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Analysis of disk testing methods and variables for GSK2140944 and linezolid against *Staphylococcus aureus, Streptococcus pneumoniae* and *Streptococcus pyogenes*

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Abstract

Background: GSK2140944 is an antimicrobial that inhibits bacterial DNA gyrase and topoisomerase IV by a novel mechanism and is being developed as an oral and IV treatment of Gram-positive (including MRSA) and bio-threat pathogens. This study was conducted to determine the effect of various testing parameters on GSK2140944 disk diffusion zones against relevant pathogens. **Methods:** 30 *S. aureus*, 15 *S. pneumoniae*, 15 *S. pyogenes* and QC isolates were tested by disk diffusion (10 µg GSK2140944 and 10 and 30 µg linezolid disks) using 3 different methods: the CLSI method using 3 separate manufacturers: Becton Dickinson (BD), Hardy Diagnostics (HDY), and Oxoid, Ltd. (OX), The European Committee on Antimicrobial Susceptibility Testing (EUCAST) method and The British Society for Antimicrobial Chemotherapy (BSAC) method. The following significant variables were studied: temperature, incubation time, atmospheric conditions, inoculum concentration, pH, calcium, magnesium, zinc, potassium, thymidine, and polysorbate 80. **Results:** GSK2140944 and comparator agent zones, on all media against all organisms, were comparable with the exception of BSAC (IsoSensitest agar [ISA] with BSAC inoculum method). Variables that most affected GSK2140944 disk zones are shown in the table. For the most part, GSK2140944 and linezolid disk zones acted similarly with variations against study isolates.

Testing Variable	GSK2140944 10 µg zones compared to reference method zones (average zone difference)		
	S.aureus	*S. pneumoniae*	*S. pyogenes*
Inoculum (10^6 CFU/mL)	↑ 4.5 mm	↑ 6.4 mm	↑ 5.6 mm
Inoculum (10^7 CFU/mL)	↑ 3.1 mm	↑ 3.7 mm	↑ 3.8 mm
pH 5.5	↓ 4.4 mm	↑ 5.8 mm	↑ 2.8 mm
pH 8.5	↑ 13.2 mm	-	-
10% CO^2	↓ 3.8 mm	↓ 5.6 mm	↓ 3.1 mm
40°C	-	↓ 3.0 mm	↑ 5.0 mm
30°C	-	-	↑ 3.9 mm
14 hours	-	-	↑ 4.2 mm
Thymidine (+1 & 5 µg/mL)	-	↓ 3.5 mm	-
- Average difference >2 mm			

Conclusions: When performing disk diffusion testing with GSK2140944 it is important to control the inoculum concentration and pH of media. Incubation atmosphere, temperature and time, and thymidine concentration may also affect GSK2140944 disk zones.

Introduction

Disk diffusion susceptibility testing of antimicrobial agents is typically performed according to standardized methods (1-3). These guidelines provide standardized procedures for controlling important testing conditions such as inoculum concentration, incubation conditions, media, and pH and cation concentration of the media, which have been shown to have an effect on disk diffusion zones. This study was performed to determine the effect of various testing parameters on the *in vitro* activity of a novel bacterial type II topoisomerase inhibitor (BTI) antimicrobial agent (GSK2140944) and a comparative agent, linezolid, against *Staphylococcus aureus, Streptococcus pneumoniae* and *Streptococcus pyogenes* using disk diffusion.

Methods

Antibiotics

Antibiotic	Disk Concentration (µg)	Disk Manufacturer
GSK2140944	10	Becton Dickinson (BD) & Mast
Linezolid	30	BD
Linezolid	10	Mast (EUCAST method only)

Microorganisms

- **30 *S. aureus* (15 MRSA and 15 MSSA)**
- **15. *S. pneumoniae***
- **15 *S. pyogenes***
- **Quality Control:**
  ***S. aureus* ATCC 29213,**
  ***S. pn eumoniae* ATCC 49619**

Disk Diffusion Methods

The reference methodology used was the standard CLSI antimicrobial disk susceptibility testing method, a summary of which is shown for each bacterial species in the Disk Reference Method Table (1). This standard method was utilized to compare Mueller Hinton Agar made from 3 different manufacturers, as well as IsoSensitest Agar (Media and Method Comparison Table). EUCAST (2) and BSAC (3) methods were also tested and compared (Media and Method Comparison Table). The variations to the standard CLSI method that were also tested in this study are shown in the Testing Variables Table.

Disk Reference Method

Organism	Media Used	Media Manufacturer	Incubation Time (hrs)	Atmospheric Condition	QC Organism (ATCC No.)
S. aureus	Mueller Hinton Agar	BD	16-18	Ambient	29213, 25922
S. pneumoniae	Mueller Hinton Agar + 5% Sheep Blood	BD	20-24	CO_2	49619
S. pyogenes	Mueller Hinton Agar + 5% Sheep Blood	BD	20-24	CO_2	49619

Media and Method Comparison

Media Manufacturer		*S. aureus*	Streptococci	Method
BD	Becton Dickinson	MHA	MHA +5% SB	CLSI (1)
OX	Oxoid	MHA	MHA +5% SB	CLSI (1)
HDY	Hardy	MHA	MHA +5% SB	CLSI (1)
EU	Becton Dickinson	MHA	MHA+5% DHB + 20 mg/L NAD	EUCAST (2)
ISA	Oxoid	ISA	ISA+5% DHB	BSAC media with CLSI inoculum (0.5 McFarland)
BSAC	Oxoid	ISA	ISA+5% DHB	BSAC media with BSAC inoculum* (3)
***BSAC Inoculum (dilution of 0.5 McFarland)**		1:10	Beta-hemolytic streptococci: 1:100 *S. pneumoniae*: 1:10	

