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Standardization of a Modified Broth Microdilution Methodology for Dalbavancin against *Neisseria gonorrhoeae*

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Abstract

Background: The CLSI recommended MIC procedure for testing *Neisseria gonorrhoeae* (NG) is an agar dilution (AD) method. Prior studies have shown higher dalbavancin MICs with AD and therefore, a broth microdilution method (BMD) with addition of 0.002% polysorbate 80 (P80) was used. Initial testing with GC broth + 0.002% P80 resulted in a precipitate that made determination of growth impossible and therefore, the medium was modified to include glucose instead of starch (GCm). GCm and 2 other broths (GC+5% LHB and an anaerobic enrichment broth, MTGE) were tested in the present study. **Methods:** Dalbavancin and comparator agents [ceftriaxone (CRO) and ciprofloxacin (CIP)] were tested against 5 strains (3 clinical strains, and NG ATCC 49226 and *S. aureus* (SA) ATCC 29213) by BMD under 5% CO_2 and anaerobic conditions (ANO_2) using GCm+0.002% P80, MTGE and GC+5% LHB broth using 2 different inoculum preparation procedures and incubated for 24 and 48 hours. **Results:** Broth microdilution endpoints showed sufficient growth using GC+5% LHB, MTGE and GC broth after 24 hour incubation in CO_2. All strains and media incubated anaerobically and all isolates tested in GCm (incubated in both CO_2 and ANO_2) grew poorly. NG dalbavancin MIC results based on BMD testing in GC+5% LHB in 5% CO_2 and incubated for 24 hours were 1-2 doubling dilutions lower compared to AD MIC results for 3 clinical isolates. MIC results for CRO and CIP were comparable for GC with and without 5% LHB and QC results were within AD MIC ranges.
Dalbavancin MIC results by medium-method:

Isolate No.-Replicate No.	Dalbavancin MIC (µg/mL)			
	GC+5% LHB-BMD[1]	MTGE-BMD[1]	GCm-BMD[1]	GC-AD[2]
1-R1	0.5	0.5	NG	2
1-R2	0.5	0.5	NG	
2-R1	0.25	0.06	NG	1
2-R2	0.25	0.06	NG	
3-R1	2	2	NG	1
3-R2	2	2	NG	
NG ATCC 49226	2	1	0.5	2
SA ATCC 29213	0.25	0.12	0.06	4

[1] BMD: direct inoculum method, 5% CO2 for 24 hours
[2]AD: results from prior study
NG = no growth

Conclusions: As antimicrobial resistance increases among NG, the development of a broth method of susceptibility testing for all agents is beneficial. For dalbavancin, a broth method using GC broth with 5% LHB or MTGE broth is promising and additional testing with more isolates is recommended for further study.

Introduction

Background: The CLSI recommended MIC procedure for testing *Neisseria gonorrhoeae* is an agar dilution (AD) methodology. Based on our prior studies, which have shown significantly higher dalbavancin MICs with AD, a broth microdilution method (BMD) with addition of 0.002% P80 is suggested for testing *Neisseria gonorrheae* against dalbavancin. Initial testing with GC broth + 0.002% P80 resulted in a precipitate in the media that made determination of growth in the wells impossible. It was suspected that the precipitate was a result of the interaction of the starch and the P80. Therefore, the media was modified to include glucose instead of starch and growth curves were performed which showed sufficient growth (>2 $\log_{10}$ increase in CFU/mL of *N. gonorrheae* from 0 to 24 hours). This modified GC broth (mGCB) was used for dalbavancin testing in a subsequent study that compared dalbavancin and ceftriaxone MIC results to agar dilution MIC results for 32 isolates. There were 8 isolates that did not grow in the mGCB and 12 isolates that did not grow in GC broth after 48 hour incubation in 5% CO_2. A subset of 4 of these isolates were retested using different inoculum procedures and were also tested against 2 additional comparator agents (cefuroxime and ciprofloxacin). Poor or no growth was still an issue with some isolates and therefore, all 32 isolates were retested using different levels of dextrose. There was no difference in MIC or growth based on the amount of dextrose and the overall conclusion was that further testing was still required to determine the optimal broth and incubations conditions for broth susceptibility testing of *N. gonorrhoeae*.

