >4-fold) after 50 daily passages. Ceftaroline MICs for *H. influenzae* and *M. catarrhalis* were 0.06 to 2 μg/ml for four strains and 8 μg/ml for a β-lactamase-positive, efflux-positive *H. influenzae* with a mutation in L22. One *H. influenzae* clone with an increased ceftaroline MIC (quinolone-resistant, β-lactamase-positive) was recovered after 20 days. The ceftaroline MIC for this isolate increased 16-fold, from 0.06 to 1 μg/ml. MICs for *S. aureus* ranged from 0.25 to 1 μg/ml. No *S. aureus* isolates tested with ceftaroline had clones with increased MIC (>4-fold) after 50 passages. Two *E. faecalis* isolates tested had ceftaroline MICs increased from 1 to 8 μg/ml after 38 days and from 4 to 32 μg/ml after 41 days, respectively. The parental ceftaroline MIC for the one *K. pneumoniae* extended-spectrum β-lactamase-negative isolate tested was 0.5 μg/ml and did not change after 50 daily passages.

Community-acquired pneumonia (CAP) is a common infection in the United States that is most frequently caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* and, less frequently, *Moraxella catarrhalis* (61). Community-acquired methicillin-resistant *S. aureus* (MRSA) can also cause fulminating and life-threatening CAP infections, particularly in patients with a history of infection with influenza A virus (62). *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila* are also causative agents in CAP, but the exact role of these pathogens in this disease is not well understood (21).

Until recently, infectious disease clinicians had a number of satisfactory antibacterials available for the treatment of CAP. However, the spectrum of resistance phenotypes recovered from CAP patient isolates is changing. Emerging resistance is the result of widespread use of both oral antibiotics and the pediatric conjugate vaccine. The latter is associated with the rise of multidrug-resistant serotype 19A *S. pneumoniae* strains for which there are no U.S. Food and Drug Administration (FDA)-approved drugs available for use in pediatric patients (45, 55).

Historically, β-lactam resistance in *H. influenzae* was largely attributed to β-lactamase production. Recently, the incidence of β-lactamase-negative, ampicillin-resistant (BLNAR) strains has begun to increase, and this incidence may be underestimated due to problems in laboratory detection (24, 28, 45, 59). The BLNAR resistance phenotype is driven by mutations in penicillin-binding protein 3 (PBP3) that cause decreased binding affinity for β-lactam antibiotics. It should be noted that *in vitro*, pharmacodynamic, and clinical otitis medium studies have shown that most *H. influenzae* strains are inherently resistant to macrolides and ketolides, probably due to one or more efflux systems (5, 54).

Problematic resistance phenotypes have also begun to appear in other CAP pathogens. The difficulty of treating MRSA infections has been exacerbated by the emergence of three vancomycin-nonsusceptible phenotypes: heteroresistant vancomycin-intermediate *S. aureus* (hVISA), vancomycin-intermediate resistant *S. aureus* (VISA), and the rarely isolated vancomycin-resistant *S. aureus* (VRSA) (2). The mechanism of resistance in VISA isolates is mediated by a thickened cell wall that often renders these isolates refractory to daptomycin, dalbavancin, and oritavancin (2). Most MRSA strains, especially those acquired in the hospital, are also quinolone resistant (2). In addition, β-lactamase-producing *M. catarrhalis*, macrolide-resistant *Streptococcus pyogenes*, and *Enterobacteriaceae* (e.g., *Klebsiella pneumoniae*) that produce a number of different β-lactamases and also prevent drug entry through mutations in genes encoding the outer membrane protein family (porins) are becoming more common (5, 27, 42).

Complicated skin and soft-structure infections (cSSSIs) are most commonly caused by *S. aureus* (including MRSA) and *S. pyogenes*, often with the same resistance phenotypes noted above (22). Diabetic ulcers of the lower leg with these pathogens are a particularly challenging problem. Elderly patients with these infection types and those who are treated with vancomycin are at particular risk of developing vancomycin-nonsusceptible MRSA as a consequence of decreased vascularization and previous exposure to antibacterial therapy (2).

