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Evaluation of the Effects of pH, Serum Protein Concentration, Media Supplements, Inoculum Size, Media Type, and Incubation Conditions on Activity of Ceftaroline Combined With NXL104

P.R. RHOMBERG[1], R.N. JONES[1], G.B. WILLIAMS[2], H.S. SADER[1]

[1]JMI Laboratories, North Liberty, Iowa; [2]Cerexa, Inc. (a wholly owned subsidiary of Forest Laboratories, Inc., New York, NY)

Helio S. Sader, MD, PhD
JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
ph. 319.665.3370
fax 319.665.3371
helio-sader@jmilabs.com

Abstract

Background: Ceftaroline (CPT) is a broad-spectrum cephalosporin and NXL104 is a novel non-β-lactam β-lactamase (βL) inhibitor that inhibits AmpC, ESBL, and KPC-type enzymes. We tested the effects of variation in susceptibility testing constituents and incubation conditions on the potency of CPT and CPT combined with NXL104 at a fixed concentration of 4 μg/mL (CXL).

Methods: 12 strains (7 species) were tested by broth microdilution (BMD) method according to CLSI (M07-A8), with the following variables: $5x10^3$ and $5x10^7$ CFU/mL inoculum concentration; medium pH of 5.0, 6.0, and 8.0; 50 and <5 mg/L of calcium (Ca); 5% CO_2 and anaerobic (ANA) incubation atmosphere, and the addition of 10% and 20% human serum in Mueller-Hinton broth (MHB). All tests were performed in triplicate and results were compared with those obtained under standard CLSI conditions.

Results: When testing CPT, 2 and 5 strains (all Gram-positive [GP]) had MIC values 2- and ≥4-fold lower at pH 5, respectively. Only *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 showed a ≥4-fold increase in CPT MIC values when tested with a high inoculum concentration ($5x10^7$ CFU/mL). No significant (>2-fold) MIC variations were noted when the test was modified for 5% CO_2 or ANA incubation, $5x10^3$ CFU/mL inoculum, pH 6 or 8, serum supplements (10% and 20%) or Ca ion content (<5 or 50 mg/L) of the MHB. When testing CXL, a ≥4-fold decrease in MIC was observed with 6 strains (all GP) at pH 5 (Table). In contrast, a slight MIC increase (2- to 4-fold) was observed when 2 *E. coli* strains were tested at pH 5. A significant increase in the CXL MIC was observed when a KPC-producing *Klebsiella* spp. strain was tested with $5x10^7$ CFU/mL inoculum concentration. No significant (>2-fold) MIC variations were noted when the test was modified for 5% CO_2 or ANA incubation, $5x10^3$ CFU/mL inoculum concentration, pH 6 or 8, serum supplements (10% and 20%) or Ca content (<5 or 50 mg/L) of the MHB.

Table: Partial list of CXL MIC results when testing conditions are varied from the standardized procedures.

Organism	Standard conditions[a]	Inoculum (CFU/mL)		pH		
		5×10^5	5×10^7	5	6	8
S. aureus						
ATCC 29213	0.12(3)[a]	0.25(3)	0.25(3)	0.06(3)	0.12, 0.25(2)	0.25(3)
MRSA	0.5(3)	0.5(3)	1(3)	0.03(3)[b]	0.5(3)	0.5(3)
MRSA	0.5(3)	0.5(3)	1(3)	0.03(3)	0.5(3)	0.5(3)
USA300	0.5(3)	0.5(3)	1(3)	0.03(3)	0.5(3)	0.5(3)
CoNS[b]						
MS-CoNS	0.06(3)	0.03(2), 0.06	0.12(3)	≤0.015(3)	0.06(3)	0.03(3)
MR-CoNS	0.5(3)	0.25(2), 0.5	0.5(2), 1	≤0.015(3)	0.5(3)	0.12(2), 0.25
E. faecalis						
ATCC 29212	0.5(3)	0.5(3)	1(3)	0.12(3)	0.5(3)	1(3)
E. coli						
ATCC 25922	0.06(3)	0.03(3)	0.06(3)	0.12(3)	0.06(3)	0.03(3)
CTX-M-15	0.06(2), 0.12	0.03, 0.06(2)	0.12(3)	0.25(3)	0.06, 0.12(2)	0.06(2), 0.12
K. pneumoniae						
KPC-2	0.5(3)	0.25(3)	8(1), 16(2)	0.5(3)	0.5(2), 1	0.25(3)

a. Number of results at each value is indicated in parenthesis.
b. Underlined values show MIC variations of ≥4-fold.