Study Objective: The purpose of this study was to test the susceptibility of dalbavancin and comparator agents (ceftriaxone and ciprofloxacin) by BMD (under 5% CO_2 and anaerobic conditions) using modified GC broth (GC broth with comparator agents), MTGE and GC +5% LHB broth with a set of 5 isolates (3 susceptible *N. gonorrhoeae,* QC strain *N. gonorrhoeae* ATCC 49226 and QC strain *S. aureus* ATCC 29212).

Methods

- All BMD methods (with exception of variations that are noted) were performed and quality control results referenced according to current CLSI methods (1-3)
- A set of 5 isolates were tested (3 susceptible *N. gonorrhoeae,* QC strain *N. gonorrhoeae* ATCC 49226 and QC strain *S. aureus* ATCC 29212)
- Each media tested was setup using both the stationary phase and direct colony methods and were incubated in both CO_2 and anaerobic conditions

Antimicrobials – Concentrations Tested (µg/mL):

Dalbavancin – 0.008 – 8
Ceftriaxone – 0.001 – 1
Ciprofloxacin – 0.0005 – 0.5

Methods (cont.)

Media Tested:

Modified GC Broth – (with P80)
MTGE Broth (provided and prepared by Anaerobe Systems – No P80 added)
GC Broth + 5% LHB – (No P80 added)
GC Broth – (comparator drug only – No P80 added)

GC Broth + LHB	Modified GC Broth	MTGE Broth	GC Broth	
			GC Broth Base	IsoVitalex
Same as GC Broth except 5% lysed horse blood is added	Same as GC Broth except 1 g dextrose is used instead of soluble starch and 1 mL 2% P-80 is added aseptically with the IsoVitalex.	Protein Mixture, 10.0 g	15 g protease peptone 3	Approximate Formula per L Purified Water
		Yeast Extract, 5.0 g	1 g soluble starch	Vitamin B_{12}, 0.01 g
		Sodium Bicarbonate, 5.0 g	4 g K2HPO4	L-Glutamine, 10 g
		Sodium Format15e, 0.5 g	1 g KH2PO4	Adenine, 1 g
		Sodium Fumarate, 1.0 g	5 g NaCl	Guanine Hydrochloride, 0.03 g
		Sodium Succinate, 0.5 g	1 L dH20	*p*-Aminobenzoic Acid, 0.013 g
		Potassium Phosphate, dibasic, 2.0 g	Aseptically supplement after autoclaving and cooling with 10 mLs of IsoVitalex	Nicotinamide Adenine Dinucleotide, 0.25 g
		Sodium Chloride, 5.0 g		Thiamine Pyrophosphate, 0.1 g
		Magnesium Sulfate, 0.1 g		Ferric Nitrate, 0.02 g
		Dextrose, 1.0 g		Thiamine Hydrochloride, 0.003 g
		Volatile Fatty Acid Mix, 3.0 ml		L-Cysteine Hydrochloride, 25.9 g
		Vitamin K_1 (1% soln), 1.0 ml		L-Cystine, 1.1 g
		Sodium Pyruvate, 0.8 g		Dextrose, 100 g
		Thiamine Pyrophosphate, 0.025 g		(BBL, Becton Dickinson, Sparks, MD)
		L-Cysteine, 0.5 g		
		Serum, 50.0 ml		
		Distilled Water, 940.0 ml		
		(Anaerobe Systems, Morgan Hill, CA)		

Results

- Broth microdilution endpoints showed sufficient growth using the MTGE, GC w/LHB and GC Broth after 24 hours incubated in CO_2.
- Dalbavancin MICs for all methods against all test strains were similar (within 1 doubling dilution of each other), with the exception of #26 which were 2 dilutions lower in MTGE broth compared to GC w/LHB MICs (Tables 1-2, Figure 1).
- Comparator MICs for all methods against all test strains were similar (within 1 doubling dilution of each other) (Figure 2)
- All strains and media incubated in anaerobic conditions and all isolates tested in modified GC broth (incubated in both CO_2 and ANO_2) had poor growth, therefore, results were not analyzed.
- Quality control strains were within the recommended CLSI agar dilution ranges for both comparators using both the GC broth and GC broth + 5% lysed horse blood.