The future spectrum of CAP and cSSSI will almost certainly include strains with the resistance phenotypes described above,

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TABLE 1. Characteristics of all strains used in the study[a]

Strain	Penicillin susceptibility (MIC, μg/ml)	Phenotype [R determinants]	Source	Yr	Origin	Source or reference
S. pneumoniae 3665	S (0.125)	Macrolide resistant [*mef*(A)]	Blood	2000	Bulgaria	33, 36, 51
S. pneumoniae 2686	S (2)	Macrolide resistant [L4 mutation]	Sputum	2000	Poland	33, 36, 51
S. pneumoniae 1077	S (0.03)	Macrolide susceptible, quinolone resistant	NA	1998	NA	33, 36
S. pneumoniae 7599	I (4)	Macrolide resistant, multiresistant [*erm*(B) and *mef*(A)]	Ear	2006	United States	This study
S. pneumoniae 3548	R (8)	Macrolide resistant [*erm*(B)]	NA	2000	Hungary	33, 36, 51
S. pyogenes 2132	S	Macrolide susceptible	Throat	2000	Bulgaria	52
S. pyogenes 2368	S	Macrolide resistant [*erm*(B)]	Throat	2001	Czech Republic	52
S. pyogenes 2011	S	Macrolide resistant [*mef*(A)]	Throat	2000	Croatia	52
S. aureus 873		HA-MRSA, hVISA	Sputum	2006	Hershey, PA	37
S. aureus 555		HA-MRSA, VISA, daptomycin resistant	Blood	2005	Hershey, PA	30
S. aureus 510		HA-MRSA, VRSA	Wound	2004	Hershey, PA	6, 10
S. aureus 1449		CA-MRSA	NA	2006	Houston, TX	29
S. aureus 543		MSSA	Wound	2006	Hershey, PA	37
H. influenzae 115		Macrolide hypersusceptible, efflux negative, β-lactamase negative	Sputum	1999	NA	26
H. influenzae 73		Macrolide resistant, [L22 mutation], efflux positive, β-lactamase positive	NA	2000	NA	26
H. influenzae 83		BLNAR	NA	2000	Japan	26
H. influenzae 44		Quinolone resistant, β-lactamase positive	NA	1998	NA	26
M. catarrhalis 36		β-Lactamase positive	NA	2002	Cleveland, OH	This study
E. faecalis 568		VSE	Blood	2007	Hershey, PA	This study
E. faecalis 609		VRE	Blood	2007	Hershey, PA	This study
K. pneumoniae 512		ESBL negative	Urine	2009	Hershey, PA	This study

[a] NA, not available; hVISA, heteroresistant vancomycin-intermediate *S. aureus*; HA-MRSA, hospital-acquired methicillin-resistant *S. aureus*; CA-MRSA, community-acquired methicillin-resistant *S. aureus*; VISA, vancomycin-intermediate-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; BLNAR, β-lactamase negative, ampicillin resistant; VSE, vancomycin-susceptible enterococci; VRE, vancomycin-resistant enterococci; ESBL, extended-spectrum β-lactamase producing.

and there is a need for drugs that cover all of these pathogens. Ceftaroline is a novel, broad-spectrum cephalosporin with activity against most Gram-positive pathogens, including MRSA (20, 22, 31, 53). Ceftaroline activity against Gram-negative species is limited, especially against extended-spectrum β-lactamase (ESBL) enzymes and cephalosporinase producers in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Ceftaroline alone is not active against AmpC-derepressed *Enterobacter cloacae*. The selection of species and resistance phenotypes for the present study was made based on recent ceftaroline FDA-approved indications of CAP and cSSSI and its activity against pathogens common in CAP and cSSSI. We attempted to test a range of resistance phenotypes of most species. We assessed the potential of ceftaroline and comparator antibiotics to select for development of resistance using a multistep resistance selection method against a panel of MRSA, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. pyogenes*, *Enterococcus faecalis*, and *K. pneumoniae* strains of different phenotypes and genotypes.

MATERIALS AND METHODS

Strains and antimicrobials. The characteristics of all of the strains tested here are shown in Table 1. All strains were selected to represent different resistotypes specific for each species. Ceftaroline (lot FMD-CEF-019) was obtained from Forest Laboratories, Inc. (New York, NY), and other antibiotics from their respective manufacturers. All susceptibility testing was conducted using Clinical and Laboratory Standards Institute broth macrodilution methods (16, 17).

