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Comparison of daptomycin Etest MICs on Mueller–Hinton, IsoSensitest and Brain–Heart Infusion agars with and without calcium supplementation to broth microdilution MICs against 20 *Staphylococcus aureus* isolates

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REVISED ABSTRACT

The Etest manufacturer (AB Biodisk) recommends BBL™ Mueller–Hinton agar (MHA) from Becton Dickinson for daptomycin testing, which historically has calcium concentrations of 25–30 mg/L. We have evaluated the effect of agar calcium concentration on daptomycin Etest minimum inhibitory concentrations (MICs) using three different types of susceptibility test agar with free calcium concentrations of 8.5–52.9 mg/L. Twenty clinical isolates of *Staphylococcus aureus* with daptomycin MICs of 0.25–2 mg/L were retested by the Clinical and Laboratory Standards Institute broth microdilution (BMD) assay and tested with the daptomycin Etest on each of the study agars. *S. aureus* ATCC 29213 was included as a quality control strain with each day's testing. The calcium concentration of each agar and broth was determined using ion-selective electrode methodology.

As expected, daptomycin MICs decreased with increasing amounts of calcium. Strains with BMD MICs of 0.5, 1, and 2 mg/L had mean Etest MICs that were 1.32-, 1.75-, and 1.16-fold higher, respectively, using MHA with a calcium concentration of 28 mg/L (Table 1). Etest MIC values closest to the BMD MICs were obtained using MHA with 42.8 mg/L calcium, and IsoSensitest agar (ISA) with a calcium concentration of 52.9 mg/L. Etest MICs using Brain–Heart Infusion agar (BHIA) were all higher than the corresponding BMD MIC and, even with a BHIA calcium concentration of 52.6 mg/L, were approximately one doubling dilution higher than the BMD gold standard.

This study supports the Etest manufacturer's recommendation of using MHA for susceptibility testing of daptomycin by Etest. Nevertheless, 87.5% of strains with BMD MICs of 1 mg/L (the daptomycin susceptible breakpoint), had Etest MICs of 1.5 or 2 mg/L using MHA with a calcium concentration of 28 mg/L. Therefore, it is suggested that Etest MICs of 1.5 or 2 mg/L using MHA containing 28 mg/L calcium are considered to be indeterminate, and should be retested by BMD. BHIA and unsupplemented ISA are not recommended for use with the daptomycin Etest strip.

INTRODUCTION

When testing the minimal inhibitory concentration (MIC) of daptomycin by broth microdilution (BMD) methodology, a physiological concentration of free calcium (50 mg/L) in the broth is recommended. In the development of the daptomycin Etest strip, calcium was added to the strip at concentrations that provided optimal correlation with BMD methodology when tested using Mueller–Hinton agar (MHA). The BBL brand of MHA is currently recommended due to its consistent concentration of calcium. Calcium concentrations of multiple BBL MHA lots determined by the Clinical and Laboratory Standards Institute (CLSI) using ion-selective electrode methodology over the past 4 years have ranged from 25 to 30 mg/L. This study was conducted to investigate the variation of daptomycin MICs that may occur with media other than MHA, such as IsoSensitest agar (ISA), and Brain–Heart Infusion agar (BHIA), and to show the effect of variation in calcium concentration.

MATERIALS and METHODS

Antibiotics

Etest daptomycin 0.016–256 µg/mL (AB Biodisk, catalog no. DPC); Batch Numbers: BH0645 (Lot 1) and BH1592 (Lot 2); Broth Microdilution Daptomycin 0.03–32 µg/mL (Trek dried panels, catalog no. LSMCH1) Batch Number: B6031A.