Table 1: Dalbavancin MIC Results (µg/mL) by method

Isolate #	24 hours				48 hours			
	MTGE		GC w/LHB		MTGE		GC w/LHB	
	Direct	Stationary	Direct	Stationary	Direct	Stationary	Direct	Stationary
14-1	0.5	0.5	0.5	0.5	0.5	0.5	1	1
14-2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
26-1	0.06	0.06	0.25	0.5	0.06	0.06	0.5	0.5
26-2	0.06	0.06	0.25	0.5	0.06	0.06	0.5	0.5
27-1	2*	1	2	2	2	1	2	2
27-2	2*	1	2	2	2	2	2	2
GC49226	1	1	2	2	1	1	2	2
SA29213	0.12	0.12	0.25	0.12	0.12	0.12	0.25	0.12

*skip in 0.06, very light growth

Table 2: Dalbavancin Dilution Difference Compared to Suggested BMD Reference Method (GC broth with 5% LHB, Direct Colony Inoculum, 24 hour incubation in CO_2) for 4 *N. gonorrhoeae* (duplicate MICs for 3 isolates and 1 MIC for QC strain)

Method*	*N. gonorrhoeae* (n=6): Dalbavancin MIC µg/mL								
	-4	-3	-2	-1	0	1	2	3	4
GC Broth w/5% LHB Stationary Phase 24 hours					4	2			
MTGE Direct Colony 24 hours			2		4				
MTGE Stationary Phase 24 hours			2	2	2				
GC Broth w/5% LHB Direct Colony 48 hours					3	3			
GC Broth w/5% LHB Stationary Phase 48 hours					2	4			
MTGE Direct Colony 48 hours			2		4				
MTGE Stationary Phase 48 hours			1	1	3				

*Compared to suggested reference method of GC broth with 5% Lysed Horse Blood, Direct Colony Inoculum, 24 hour incubation in CO_2

Results (cont.)

Figure 1: Dalbavancin MICs (µg/mL) after 24 hour Incubation in CO_2 by Isolate Number

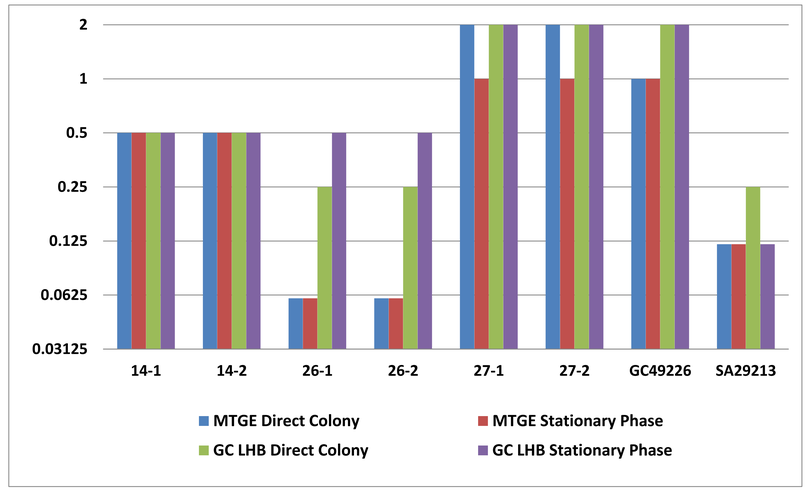
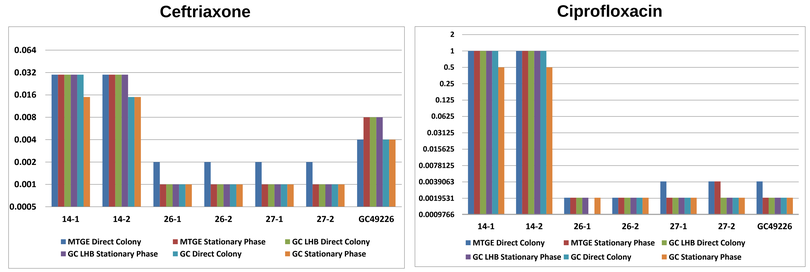


Figure 2: Comparator MICs (µg/mL) after 24 hour Incubation in CO_2 by Isolate Number



Conclusions

- Both GC broth+5% lysed horse blood (LHB) and MTGE broth provides sufficient growth and reproducible dalbavancin, ceftriaxone and ciprofloxacin MIC results of *N. gonorrhoeae*.
- Although growth was sufficient in GC broth (without addition of blood) for the isolates tested in this study, prior studies demonstrated poor or no growth in GC Broth.
- Additional testing with a larger set of isolates against dalbavancin and comparator agents using the GC with 5% lysed horse blood and MTGE with and without P80 compared to agar dilution and GC broth (comparator agents only) is recommended.

References

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