Multistep studies. Serial passages were performed daily in Mueller-Hinton broth (with the addition of 5% lysed horse blood for pneumococci and group A streptococci) or in freshly prepared *Haemophilus* test medium for *H. influenzae*. Each strain was exposed to 2-fold dilution series of concentrations of the tested antimicrobials. For each subsequent daily passage, a 10-μl aliquot was taken from the tube with concentrations one to two dilutions below the MIC that matched the turbidity of a growth control tube and was used to inoculate the dilution series for the next day. Daily passages were performed until a significant increase in MIC (>4-fold or MICs ≥ 32 μg/ml) was obtained or until 50 consecutive passages were completed. The minimum number of passages was 20 passages. The stability of acquired resistance in isolates with a >4-fold MIC increase was evaluated by MIC determinations after 10 daily passages on antibiotic-free blood agar (chocolate agar for *H. influenzae* and *M. catarrhalis*). A stable clone was defined as one that had the same elevated MIC after 10 daily drug-free passages (±1 doubling dilution). All isolates with relevant MIC changes and all isolates passaged on ceftaroline were tested for cross-resistance to the other antimicrobial agents used here. In addition, parent and resistant clone identification was tested by pulsed-field gel electrophoresis, as described previously (13, 33).

Molecular characterization of azithromycin-resistant clones. The deduced amino acid sequence of L4 and L22 proteins and nucleotide sequence of domain II and V of 23S rRNA were determined in *S. pneumoniae* clones with elevated MICs selected in the presence of azithromycin, as well as for their parent strain, as previously described (43).

RESULTS

Initial ceftaroline MICs for *S. pneumoniae* and *S. pyogenes* isolates ranged from 0.004 to 0.25 μg/ml (Table 2). Ceftaroline activity was maintained (0.125 to 0.25 μg/ml) against three β-lactam-resistant pneumococcal strains with penicillin G MICs of 2 to 8 μg/ml, amoxicillin-clavulanate MICs of 8 μg/ml, and ceftriaxone MICs of 2 μg/ml. None of the *S. pneumoniae* or *S. pyogenes* strains tested yielded clones with >4-fold-increased MICs to ceftaroline, ceftriaxone, amoxicillin-clavulanate, or linezolid. Azithromycin-resistant clones were recovered from 2 to 35 days with two *S. pneumoniae* and two *S. pyogenes* strains (8- to >1,024-fold increases in the MIC). Increased moxifloxacin MICs (8- to 16-fold) were observed after 20 to 29 days with four *S. pneumoniae* isolates

TABLE 2. *Streptococcus pneumoniae* and *S. pyogenes* multistep selection results[a]

