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Correlation between Broth Microdilution and Disk Diffusion Methods Results When Testing Ceftaroline against Methicillin-Resistant *Staphylococcus aureus* and Molecular Characterization of Isolates with Elevated Ceftaroline MIC Results

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Introduction

- According to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M23, 2018), disk diffusion breakpoints are established after MIC breakpoints have been determined by plotting a scattergram of zone diameters versus MIC values for isolates tested by both methods; thus, the zone diameter interpretative criteria that provide the lowest inter-method error rates (or discrepancy rates) are selected using a statistical method denominated error-rate-bounded method
- The discrepancy rates, very major (VM), major (Ma), and minor (Mi) error rates, are directly proportional to the percentage of isolates with MIC values in the range of plus or minus 1 doubling dilution of the breakpoints; therefore, the accuracy of the disk diffusion test will vary according to the susceptibility of the organism collection to the antimicrobial agent being tested
- SCC*mec* plays an important role in the antimicrobial resistance characteristics, molecular epidemiology, and evolution of methicillin-resistant *Staphylococcus aureus* (MRSA), and ceftaroline potency (MIC value) against MRSA varies according to the SCC*mec* type
- Data from the SENTRY Program for isolates consecutively collected worldwide in 2016 and 2017 shows ceftaroline susceptibility rates against MRSA varying from 95.1% in the United States to 88.7% in Europe, 77.8% in the Asia-Pacific region, and 74.7% in Latin America (Table 1)
- The main objective of this investigation was to evaluate the performance of the disk diffusion tests when testing ceftaroline against MRSA isolates. Secondary objectives included:
  - Assessing the impact of the ceftaroline susceptibility rates on the performance of the disk diffusion test
  - Assessing the influence of the disk and Mueller-Hinton agar reagents on the performance of the disk diffusion test
  - Characterizing a subset of MRSA isolates and evaluating the ability of susceptibility testing methods to segregate wild-type from non-wild-type populations

Table 1 Summary of ceftaroline activity tested against MRSA stratified by geographic region (SENTRY Program, 2016–2017)

Geographic region (no. tested)	No. of isolates (cumulative %) inhibited at ceftaroline MIC (µg/mL) of: 0.25	0.5	1	2	4	8	16	%S	%I	%R
North America (5,454)	177 (3.2)	2,967 (57.6)	2,042 (95.1)	265 (99.9)	3 (100.0)			95.1	4.9	0.1
Europe (1,497)	44 (2.9)	622 (44.5)	662 (88.7)	169 (100.0)				88.7	11.3	0.0
Latin America (495)	3 (0.6)	173 (35.6)	194 (74.7)	116 (98.2)	9 (100.0)			74.7	23.4	1.8
Asia-Pacific (666)	8 (1.2)	305 (47.0)	205 (77.8)	105 (93.5)	37 (99.1)	1 (99.2)	5 (100.0)	77.8	15.8	6.5
All isolates (8,112)	232 (2.9)	4,067 (53.0)	3,103 (91.2)	655 (99.3)	49 (99.9)	1 (99.9)	5 (100.0)	91.2	8.1	0.7

Materials and Methods

Organism collection

- The organism collection included 158 clinical MRSA isolates and was enriched with a 52-isolate subset that had elevated ceftaroline MIC values (1–16 µg/mL)
- Ceftaroline susceptibility and resistance rates were 62.0% and 15.8%, respectively; furthermore, 68.4% of isolates (108/158) had ceftaroline MIC values at the CLSI and FDA breakpoints (1–4 µg/mL)

Antimicrobial susceptibility testing

- Isolates were tested for susceptibility to ceftaroline by reference broth microdilution and disk diffusion methods as described by CLSI
- Each isolate was tested by the disk diffusion method using ceftaroline 30-µg disks from 2 manufacturers (disks A and B) and Mueller-Hinton agar (MHA) from 2 manufacturers (MHA 1 and 2); therefore, there were 4 zone diameter values for each MIC value
- Ceftaroline 30-µg disks were obtained from Hardy/Mast (disk A) and Becton Dickinson-BBL (disk B); MHA was obtained from Remel (MHA 1) and Becton Dickinson-BBL (MHA 2)
- *S. aureus* ATCC 29213 and 25923 were tested in each experiment and all QC values were within the expected range