Media

Etest

Agar plates (100×15 mm) unsupplemented, and supplemented with various concentrations of calcium (Tables 1–3)

- Mueller–Hinton agar (MHA) – Mast Diagnostics, Lot # 211678/210835
- IsoSensitest agar (ISA) – Oxoid Ltd, Lot # 517506
- Brain–Heart Infusion agar (BHIA) – Difco, Lot # 128426

Broth microdilution

Trek dried panels with cation-adjusted Mueller–Hinton broth (MHB)

Microorganisms

Quality control (QC) strain: Staphylococcus aureus (ATCC 29213)

Clinical isolates: Twenty clinical isolates of *S. aureus* submitted to Laboratory Specialists, Inc. as part of on-going post-marketing reference testing in the US. Isolates with BMD MICs of 0.5 mg/L (6 isolates), 1 mg/L (7 isolates), and 2 mg/L (7 isolates) were selected as a representative sample of strains with BMD MICs at the daptomycin susceptible breakpoint (1 mg/L), as well as at ±1 dilution.

Calcium analysis of agar and MIC plate broth[1]

A sample of the prepared agar was weighed and macerated. A 2:1 volume of sterile water to agar was added and mixed. The mixture was refrigerated overnight, centrifuged and the supernatant used for analysis.

The agar supernatant and broth from reconstituted daptomycin wells of the Trek panels were analyzed for free calcium using ion-selective methodology.

Broth microdilution procedure[2]

The QC strain and each study strain were tested four times (using separate inocula) to verify initial reference MIC values. Inocula (0.5 McFarland standard) were prepared by the direct colony suspension method from blood agar plates incubated for 18–20 hours, and were diluted in cation-adjusted MHB to achieve a final well concentration of 5×10^5 colony forming units. Colony counts of the final inocula were performed for each replicate. A total of 100 µL of the final inoculum was dispensed into each well of the MIC panels, and panels were incubated under ambient conditions at 35°C for 24 h. The MIC was defined as the lowest drug concentration showing no growth.

Etest procedure

The QC strain and each study strain were tested with two different lots of Etest strips on each of the agars. One of the four inocula used for the BMD replicates was selected for the Etest. Daptomycin Etest strips were applied to the inoculated plates and incubated for 16–18 hours at 35°C. Etest results were read according to the manufacturer's instructions.

Comparison of MICs

The doubling dilution difference in the MIC (as shown in Tables 2–4) was calculated using the formula: ($\log_2$Etest MIC)+10 – ($\log_2$BMD MIC)+10.

MIC frequency distributions (as shown in Figures 1–3) were calculated by rounding dilution MICs to the next doubling dilution (e.g. an MIC of 1.5 mg/L was designated 2 mg/L).

RESULTS

- The BMD MIC values presented are the retested MICs. Retested MICs were consistent with the initial MIC values with three exceptions: three strains with initial BMD MIC values of 0.5, 1, and 2 mg/L had retest BMD MIC values of 0.25, 0.5, and 1 mg/L, respectively
- The final calcium concentration of broth with daptomycin in the BMD panels was 55.6 mg/L. The calcium concentrations of 14 selected susceptibility test agars are shown in Table 1
- Mean daptomycin MICs are shown in Table 1
  - Overall, Etest daptomycin MICs decreased with increasing amounts of calcium on all media (Table 1), and minimal lot-to-lot variation between Etest MICs was noted (data not shown)
  - MIC values closest to the BMD MICs were achieved using MHA with 42.8 mg/L calcium and ISA with a calcium concentration of 52.9 mg/L
  - Strains with BMD MICs of 0.5, 1, and 2 mg/L had mean Etest MICs that were 1.32-, 1.75-, and 1.16-fold higher, respectively, using MHA with a calcium concentration of 28 mg/L
- The frequency distributions of BMD with CLSI-recommended calcium concentration and Etest MICs on media are shown in Figures 1–3
  - These data clearly show falsely elevated MICs with BHIA, even when supplemented with 52.6 mg/L calcium