Strain	Pen MIC (μg/ml)	Phenotype [R determinant(s)]	Antibiotic	Initial MIC (μg/ml)	Selected resistance		Retest MIC (μg/ml) after 10 antibiotic-free subcultures						
					MIC	No. of passages	CPT	CRO	A/C	TGC	LZD	AZM	MOX
S. pneumoniae 3665	S (0.125)	Macrolide resistant [*mef*(A)]	CPT	0.016	0.03	50	0.03	0.125	0.25	0.06	1	8	0.125
			CRO	0.125	0.25	50							
			A/C	0.25	1	50							
			TGC	0.125	4	20	0.03	0.125	0.5	0.125	1	8	0.125
			LZD	1	2	50							
			AZM	8	16	50							
			MOX	0.125	0.5	50							
S. pneumoniae 2686	S (2)	Macrolide resistant [L4 mutation]	CPT	0.125	0.5	50	0.5	4	8	0.016	1	>64	0.06
			CRO	2	8	50							
			A/C	8	16	50							
			TGC	0.03	0.125	50							
			LZD	2	4	50							
			AZM	>64	NT	NT							
			MOX	0.125	2	29	0.125	4	8	0.03	2	>64	4
S. pneumoniae 1077	S (0.03)	Macrolide susceptible, quinolone resistant	CPT	0.008	0.03	50	0.03	0.125	0.03	0.03	2	0.06	2
			CRO	0.06	0.125	50							
			A/C	0.03	0.03	50							
			TGC	0.06	0.06	50							
			LZD	2	2	50							
			AZM	0.03	>64	29	0.004	0.03	0.03	0.03	2	>64	2
			MOX	4	32	21	0.008	0.03	0.016	0.06	2	0.06	16
S. pneumoniae 7599	I (4)	Macrolide resistant, multiresistant [*erm*(B) and *mef*(A)]	CPT	0.125	0.25	50	0.25	2	8	0.06	0.5	>64	0.125
			CRO	2	4	50							
			A/C	8	16	50							
			TGC	0.125	0.5	50							
			LZD	1	2	50							
			AZM	>64	NT	NT							
			MOX	0.25	>2	20	0.125	1	8	0.25	1	>64	2
S. pneumoniae 3548	R (8)	Macrolide resistant [*erm*(B)]	CPT	0.25	1	50	0.5	4	8	0.06	0.5	2	0.06
			CRO	2	4	50							
			A/C	8	16	50							
			TGC	0.125	2	20	0.25	2	4	0.5	0.5	>64	0.06
			LZD	1	1	50							
			AZM	4	>32	2	0.25	2	4	0.125	1	>32	0.06
			MOX	0.125	2	29	0.25	2	8	0.06	0.5	4	2
S. pyogenes 2132	S	Macrolide susceptible	CPT	0.004	0.004	50	0.004	0.03	0.016	0.03	1	0.125	0.25
			CRO	0.03	0.03	50							
			A/C	0.016	0.016	50							
			TGC	0.03	0.03	50							
			LZD	1	1	50							
			AZM	0.06	1	28	0.004	0.016	0.008	0.03	1	1	0.25
			MOX	0.25	0.5	50							
S. pyogenes 2368	S	Macrolide resistant [*erm*(B)]	CPT	0.004	0.004	50	0.004	0.03	0.016	0.03	1	>64	0.125
			CRO	0.03	0.03	50							
			A/C	0.016	0.016	50							
			TGC	0.03	0.03	50							
			LZD	1	2	50							
			AZM	>64	NT	NT							
			MOX	0.25	0.5	50							
S. pyogenes 2011	S	Macrolide resistant [*mef*(A)]	CPT	0.004	0.004	50	0.004	0.03	0.016	0.03	1	8	0.25
			CRO	0.03	0.03	50							
			A/C	0.016	0.016	50							
			TGC	0.03	0.06	50							
			LZD	1	1	50							
			AZM	4	32	35	0.004	0.03	0.008	0.03	1	16	0.25
			MOX	0.5	4	24	0.004	0.03	0.016	0.06	2	8	4

[a] Pen, penicillin G; NT, not tested; CPT, ceftaroline; CRO, ceftriaxone; A/C, amoxicillin-clavulanate; TGC, tigecycline; LZD, linezolid; AZM, azithromycin; MOX, moxifloxacin.

and one *S. pyogenes* isolate. Tigecycline yielded two *S. pneumoniae* isolates with MICs that were increased 16- and 32-fold, but which were not stably maintained in the absence of a selection agent. One of these isolates (from strain *S. pneumoniae* 3548) was cross-resistant with azithromycin (MIC > 64 μg/ml). To further characterize this isolate, the macrolide resistance determinants

L4, L22, and 23S rRNA were sequenced. This analysis revealed no changes in deduced amino acid sequences of L4 and L22 proteins or in the nucleotide sequences of domains II and V of 23S rRNA. An understanding of the molecular basis for azithromycin resistance in this isolate awaits further study.

The ceftaroline MICs for *H. influenzae* and *M. catarrhalis*

TABLE 3. *H. influenzae* and *M. catarrhalis* multistep selection results[a]