Data analysis

- Ceftaroline breakpoints of ≤1/≥4 µg/mL (susceptible/resistant) for MIC and ≥24/≤20 mm (susceptible/resistant) for disk diffusion were applied as established by the CLSI and US FDA
- Discrepancy rates between MIC values and zone diameter test results were calculated according to the CLSI M23 (2018) document
- Discrepancies involving false susceptible disk results were defined as VM errors, whereas false resistant disk diffusion results were defined as Ma errors; discrepancies involving the intermediate (I) category were defined as Mi errors
- Optimal disk breakpoints were determined by the error-rate bounded method according to CLSI M23 (2018) using a software developed by JMI Laboratories based on the dBETS software
- Four subsets with ceftaroline intermediate/resistant rates of 5%/0%, 10%/2%, 15%/4%, and 20%/8% were evaluated to assess the influence of the frequency of nonsusceptible isolates on the discrepancy error rates, and these subsets were randomly selected from the entire isolate collection

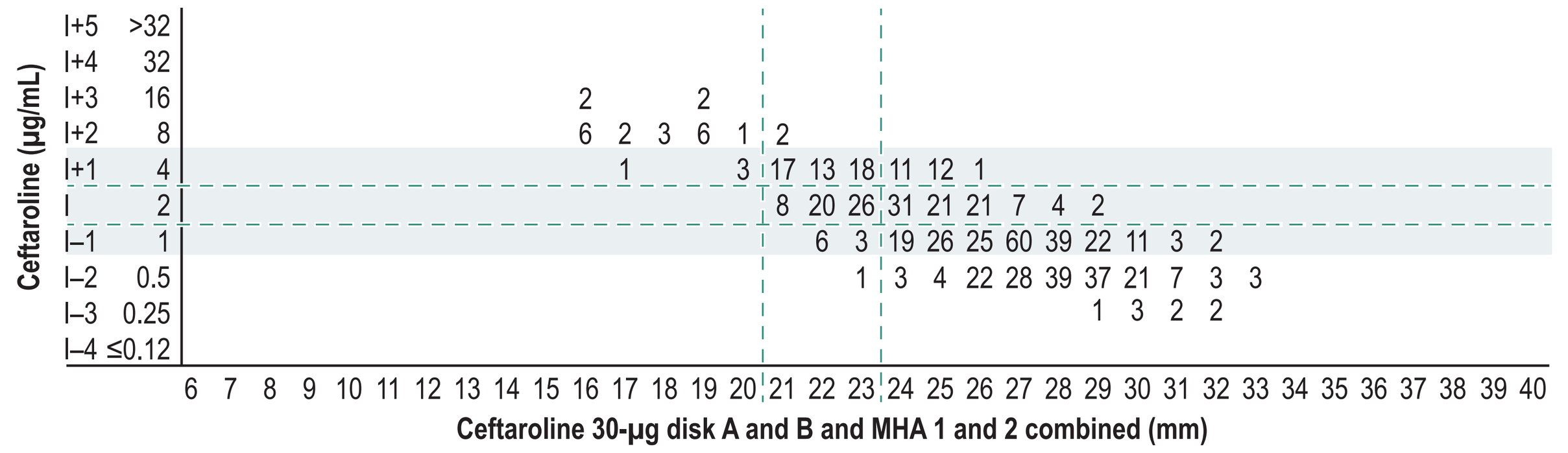
Molecular characterization of selected isolates

- A total of 50 isolates were submitted to whole genome sequencing, including most (24 of 25) ceftaroline-resistant isolates (MIC result ≥4 µg/mL), 15 of 35 ceftaroline-intermediate (MIC, 2 µg/mL), and 11 of 54 isolates with a ceftaroline MIC of 1 µg/mL
- Genomes were sequenced on a MiSeq Sequencer (JMI Laboratories). Genomic DNA of isolates was extracted using the Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor (Cleveland, Ohio) and used as input material for library construction. DNA libraries were prepared using the NexteraXT™ library construction protocol (Illumina, San Diego, California) following the manufacturer's instructions