Table 1: Mean daptomycin Etest MICs

Media	[Ca^{2+}] (mg/L)	Mean daptomycin Etest MIC (mg/L)* for strains with BMD MICs of: 0.5 mg/L** (n=6)	1 mg/L (n=7)	2 mg/L (n=7)
MHA	9.4	1.58	2.81	3.80
MHA	20.3	1.81	2.11	3.01
MHA***	28	0.66	1.75	2.32
MHA	42.8	0.53	1.37	2.06
MHA	51.9	0.31	1.36	1.83
ISA (unsupplemented)	8.5	1.63	2.93	4.67
ISA	22.6	1.13	2.12	3.61
ISA	30.5	0.85	1.74	2.58
ISA	41.9	0.72	1.42	2.32
ISA	52.9	0.53	1.24	1.98
BHIA (unsupplemented)	21.9	2.17	3.73	5.60
BHIA	30.8	1.93	3.21	4.98
BHIA	42.5	1.38	2.83	4.02
BHIA	52.6	1.07	2.23	3.55

*Each strain tested with two different lots of Etest strips
**Includes one strain with retested BMD MIC of 0.25 mg/L
***Calcium concentration equivalent to BBL unsupplemented MHA

- The dilution differences of Etest MICs compared with BMD MICs are shown in Tables 2–4
  - In total, 100% of strains with BMD MICs of 1 mg/L had MHA Etest MICs >1 mg/L using MHA with <20 mg/L calcium, and this value was 85.7% for Etests using MHA with a calcium concentration of 28 mg/L calcium (Table 2)
  - In total, 100% of strains with BMD MICs of 1 mg/L and 91.7% of strains with BMD MICs of 0.5 mg/L had Etest MICs >1 mg/L using ISA with <20 mg/L calcium (Table 3)
  - Etest MICs on BHIA were higher than BMD MICs and, even with a BHIA calcium concentration of 52.6 mg/L, were approximately one doubling dilution higher than the BMD gold standard. All strains with BMD MICs ≤1 mg/L had Etest MICs of 2.0 mg/L or greater when tested using unsupplemented BHIA (Table 4)

Table 2: Distribution of Etest MICs using calcium supplemented and unsupplemented MHA, relative to BMD MICs (Etest lots 1 and 2 combined)

BMD MIC (mg/L)	MHA [Ca^{2+}] (mg/L)	Number of Etest MICs with a doubling dilution difference compared with BMD MICs of: −3	−2.5	−2	−1.5	−1	−0.5	0	0.5	1	1.5	2	2.5	3
0.5*	<20									1	6	3	1	1
	20								5	5	2			
	25–30						1	3	5	3				
	40					2	1	4	5					
	50					7	3	1						
1	<20									3	10	1		
	20								2	9	3			
	25–30							2	2	10				
	40							4	9	1				
	50**					1	1	4	7	1				
2	<20								4	9	1			
	20							3	7	4				
	25–30							9	5					
	40						3	8	3					
	50***					1	3	8	1					
Total	<20								4	13	17	4	1	1
	20							3	14	18	5			
	25–30						1	14	12	13				
	40					2	4	16	17	1				
	50					8	6	13	9	1				

*Includes one isolate with an MIC of 0.25 mg/L
**2 results not available
***1 result not available

Table 3: Distribution of Etest MICs using calcium supplemented and unsupplemented ISA, relative to BMD MICs (Etest lots 1 and 2 combined)

BMD MIC (mg/L)	ISA [Ca^{2+}] (mg/L)	Number of Etest MICs with a doubling dilution difference compared with BMD MICs of: −3	−2.5	−2	−1.5	−1	−0.5	0	0.5	1	1.5	2	2.5	3
0.5*	<20									1	5	4	1	1
	20									8	1	3		
	25–30								7	2	2	1		
	40								2	7	1	1		
	50						2	6	2	2				
1	<20									3	8	3		
	20									12	2			
	25–30								7	7				
	40							4	7	3				
	50					1		5	7	1				
2	<20								2	6	5	1		
	20								5	9				
	25–30							6	7	1				
	40							9	5					
	50**						2	9	1					
Total	<20								2	10	18	8	1	1
	20								5	29	3	3		
	25–30							6	21	10	2	1		
	40							15	19	4	2			
	50					1	4	20	10	3				