Conclusions: CPT or CXL MIC results were minimally influenced by most variations in CLSI BMD testing conditions, except for high-inoculum and low-media pH.

Introduction

Ceftaroline, the active form of the prodrug ceftaroline fosamil, is a novel, broad-spectrum cephalosporin exhibiting bactericidal activity against resistant Gram-positive organisms, including *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA), as well as common Gram-negative organisms. Similar to other cephalosporins, ceftaroline is less active against some β-lactamase-producing Gram-negative organisms. However, when ceftaroline is combined with NXL104, a potent non-β-lactam β-lactamase inhibitor, its spectrum of activity includes strains producing AmpC, extended-spectrum β-lactamases (ESBLs), and KPC-type enzymes.

We evaluated the effects of varying the methods recommended by the Clinical and Laboratory Standards Institute (CLSI) for in vitro susceptibility testing parameters on the MIC test results of ceftaroline alone and in combination with NXL104 at a fixed concentration of 4 μg/mL.

Methods

A collection of 12 bacterial strains were tested against ceftaroline alone and in combination with NXL104 at a fixed concentration of 4 μg/mL to determine MIC results using CLSI reference broth microdilution methods. The strains tested were: *S. aureus* (4; 2 wild-type MRSA, 1 community-acquired MRSA USA300, and 1 ATCC QC strain 29213); coagulase-negative staphylococci (2; 1 methicillin-resistant); *Enterococcus faecalis* ATCC 29212; Enterobacteriaceae (3; 1 *Escherichia coli* ATCC 25922, 1 *E. coli* CTX-M-15 producing, and 1 *Klebsiella pneumoniae* KPC-2 producing); *S. pneumoniae* (2; 1 ATCC 49619) and 1 *Haemophilus influenzae* ATCC 49247.

Modifications of the standard CLSI test conditions (M07-A8, 2009) were investigated and the MIC values of these tests were compared with the reference broth microdilution results. All tests were performed in triplicate. The following modifications of media testing parameters were evaluated:

Inoculum effects: Inoculum concentration was tested at 5×10^5 CFU/mL, 5×10^7 CFU/mL, and 5×10^5 CFU/mL (control)

pH effects: Isolates were tested in Mueller-Hinton Broth (MHB) adjusted to pH values of 5.0, 6.0, 7.2-7.4 (standard CLSI method), and 8.0

Effects of added serum: Isolates were tested in MHB containing 10% and 20% of pooled human serum (inactivated), in addition to the standard CLSI method (no human serum)

Divalent calcium cation concentration: Isolates were tested in MHB containing 3 distinct calcium concentrations: i) trace (<5 mg/L); ii) 25 mg/L, as recommended by the CLSI (2009); and iii) 50 mg/L

Incubation conditions: Variations in incubation environments included anaerobic conditions, 5% CO_2, and ambient air as recommended by the CLSI (2009)

Media variations: Haemophilus Test Medium (HTM) and MHB supplemented with 2-5% lysed horse blood (LHB) were tested using all strains, including QC strains *H. influenzae* ATCC 49247 and *S. pneumoniae* ATCC 49619

All tests were performed in triplicate.