Strain	Phenotype [R determinants]	Antibiotic	Initial MIC (μg/ml)	Selected resistance		Retest MIC (μg/ml) after 10 antibiotic-free subcultures						
				MIC	No. of passages	CPT	CRO	A/C	TGC	LZD	AZM	MOX
H. influenzae 115	Macrolide hypersusceptible, efflux negative, β-lactamase negative	CPT	2	4	50	2	0.03	0.25	0.5	8	0.06	0.016
		CRO	0.03	0.03	50							
		A/C	0.5	1	50							
		TGC	0.5	0.5	50							
		LZD	8	32	20	2	0.125	0.5	0.5	32	0.25	0.016
		AZM	0.06	2	20	2	0.06	0.5	0.5	8	2	0.016
		MOX	0.016	0.125	24	2	0.125	0.5	0.25	8	0.06	0.125
H. influenzae 73	Macrolide resistant [L22 mutation], efflux positive, β-lactamase positive	CPT	8	8	50	8	0.125	2	0.5	32	>64	0.03
		CRO	0.03	0.03	50							
		A/C	2	4	50							
		TGC	1	2	50							
		LZD	32	NT	NT							
		AZM	64	NT	NT							
		MOX	0.03	0.25	47	8	0.125	4	1	32	64	0.25
H. influenzae 83	BLNAR	CPT	0.125	0.125	50	0.125	0.25	4	0.5	8	1	0.016
		CRO	0.25	0.25	50							
		A/C	8	8	50							
		TGC	1	2	50							
		LZD	16	32	22	0.125	0.25	8	0.5	16	2	0.016
		AZM	1	4	50							
		MOX	0.016	0.125	30	0.5	0.5	8	1	32	2	0.25
H. influenzae 44	Quinolone-resistant, β-lactamase positive	CPT	0.06	1	20	1	0.016	4	1	32	2	8
		CRO	0.004	0.016	50							
		A/C	1	1	50							
		TGC	1	2	50							
		LZD	16	32	7	0.06	0.004	1	0.5	16	2	4
		AZM	2	4	50							
		MOX	4	32	35	0.125	0.008	1	1	16	2	32
M. catarrhalis 36	β-Lactamase positive	CPT	0.5	0.5	50	0.5	0.5	0.25	0.5	4	0.06	0.06
		CRO	1	2	50							
		A/C	0.25	0.25	50							
		TGC	1	8	20	0.25	1	0.25	1	8	0.06	0.06
		LZD	4	8	50							
		AZM	0.03	0.03	50							
		MOX	0.06	0.06	50							

[a] NT, not tested; CPT, ceftaroline; CRO, ceftriaxone; A/C, amoxicillin-clavulanate; TGC, tigecycline; LZD, linezolid; AZM, azithromycin; MOX, moxifloxacin; BLNAR, β-lactamase negative, ampicillin resistant.

were 0.06 to 8 μg/ml (Table 3). One *H. influenzae* strain 44 (quinolone-resistant, β-lactamase-positive) derived clone with stable increases in ceftaroline MIC was recovered after passage day 20. The MIC for this isolate increased from 0.06 to 1 μg/ml (a 16-fold increase). No *H. influenzae* or *M. catarrhalis* strain tested with amoxicillin-clavulanate or ceftriaxone yielded resistant clones after 50 daily passages. One β-lacta-mase-positive *M. catarrhalis* strain produced an unstable tige-cycline-resistant clone after 20 days (MIC increased 8-fold). No tigecycline-resistant clones of *H. influenzae* were recovered in the present study. *H. influenzae* with linezolid-resistant phe-notypes (MIC of 32 μg/ml) were recovered from each strain tested and were stable upon antibiotic-free passage. Linezolid did not select resistant clones in the *M. catarrhalis* strain. Azi-thromycin yielded 1 *H. influenzae* clone with a 32-fold increase in MIC after 20 passages (MIC of 2 μg/ml). Moxifloxacin yielded resistant clones after 24 to 47 days with four of five *H. influenzae* and *M. catarrhalis* strains tested. MICs increased

8-fold in these isolates. Cross-resistance was not observed be-tween any agents tested in these species.

The results of susceptibility testing and multipassage resis-tance selection for ceftaroline and comparator agents against *S. aureus* and *E. faecalis* are summarized in Table 4. All resis-tant clones obtained during multipassage selection had pulsed-field gel electrophoresis profiles identical to those of their parental strains, indicating that no contamination occurred during passaging. Ceftaroline was among the most active agents tested against *S. aureus*, with an MIC range of 0.25 to 1 μg/ml. No staphylococcal strains tested with ceftaroline yielded a clone with an MIC increased >4-fold after 50 daily passages. Interestingly, the azithromycin MIC for one *S. aureus* isolate (isolate 1449) decreased from >64 to 4 μg/ml when passaged for 50 consecutive days in the presence of ceftaroline. Tigecy-cline was the only other agent tested that had consistently low MICs for *S. aureus*; however, all isolates tested yielded resis-tant clones with stable 8-fold increases in MIC within 20 to 35