*Includes one isolate with an MIC of 0.25 mg/L
**2 results not available

Table 4: Distribution of Etest MICs using calcium supplemented and unsupplemented BHIA, relative to BMD MICs (Etest lots 1 and 2 combined)

BMD MIC (mg/L)	BHIA [Ca^{2+}] (mg/L)	Number of Etest MICs with a doubling dilution difference compared with BMD MICs of: −3	−2.5	−2	−1.5	−1	−0.5	0	0.5	1	1.5	2	2.5	3
0.5*	<25											8	2	2
	25–30									2	2	3	2	3
	40**									4	1	3	2	
	50								2	5	3	1	1	
1	<25										5	8	1	
	25–30									4	3	6	1	
	40								1	3	8	1	1	
	50								2	7	5			
2	<25									4	8	2		
	25–30								2	5	6		1	
	40							1	3	7	2	1		
	50							2	3	8	1			
Total	<25									4	13	18	3	2
	25–30								2	11	11	9	4	3
	40							1	4	14	11	5	3	
	50							2	7	20	9	1	1	

*Includes one isolate with an MIC of 0.25 mg/L
**2 results not available

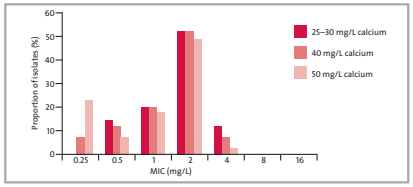

Figure 1. Frequency distribution of daptomycin Etest MICs using MHA with varying concentrations of calcium (lots 1 and 2 combined)

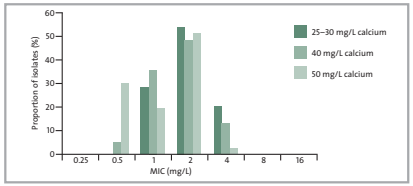

Figure 2. Frequency distribution of daptomycin Etest MICs using ISA with varying concentrations of calcium (lots 1 and 2 combined)

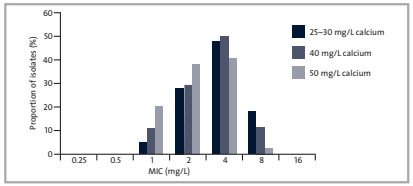

Figure 3. Frequency distribution of daptomycin Etest MICs using BHIA with varying concentrations of calcium (lots 1 and 2 combined)

CONCLUSIONS

- This study supports the Etest manufacturer's recommendation to use MHA for susceptibility testing of daptomycin by Etest; however, MIC values closest to the BMD MICs were achieved using MHA with a calcium concentration of 42.8 mg/L
- A large proportion (87.5%) of strains with BMD MICs of 1 mg/L (the daptomycin susceptibility breakpoint) had Etest MICs of 1.5 or 2 mg/L when tested using MHA with a calcium concentration of 25–30 mg/L (similar to that of unsupplemented BBL MHA). **Note:** *S. aureus* with a daptomycin MIC of 1 mg/L form approximately 1% of surveillance isolates; therefore, the occurrence of such isolates in hospital laboratories should be rare. The data for *S. aureus* with a daptomycin MIC of 0.5 mg/L (the MIC_{90} for this drug) did not demonstrate any shift to an MIC >1 mg/L
- Because of this observed discrepancy at Etest MICs just above the daptomycin breakpoint and the possibility of misclassifying strains as resistant, it is suggested that clinical isolates assigned Etest MICs of 1.5 or 2 mg/L are considered to be indeterminate and should be retested by BMD methodology
- The majority of strains designated susceptible to daptomycin using the BMD assay would be considered resistant according to BHIA and unsupplemented ISA Etest results. Therefore, both BHIA and unsupplemented ISA are not recommended for use with the daptomycin Etest strip

REFERENCES

1. Koeth L *et al. Int J Antimicrob Agents* 2004; 23:17–24.
2. CLSI document M7–A7. Vol 26, No. 2.

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