Results

- Tables 1 and 2 summarize the ceftaroline and ceftaroline/NXL104 (fixed 4 μg/mL) MIC results for the method modifications, which included variations in pH (4), medium serum content (3), calcium ion content (3), media types (3), inoculum concentrations (3), and incubation conditions (3)

Ceftaroline Results

- When tested in MHB at pH 5, 5 strains (3 *S. aureus*, 1 coagulase-negative staphylococci [CoNS], and 1 *E. faecalis*) had MIC values ≥4-fold lower, and 2 strains (1 *S. aureus* and 1 CoNS) exhibited MIC values 2-fold lower, than those obtained under standard conditions (Table 1)

- *E. coli* ATCC 25922 and CoNS 015-7427X showed a marked increase in ceftaroline MIC values (≥4-fold) when tested with a high inoculum concentration (5×10^7 CFU/mL). A 2-fold increase in the MIC was noted with all 4 *S. aureus* strains and 1 CoNS when tested with the high inoculum concentration (Table 1)

- No significant (>2-fold) MIC variations were noted for ceftaroline compared with standardized test conditions when the susceptibility test was modified for 5% CO_2 or anaerobic incubation, 5×10^3 CFU/mL inoculum concentration, LHB or HTM medium, pH 6 or 8, serum supplements (10% and 20%), or calcium concentration (<5 or 50 mg/L) (Table 1)

Ceftaroline/NXL104 Results

- A ≥4-fold decrease in the ceftaroline/NXL104 MIC values was observed with 6 strains (3 *S. aureus*, 2 CoNS, and 1 *E. faecalis*) when tests were performed in MHB at pH 5. In contrast, a slight increase in MIC (2- to 4-fold) was observed with both *E. coli* strains tested (Table 2)

- A significant increase in the ceftaroline/NXL104 MIC values was observed when KPC-producing *Klebsiella* spp. strain (02-502M) was tested with a high inoculum concentration (5×10^7 CFU/mL). Furthermore, a tendency toward higher MIC values (up to 1 $\log_2$ dilution higher) with high inoculum concentration was observed with 7 of 8 Gram-positive strains tested (Table 2)

- For ceftaroline/NXL104, no significant (>2-fold) MIC variations were noted compared with standardized test conditions when the test was modified for 5% CO_2 or anaerobic incubation, 5×10^3 CFU/mL inoculum concentration, LHB or HTM medium, pH 6 or 8, serum supplements (10% and 20%), or calcium concentration (<5 or 50 mg/L) (Table 2)

Table 1. Ceftaroline Reference Broth Microdilution MIC Results Tested in Triplicate When Testing Conditions Are Varied From the Standardized Procedures (CLSI M07-A8, 2009)

Organism/ strain no.	Source	Standard conditions[a]	Atmosphere		Inoculum (CFU/mL)		Media		pH			Serum (%)		Calcium concentration (mg/L)	
			Anaerobic	5% CO_2	5×10^3	5×10^7	LHB[b]	HTM[b]	5	6	8	10	20	<5	50
S. aureus															
ATCC 29213	ATCC	0.12(3)[c]	0.12(3)	0.12(3)	0.12(2), 0.25	0.25(3)	0.25(3)	0.25(3)	0.06(3)	0.25(3)	0.12(2), 0.25	0.25(3)	0.25(3)	0.25(2), 0.12	0.25(3)
3498J	MRSA	0.5(3)	0.25(3)	0.5(3)	0.5(3)	1(3)	0.5(2), 1	0.5(2)	0.06(3)[d]	0.5(3)	0.5(3)	0.5(3)	0.5(3)	0.25(3)	0.5(3)
3456J	MRSA	0.5(3)	0.25(3)	0.5(3)	0.5(3)	1(3)	1(3)	0.5(2)	0.06(3)	1(3)	0.5(2), 1	0.5(2), 1	0.5(3)	0.5(3)	1(3)
3544J	USA-300 CA	0.5(3)	0.25(3)	0.5(3)	0.5(3)	1(3)	1(3)	1(2)	0.06(3)	0.5(3)	1(3)	1(3)	0.5(2), 1	0.5(3)	1(3)
CoNS[b]															
081-4627X	MS-CoNS	0.06(3)	0.06(3)	0.06(3)	0.06(3)	0.12(3)	0.06(3)	0.06(2)	≤0.03(3)	0.06, 0.12(2)	0.06(3)	0.06(2), 0.12	0.12(2), 0.25	0.06(3)	0.06(3)
015-7427X	MR-CoNS	0.5(3)	0.5(3)	0.5(3)	0.25(2), 0.5	0.5(2), 1	0.5(3)	0.5(3)	≤0.03(3)	0.5(3)	0.25, 0.5(2)	0.5(3)	0.5(3)	0.25(3)	0.5(3)
E. faecalis															
ATCC 29212	ATCC	0.5(3)	0.5(3)	0.5(3)	0.5(3)	2(3)	0.5, 1(2)	0.25(3)	0.12(3)	0.5(3)	1(3)	1(3)	1(3)	1(3)	0.5(3)
E. coli															
ATCC 25922	ATCC	0.06(2), 0.12	0.06(3)	0.06, 0.12(2)	0.06(3)	1(3)	0.12, 0.25(2)	0.06(3)	0.12(3)	0.06(3)	0.06(3)	0.06(3)	0.06(2), 0.12	0.06(3)	0.06, 0.12(2)
024-1310A	CTX-M-15	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)	>2(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)
K. pneumoniae															
02-502M	KPC-2	>64(3)	>64(3)	>64(3)	64(3)	>64(3)	>2(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)	>64(3)
S. pneumoniae															
ATCC 49619	ATCC	-[e]	-	-	-	-	0.015(3)[e]	≤0.03(3)	-	-	-	-	-	-	-
H. influenzae															
ATCC 49247	ATCC	-[f]	-	-	-	-	0.12(3)	0.06(3)[f]	-	-	-	-	-	-	-