TABLE 4. *S. aureus*, *E. faecalis*, and *K. pneumoniae* multistep selection results[a]

Strain	Phenotype (R determinants)	Antibiotic	Initial MIC (µg/ml)	Selected resistance		Retest MIC (µg/ml) after 10 antibiotic-free subcultures						
				MIC	No. of passages	CPT	CRO	A/C	TGC	LZD	AZM	MOX
S. aureus 873	hVISA, HA-MRSA	CPT	1	2	50	2	>64	64	0.5	2	>64	4
		CRO	>64	NT	NT							
		A/C	32	NT	NT							
		TGC	0.5	4	24	0.5	>64	32	4	2	>64	8
		LZD	2	8	50							
		AZM	>64	NT	NT							
		MOX	8	8	50							
S. aureus 555	VISA, HA-MRSA, Daptomycin R	CPT	0.5	0.5	50	0.5	>64	8	0.5	2	2	4
		CRO	64	NT	NT							
		A/C	4	32	5	1	>64	32	0.5	2	2	4
		TGC	0.5	4	35	1	>64	32	4	2	2	4
		LZD	2	2	50							
		AZM	2	>16	20	0.25	64	8	0.5	2	32	4
		MOX	4	8	50							
S. aureus 510	VRSA, HA-MRSA	CPT	1	4	50	2	>64	32	0.5	2	>64	4
		CRO	>64	NT	NT							
		A/C	32	NT	NT							
		TGC	0.5	4	26	1	>64	64	4	2	>64	4
		LZD	2	4	50							
		AZM	>64	NT	NT							
		MOX	4	8	50							
S. aureus 1449	CA-MRSA	CPT	0.5	0.5	50	0.5	>64	16	0.5	4	4	0.06
		CRO	>64	NT	NT							
		A/C	16	32	5	1	>64	32	0.5	4	>64	0.06
		TGC	1	8	20	0.5	64	16	8	4	>64	0.06
		LZD	4	8	50							
		AZM	>64	NT	NT							
		MOX	0.06	>1	21	0.5	64	8	1	4	4	0.125
S. aureus 543	MSSA	CPT	0.25	1	50	0.5	8	8	0.5	4	>64	0.016
		CRO	4	32	8	0.5	16	4	0.5	2	>64	0.016
		A/C	4	8	50							
		TGC	1	8	26	0.5	4	8	8	4	>64	0.016
		LZD	4	32	15	0.25	4	2	0.5	32	>64	0.03
		AZM	>64	NT	NT							
		MOX	0.03	0.03	50							
E. faecalis 568	VSE	CPT	4	32	41	32	>64	1	0.25	2	>64	16
		CRO	>64	NT	NT							
		A/C	0.5	0.5	50							
		TGC	0.5	4	44	8	>64	0.5	1	2	>64	32
		LZD	2	32	35	4	>64	0.5	0.25	32	1	32
		AZM	>64	NT	NT							
		MOX	32	NT	NT							
E. faecalis 609	VRE	CPT	1	8	38	8	>64	0.5	0.25	2	>64	16
		CRO	>64	NT	NT							
		A/C	0.5	>32	6	1	>64	0.5	0.5	2	>64	16
		TGC	0.5	4	46	1	>64	0.5	0.5	2	>64	16
		LZD	2	16	21	1	>64	0.5	0.5	16	>64	16
		AZM	>64	NT	NT							
		MOX	16	16	50							
						CPT	CRO	IMI				
K. pneumoniae 512	ESBL negative	CPT	0.5	0.5	50	0.5	0.25	0.25				
		CRO	0.5	8	30	2	4	0.25				
		Imipenem	0.5	4	20	0.5	0.5	0.5				

[a] NT, not tested; CPT, ceftaroline; CRO, ceftriaxone; A/C, amoxicillin-clavulanate; TGC, tigecycline; LZD, linezolid; AZM, azithromycin; MOX, moxifloxacin; IMI, imipenem; hVISA, heteroresistant vancomycin-intermediate *S. aureus*; HA-MRSA, hospital-acquired methicillin-resistant *S. aureus*; CA-MRSA, community-acquired methicillin-resistant *S. aureus*; VISA, vancomycin-intermediate-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; BLNAR, β-lactamase negative, ampicillin resistant; VSE, vancomycin-susceptible enterococci; VRE, vancomycin-resistant enterococci; ESBL, extended-spectrum β-lactamase producing.