Results

- Error rates were relatively high when testing the collection enriched with ceftaroline-nonsusceptible isolates (entire collection, n=158), with the VM error rate at ≥I+2 of 8.3%, which is greater than the acceptable rate of <5%, and overall VM, Ma, and Mi error rates of 3.8%, 0.0%, and 23.1%, respectively (Figure 1 and Table 2)
- Error rates would improve slightly and all discrepancy rates would be within the CLSI acceptable ranges if the disk breakpoint was moved 1 mm upward from ≥24 mm/≤20 mm to ≥25 mm/≤21 mm, but the overall VM, Ma, and Mi errors would still be elevated with rates of 2.1%, 0.0%, and 21.7%, respectively (Table 2)
- The overall VM and Mi error rates varied from 0.0% and 5.6% when percentages of intermediate/resistance were 5%/0% to 2.6% and 19.1%, respectively, when intermediate/resistance rates increased to 20%/8% (Table 3)
- The mean, geometric mean, and median values for zone diameters varied approximately 2 mm between disk A and disk B, independent of the MHA used, and this difference was statistically significant (*p*<0.001; Table 4)
- The majority of isolates selected for molecular characterization belonged to clonal complex (CC) 5 (42/50; 84.0%), and the majority of CC5 isolates (38/42; 90.5%) showed ceftaroline MIC values of ≥2 µg/mL (data not shown)
- Most isolates (26/39; 66.7%) exhibiting ceftaroline MIC values of ≥2 µg/mL were recovered from countries in the Asia-Pacific region (Japan, Korea, Taiwan, and Thailand; data not shown)

Figure 1 Scattergram comparing the results of ceftaroline broth microdilution MIC values (µg/mL) and disk diffusion zone diameters (mm) for a 30-µg disk when testing all MRSA isolates[a]



MIC range	Number	Very major (%)	Major (%)	Minor (%)
≥I+2	24	0	N/A	2 (8.33)
I±1	432	24 (5.56)	0	143 (33.1)
≤I–2	176	N/A	0	1 (0.57)
Total	632	24 (3.8)	0	146 (23.1)

[a] Each isolate was tested with ceftaroline disks from 2 manufacturers (disk A and disk B) and in Mueller-Hinton agar from 2 manufacturers (MHA 1 and MHA 2). Horizontal and vertical broken lines indicate ceftaroline breakpoints (US FDA and CLSI). The table at the bottom displays the number of isolates tested (n) and very major and major error rates for each category.

Table 2 Error rates for possible disk breakpoints (30-µg disk) when applying current CLSI and EUCAST MIC breakpoints of ≤1 µg/mL for susceptible and >2 µg/mL for resistant

Disk breakpoints	No. of test results	Error rate: Very major (%)	Major (%)	Minor (%)
≥23 mm (S) / ≤19 mm (R)				
≥I+2	24	0	N/A	3 (12.5)
I±1	432	42 (9.72)	0	151 (34.95)
≤I-2	176	N/A	0	0
Total	632	42 (6.65)	0	154 (24.37)
≥24 mm (S) / ≤20 mm (R)				
≥I+2	**24**	**0**	**N/A**	**2 (8.33)**
I±1	**432**	**24 (5.56)**	**0**	**143 (33.1)**
≤I-2	**176**	**N/A**	**0**	**1 (0.57)**
Total	**632**	**24 (3.8)**	**0**	**146 (23.1)**
≥25 mm (S) / ≤21 mm (R)				
≥I+2	24	0	N/A	0
I±1	432	13 (3.01)	0	133 (30.79)
≤I-2	176	N/A	0	4 (2.27)
Total	632	13 (2.06)	0	137 (21.68)

Values in bold indicate breakpoints and error rates for current CLSI disk breakpoints (CLSI M100, 2018).