a. Ambient air, 5 x 10^5 CFU/mL inoculum, Mueller-Hinton broth (MHB), pH 7.2-7.4, no serum and calcium at 25 mg/L.
b. Abbreviations: LHB = lysed horse blood; HTM = Haemophilus test media; CoNS = coagulase-negative staphylococci.
c. Results from triplicate testing. Number of results at each value is indicated in parenthesis.
d. Underlined values show MIC variations of ≥4-fold.
e. *S. pneumoniae* was tested in ambient air, 5 x 10^5 CFU/mL inoculum, pH 7.2-7.4, no serum and calcium at 25 mg/L. The only variable was the media: LHB (standard condition) and HTM.
f. *H. influenzae* was tested in ambient air, 5 x 10^5 CFU/mL inoculum, pH 7.2-7.4, no serum and calcium at 25 mg/L. The only variable was the media: LHB and HTM (standard condition).

Table 2. Ceftaroline Combined With NXL-104 (Fixed 4 μg/mL) Reference Broth Microdilution MIC Results Tested in Triplicate When Testing Conditions Are Varied From the Standardized Procedures (CLSI M07-A8, 2009)

Organism/ strain no.	Source	Standard conditions[a]	Atmosphere		Inoculum (CFU/mL)		Media		pH			Serum (%)		Calcium concentration (mg/L)	
			Anaerobic	5% CO_2	5×10^3	5×10^7	LHB[b]	HTM[b]	5	6	8	10	20	<5	50
S. aureus															
ATCC 29213	ATCC	0.12(3)[c]	0.12(3)	0.12(3)	0.25(3)	0.25(3)	0.25(3)	0.12(3)	0.06(3)	0.12, 0.25(2)	0.25(3)	0.25(3)	0.25(3)	0.12(3)	0.12(3)
3498J	MRSA	0.5(3)	0.25(3)	0.5(3)	0.5(3)	1(3)	>0.5(3)	0.25(3)	0.03(3)[d]	0.5(3)	0.5(3)	0.5(3)	0.5(3)	0.25(3)	0.5(3)
3456J	MRSA	0.5(3)	0.25(3)	0.5(3)	0.5(3)	1(3)	>0.5(3)	0.25(3)	0.03(3)	0.5(3)	0.5(3)	0.5(3)	0.5(3)	0.5(3)	0.5(3)
3544J	USA-300 CA	0.5(3)	0.25(3)	0.5(3)	0.5(3)	1(3)	>0.5(3)	0.5(3)	0.03(3)	0.5(3)	0.5(3)	0.5(3)	0.5(3)	0.5(3)	0.5(3)
CoNS[b]															
081-4627X	MS-CoNS	0.06(3)	0.06(3)	0.06(3)	0.03(2), 0.06	0.12(3)	0.12(3)	0.06(3)	≤0.015(3)	0.06(3)	0.03(3)	0.06(3)	0.12(3)	0.06(3)	0.06(3)
015-7427X	MR-CoNS	0.5(3)	0.5(3)	0.5(3)	0.25(2), 0.5	0.5(2), 1	0.5(3)	0.5(3)	≤0.015(3)	0.5(3)	0.12(2), 0.25	0.5(3)	0.5(3)	0.25(3)	0.5(3)
E. faecalis															
ATCC 29212	ATCC	0.5(3)	0.5(3)	0.5(3)	0.5(3)	1(3)	>0.5(2), 0.5	0.5(3)	0.12(3)	0.5(3)	1(3)	1(3)	1(3)	0.5, 1(2)	1(3)
E. coli															
ATCC 25922	ATCC	0.06(3)	0.03(2), 0.06	0.03(2), 0.06	0.03(3)	0.06(3)	0.12(3)	0.06(3)	0.12(3)	0.06(3)	0.03(3)	0.06(3)	0.03(3)	0.03(3)	0.03(2), 0.06