days. Ceftriaxone, linezolid, amoxicillin-clavulanate, and azithromycin each produced stably resistant clones of *S. aureus*. The MICs for methicillin-susceptible *S. aureus* (MSSA) isolate 543 increased from 4 to 32 μg/ml within 8 days for ceftriaxone and within 15 days for linezolid. The amoxicillin/clavulanate-resistant MRSA and *E. faecalis* were recovered after day 5 and day 6 passages, with MICs increasing 8-fold and >64-fold, respectively. The vancomycin-intermediate, daptomycin-resistant *S. aureus* strain 555 yielded a stably resistant azithromycin clone after 20 days. Azithromycin MICs increased >8-fold from 2 to >16 μg/ml for this isolate. Large increases in MIC (>16-fold) were observed for moxifloxacin with *S. aureus* strain 1449, but the resistance phenotype was unstable upon removal of selective pressure.

Ceftaroline was modestly active against the 2 *E. faecalis* strains tested. Initial ceftaroline MICs were 4 μg/ml for the vancomycin-susceptible strain (VSE) and 1 μg/ml for the vancomycin-resistant strain (VRE). An 8-fold increase in MIC was observed for VSE and VRE after 41 and 38 days, respectively, of passage on ceftaroline. Linezolid-resistant *E. faecalis* isolates were also recovered, with an 8-fold MIC increase at day 21 for VSE and a 16-fold increase at day 35 for VRE. *E. faecalis* isolates resistant to ceftaroline or linezolid were stable upon passage on antibiotic-free medium. No *E. faecalis* clones with a stable resistant phenotype to tigecycline, amoxicillin-clavulanate, or moxifloxacin were found.

One *K. pneumoniae* strain with a ceftaroline MIC of 0.5 μg/ml was tested in the present study (Table 4). The MIC for this strain did not change after 50 daily passages with ceftaroline. One ceftriaxone-resistant clone was recovered after 30 days. The MIC for this isolate increased 16-fold, from 0.5 to 8 μg/ml. Imipenem selection produced a *K. pneumoniae* clone with 8-fold-increased MIC, although this MIC was unstable in the absence of selection, and cross-resistance to the other β-lactams tested was not observed.

DISCUSSION

After 50 serial subcultures, ceftaroline did not yield resistant clones and demonstrated MICs consistent with previously published reports against all strains of *S. aureus*, *S. pneumoniae*, *S. pyogenes*, *M. catarrhalis*, and *K. pneumoniae* tested (8, 20, 22, 31, 46, 49, 50, 53, 57). One of four *H. influenzae* strains tested (a quinolone-resistant isolate) produced a ceftaroline clone with increased MIC of 0.06 to 1 μg/ml after 20 days of passaging. Possible factors responsible for resistance development in *H. influenzae* may include production of β-lactamases or alterations in the penicillin-binding proteins PBP3a and PBP3b (23, 48, 60). In addition, PBP5 and PBP6 may also be involved since these proteins have been reported to be associated with low levels of β-lactamase activity, and their loss results in β-lactam hypersusceptibility (23). However, the clinical incidence of quinolone-resistant *H. influenzae* strains is very low (65).

The tendencies of amoxicillin-clavulanate, ceftriaxone, azithromycin, tigecycline, linezolid, and moxifloxacin to select resistant mutants in *S. pneumoniae* and *H. influenzae* (with the exception of tigecycline) were similar to those observed in previous studies (3, 4, 13–15, 33, 38, 39, 43, 47). A possible mechanism of resistance development to macrolides in *H. influenzae* may be due to mutations in the ribosomal protein

genes L4 and L22, as described previously in our laboratory (54). Resistance to macrolides that developed in two *S. pneumoniae* strains may be due to mutations in L4, L22, and 23S rRNA genes (strain 1077, Table 2). Most probably, mutation in 23S rRNA occurred in the latter strain, which previously showed resistance to azithromycin due to 23S rRNA mutation ($A_{2058}G$) (35) after azithromycin selection. No mutations in L4, L22, or 23S rRNA genes in strain 3548 (Table 4) have been observed in previous studies, which may suggest alteration within the −10, −35 putative promoter region of *erm*(B) (39). Resistance development to moxifloxacin in *S. pneumoniae* and *H. influenzae* is likely to be brought about by gene alterations in the quinolone resistance-determining regions encoded by the *parC*, *parE*, *gyrA*, and *gyrB*, as has been described previously by our group (9, 18, 35).