Table 3 Correlation between error rates and ceftaroline susceptibility rates for the isolate collection used in the analysis

Ceftaroline susceptibility rates	No. of test results	Error rate[a]: Very major (%)	Major (%)	Minor (%)
5%I and 0%R				
≥I+2	0	0	N/A	0
I±1	236	0	0	22 (9.32)
≤I-2	176	N/A	0	1 (0.57)
Total	412	0	0	23 (5.58)
10%I and 2%R				
≥I+2	0	0	N/A	0
I±1	268	4 (1.49)	0	41 (15.3)
≤I-2	176	N/A	0	1 (0.57)
Total	444	4 (0.9)	0	42 (9.46)
15%I and 4%R				
≥I+2	0	0	N/A	0
I±1	308	5 (1.62)	0	69 (22.4)
≤I-2	176	N/A	0	1 (0.57)
Total	484	5 (1.03)	0	70 (14.46)
20%I and 8%R				
≥I+2	0	0	N/A	0
I±1	368	14 (3.8)	0	103 (27.99)
≤I-2	176	N/A	0	1 (0.57)
Total	544	14 (2.57)	0	104 (19.12)
CLSI acceptable discrepancy rates[a]				
≥I+2		<2%	N/A	<5%
I±1		<10%	<10%	<40%
≤I-2		N/A	<2%	<5%

[a] CLSI M23, 2018.

Table 4 Summary of zone diameter results for 30-µg disk stratified by disk and MHA manufacturers

Organism	Zone diameter (mm): Disk A, MHA 1	Disk A, MHA 2	Disk B, MHA 1	Disk B, MHA 2	Overall
Study collection (n=158)					
Mean[a]	24.9	24.7	26.8	26.7	25.8
Median	25	25	27	27	26
Mode	27	27	28-29	28	27
Geometric mean	24.8	24.5	26.6	26.6	25.6
Range	16-32	16-32	19-33	18-33	16-33
S. aureus ATCC 25923					
Mean[b]	28.3	29.7	29.5	31.7	29.8

[a] Difference between mean values for disk A and disk B were statistically significant independent of the MHA.
[b] Nine results for each disk-MHA manufacturer combination. All results were within the acceptable range published by CLSI (26-35 mm).

Table 5 Correlation of ceftaroline MIC result with alterations detected in PBP2a and the upstream region of *pbp4* among isolates selected for further molecular characterization

PBP2a status (CC)[a]	No. of isolates at each ceftaroline MIC (µg/mL) (Alteration observed at *pbp4* [b]): 1	2	4	8	16
WT (CC5, 8, 22, 45, and 239)	8 [4 Del, 4 Mutations]	5 [3 WT, 2 Del]	1 [WT]		
N146K (CC5 and 239)	1 [WT]	1 [Del]			
K565N (CC5)[c]	1 [G105T]				
E246G (CC22)		1 [Mutations]			
E150K (CC5)	1 [Del]	5 [1 Del, 4 WT]	1 [WT]		
N146K, L357I (CC5)		1 [WT]			
N146K, L357I, I563T (CC5)		2 [WT]	2 [WT]		
A228V, L357I, I563T (CC5)			6 [WT]		
N146K, L357I, M411I, I563T (CC5)			1 [WT]		
E447K (CC5)			5 [2 Del, 3 WT]		
N104K, V470I (CC5)			1 [WT]		
K146N, E239K, E447K (CC5)			1 [Del]	1 [WT]	
N104K, V117I, E447K, I563T, S649A (CC5)				4 [WT]	1 [WT]
Location of alteration in PBP2a					
Allosteric site	2	7	1		
Allosteric and transpeptidase sites		3	11	5	1
Transpeptidase site	1[c]		6		
Total	11	15	18	5	1

[a] PBP2a, penicillin-binding protein 2a; WT, wild type; allosteric site represented by amino acid residues 27–326; transpeptidase domain represented by residues 327–668. Ceftaroline breakpoints of ≤1 (light blue cells; susceptible)/≥4 µg/mL (dark blue cells; resistant) for MIC were applied as established by the CLSI and US FDA.
[b] WT, wild type; Del, deletion in the region upstream of *pbp4*; A single G105T alteration in the position 105 upstream of *pbp4*; "Mutations" are represented by several nucleotide alterations within 400 bp upstream of *pbp4.*
[c] K565N alteration at PBP2a.