024-1310A	CTX-M-15	0.06(2), 0.12	0.03(3)	0.06(3)	0.03, 0.06(2)	0.12(3)	0.12(3)	0.12(3)	0.25(3)	0.06, 0.12(2)	0.06(2), 0.12	0.12(3)	0.06(3)	≤0.015, 0.03, 0.06	0.06(3)
K. pneumoniae															
02-502M	KPC-2	0.5(3)	1(3)	0.5(2), 1	0.25(3)	8(1), 16(2)	0.5(3)	0.25(3)	0.5(3)	0.5(2), 1	0.25(3)	1(3)	0.25(3)	0.12, 0.25(2)	0.25(3)
S. pneumoniae															
ATCC 49619	ATCC	-[e]	-	-	-	-	0.015(3)[e]	≤0.015(3)	-	-	-	-	-	-	-
H. influenzae															
ATCC 49247	ATCC	-[f]	-	-	-	-	0.03(3)	≤0.015(2), 0.03[f]	-	-	-	-	-	-	-

a. Ambient air, 5 x 10^5 CFU/mL inoculum, Mueller-Hinton broth (MHB), pH 7.2-7.4, no serum and calcium at 25 mg/L.
b. Abbreviations: LHB = lysed horse blood; HTM = Haemophilus test media; CoNS = coagulase-negative staphylococci.
c. Results from triplicate testing. Number of results at each value is indicated in parenthesis.
d. Underlined values show MIC variations of ≥4-fold.
e. *S. pneumoniae* was tested in ambient air, 5 x 10^5 CFU/mL inoculum, pH 7.2-7.4, no serum and calcium at 25 mg/L. The only variable was the media: LHB (standard condition) and HTM.
f. *H. influenzae* was tested in ambient air, 5 x 10^5 CFU/mL inoculum, pH 7.2-7.4, no serum and calcium at 25 mg/L. The only variable was the media: LHB and HTM (standard condition).

Conclusions

- Ceftaroline and ceftaroline/NXL104 (fixed 4 μg/mL) MIC results were minimally affected by variations in the following CLSI broth microdilution testing conditions: incubation atmosphere (anaerobic or 5% CO_2), medium serum content (10% and 20%), and calcium divalent ion concentration (<5 and 50 mg/L)

- No significant MIC variations were observed when tests were performed with media at pH 6 or 8 compared with standard pH (7.2-7.4). In contrast, a clear tendency toward lower MIC values was observed when Gram-positive organisms (staphylococci and *E. faecalis*) were tested in MHB at pH 5. Inhibition of bacterial growth as a result of the low pH may explain these findings

- When ceftaroline and ceftaroline/NXL104 were tested with a high inoculum, MIC values were generally identical or 2-fold higher than those obtained with CLSI standard inoculum

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