Tigecycline resistance phenotype of *S. pneumoniae*, *M. catarrhalis* and *E. faecalis* mutant clones was unstable and this may suggest that cell wall thickness plays a role as a barrier, which prevents transport of antibiotic (such as vancomycin or daptomycin) inside the cell (2). Existence of undefined efflux pumps is also possible (58).

Resistance to linezolid developed in *H. influenzae* isolates may be due to mutations in the 23S rRNA, and the level of resistance may correlate with the number of mutated copies of the rRNA operons. Such mechanisms have been described in other bacterial species (56). Selection of linezolid-resistant mutants in *S. aureus* and *E. faecalis* strains has been demonstrated previously (12, 37, 39), and in the present study a similar propensity to select resistant clones was observed. A resistance mechanism to linezolid in *S. aureus* and *E. faecalis* has been described before and involves mutation in the central loop of domain V of the 23S rRNA ($G_{2576}T$ was the most common substitution), with the level of resistance depending on the number of mutated 23S rRNA copies. By analogy to staphylococci, possible alterations in ribosomal proteins L3 and L4 proteins in *E. faecalis* may be responsible for linezolid resistance development (41, 64). To our knowledge, the ability to select clones with increased amoxicillin-clavulanate, ceftriaxone, azithromycin, tigecycline, or moxifloxacin MICs in *S. aureus*, *S. pyogenes*, and *E. faecalis* had not been reported, and *K. pneumoniae* has not been previously tested in multiple passaging experiments. β-Lactam resistance in *S. aureus*, which includes penicillinase and cephalosporins, is caused by alteration in PBP2a protein, encoded by *mecA*, resulting in decreased PBP affinity to and/or the production of β-lactamases (25, 32). Other PBPs may also be involved, especially in MSSA (11). Resistance to amoxicillin-clavulanate in *E. faecalis* may be caused by alterations in PBP4, resulting in decreased affinity or protein overproduction (56). Resistance development to ceftriaxone and imipenem in ESBL-negative *K. pneumoniae* may be due to the presence of AmpC β-lactamase and/or the loss of an outer membrane protein (7).

Macrolide resistance in *S. aureus*, *S. pyogenes*, and *E. faecalis* may be caused by alterations in ribosomal proteins (L4 and L22) and 23S rRNA genes, as well as by the presence of an efflux pump (34, 40, 63).

Stable tigecycline-resistant clones developed in all *S. aureus* strains may be caused by mutations in genes coding for efflux pumps. Tigecycline-resistant *S. aureus* mutants have been selected *in vitro* previously. McAleese et al. associated tigecycline

resistance development with overexpression of the *mepA* gene, coding for a novel single protein efflux pump belonging to the multidrug and toxin extrusion (MATE) family (44). In *S. pyogenes* and *E. faecalis*, fluoroquinolone resistance may be caused by mutations in the *gyrA* or *parC* (a subunit of topoisomerase IV) gene (19).

A decrease in azithromycin MICs during ceftaroline and moxifloxacin selection with *S. aureus* or linezolid selection with *E. faecalis* may be explained by the absence of antibiotic selection pressure. The direct association between antibiotic selection pressure and macrolide resistance development has been described in streptococci (1). In some instances, cross-resistance was observed between tigecycline and other mechanistically unrelated antibiotics. Growth in the presence of tigecycline appears to have selected for resistance to amoxicillin-clavulanate in *S. aureus* and to azithromycin in *S. pneumoniae*. Reasons for cross-resistance between these agents are unclear and would require further investigation to more fully explain.

In summary, of all the species and isolates tested in the present study, ceftaroline only selected clones with an increased MIC in one rare quinolone-resistant *H. influenzae* isolate and two *E. faecalis* strains. In conclusion, prolonged selection in the presence of ceftaroline demonstrated no evidence of resistance development for the majority of isolates and lack of cross-resistance with other antibiotic classes among tested species important in cSSSI and CAP.

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