- The most common SCC*mec* type observed among the 50 characterized isolates was type II (n=34; 68.0%), followed by types I (n=8; 16.0%), IV (n=7; 14.0%) and III (n=1; 2.0%; data not shown)
- Among those isolates having a wild-type sequence for PBP2a, the ceftaroline MIC results varied from 1 to 4 µg/mL, with a modal MIC value at 1 µg/mL (Table 5)
- The ceftaroline MIC range was also 1–4 µg/mL when testing MRSA isolates demonstrating alterations within the allosteric site (nonpenicillin binding domain [nPBD] residues 27–326), with a clear modal MIC value of 2 µg/mL (Table 5)
- MRSA isolates with alterations at the allosteric and transpeptidase sites showed a ceftaroline modal MIC value of 4 µg/mL, as did those isolates with mutations at the transpeptidase site only (Table 5)
- Isolates exhibiting ceftaroline MIC values of 4–16 µg/mL did not tend to have alterations in the upstream region of *pbp4* (3/24; 12.5%), while those MRSA with MIC results of 1–2 µg/mL had greater chances (15/26; 57.7%) of having alterations in this sequence (Table 5)
- Among isolates showing a wild-type sequence for PBP2a, 78.6% and 21.4% of ceftaroline disk diffusion results were categorized as susceptible and intermediate, respectively (Table 6)

Table 6 Correlation of ceftaroline MIC, disk zone results, and with PBP2a alterations detected among isolates selected for further molecular characterization

PBP2a status / Ceftaroline MIC (µg/mL)	Disk zone diameter (mm): 16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	%S	%I	%R
WT																				
1							3	1	4		1	3	6	3	8	1	2	78.6	21.4	0.0
2							2	3	1	4	5	3	2							
4						1	1	1	1											
Allosteric site																				
1								1	3	2	2							55.0	45.0	0.0
2						5	3	5	9	2	2		2							
4						2	1	1												
Allosteric and transpeptidase sites																				
2							2	2		3	3			2				28.8	41.2	30.0
4		1			1	9	7	11	8	6	1									
8	6	2	3	6	1	2														
16	2			2																
Transpeptidase sites																				
1							1				1		2					45.8	45.8	8.3
4					2	3	4	3	2	6										

This table depicts 4 zone diameter results for each MIC value (2 manufacturers for ceftaroline disks and 2 manufacturers for Mueller-Hinton agar).
WT, wild type; allosteric site represented by amino acid residues 27–326; transpeptidase domain represented by residues 327–668. Ceftaroline breakpoints of ≤1/≥4 µg/mL (susceptible/resistant) for MIC and ≥24 mm (light blue cells; susceptible)/≤20 mm (dark blue cells; resistant) for disk diffusion were applied as established by CLSI and US FDA.

Conclusions

- Reference broth microdilution and disk diffusion methods do not properly separate ceftaroline-susceptible MRSA from nonsusceptible populations using current CLSI and FDA breakpoints
- Isolates having a wild-type PBP2a exhibited ceftaroline MIC results (1–4 µg/mL) that overlapped with those carrying single (1–4 µg/mL) or multiple mutations (2–16 µg/mL)
- Disk diffusion did not satisfactorily separate the MRSA isolates with wild-type PBP2a from those with PBP2a alterations and those subsets with alteration(s) at different regions of PBP2a
- Error rates varied according to the percentage of ceftaroline-nonsusceptible isolates in the collection
- Error rates would improve slightly and all discrepancy rates would be within the CLSI acceptable ranges if the disk breakpoint was moved 1 mm upward from ≥24 mm/≤20 mm to ≥25 mm/≤21 mm
- CC5 isolates were dominant among ceftaroline-nonsusceptible (MIC ≥2 µg/mL) isolates, and these isolates originated mostly from countries in the Asia-Pacific region

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