ISA – IsoSensitest agar
MHA – Mueller Hinton agar
SB – sheep blood
DHB – defibrinated horse blood

Testing Variables

Variable Description	Reference Method Condition	Specific Variables Tested
Temperature	**35±2°C**	30, 35, and 40°C
Incubation time	*S. aureus*: 16-18 hours Streptococci: 20-24 hours	*S. aureus*: 14, 16-18, 24, 48 hours Streptococci: 16, 18, 20-24, 48 hours
Atmospheric conditions	Ambient	Ambient, 5% and 10% CO2
Inoculum	10^8 CFU/mL	10^4, 10^5, 10^6, 10^7, 10^8 CFU/mL
Calcium	20-25 mg/L	25, 50 and 100 mg/L
Magnesium	10-12.5 mg/L (for broth MIC method)	12.5, 25 and 50 mg/L
pH	7.2-7.4	5.5, 6.5, 7.2-7.4 and 8.5
Polysorbate 80	none	0.002%
Thymidine	not defined	1 and 5 mg/L added
Zinc	not defined	2, 5 and 10 mmol/L added
Potassium	not defined	12.5, 25 and 50 mmol/L added

Results

Differences in disk diffusion zones for GSK219044 as a result of testing variables were observed for *S. aureus, S. pneumoniae* and *S. pyogenes*. Those method variations that resulted in >2 mm average zone difference and less than 90% agreement with the reference method are summarized below and in Tables 1-3 for GSK2140944 and in Tables 4-6 for linezolid.

Variable	GSK2140944 10 µg zones compared to reference method zones (average zone difference*)					
	30 *S. aureus*		15 *S. pneumoniae*		15 *S. pyogenes*	
	BD disk (mm)	Mast disk (mm)	BD disk (mm)	Mast disk (mm)	BD disk (mm)	Mast disk (mm)
Oxoid MHA	↓ 2.4	↓ 2.8	-	-	-	-
BSAC method	↑ 2.1	-	↑ 3.6	↑ 3.7	↑ 5.5	↑ 5.3
pH 5.5	↓ 4.0	↓ 5.2	↑ 6.1	↑ 5.4	-	↑ 3.0
pH 8.5	↑ 13.6	↑ 12.8	-	↑ 2.3	-	-
Inoculum 10^7 CFU/mL	↑ 3.2	↑ 3.0	↑ 3.9	↑ 3.5	↑ 3.8	↑ 3.8
Inoculum 10^6 CFU/mL	↑ 4.5	↑ 4.5	↑ 6.6	↑ 6.1	↑ 5.5	↑ 5.6
10% CO_2	↓ 3.6	↓ 3.9	↓ 5.6	↓ 5.5	↓ 3.4	-
Thymidine 1 µg/mL	-	-	↓ 3.7	↓ 3.1	-	-
Thymidine 5 µg/mL	-	-	↓ 3.6	↓ 3.4	-	-
30°C	-	-	-	-	↑ 4.2	↑ 3.6
40°C	-	-	↓ 3.1	↓ 2.8	↑ 5.2	↑ 4.7
14 hr. incubation	-	-	-	-	↑ 4.4	↑ 3.9

*Variables with average zone differences >2 mm

Table 1. *S. aureus* (n = 30) GSK2140944 10 µg disk zone differences (compared to reference result on same day) by variable

Variable	Disk Mfr.	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ± 4mm
Reference Day 1*	BD										2	8	11	8	1								100
	MAST						1				3	9	11	1	4		1						93.3
Oxoid MHA	BD						1	9	7	7	2	1		2	1								96.7
	MAST						5	8	8	3	2	2	1			1							83.3
BSAC	BD								1	1	4	3	5	5	4	2	2			1	1	1	83.3
	MAST									4	4	3	8	4	2	2			1	2			90.0
Reference Day 2*	BD							1	1		12	4	5	3	2	2							100
	MAST				1					3	7	9	8		2								96.7
5.5 pH	BD	2		1	2	6	4	5	1	2	3	3	1										50.0
	MAST	2	2	1	1	5	10	7			1		1										30.0
8.5 pH**	BD																			1	3	19	0
	MAST																			2	5	16	0
Reference Day 4*	BD			1					1	1	6	12	7	1		1							96.7
	MAST						1		2	1	6	10	9	1									96.7
10^6 CFU/mL	BD											1		1	3	15	6	1	1			2	66.7
	MAST													3	6	5	11	3			1	1	46.7
10^7 CFU/mL	BD											1	2	7	12	3	2	1	1	1			83.3
	MAST												4	10	7	4	2	2		1			83.3
10% CO_2	BD				2	4	4	6	9	3					1		1						63.3
	MAST				1	5	6	9	6	1				1	1								60.0

*Reference for each day compared to the mean of all reference results (n = days 1-5)
**No growth for 7 isolates

Table 4. *S. aureus* (n = 30) linezolid disk zone differences (compared to reference result on same day) by variable

Variable	Disk Conc.	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference Day 1*	30										3	7	6	8	2	3		1					96.7
BSAC	30											1	3	6	3	4	3	4	3		1	2	56.7
BSAC	10										1	4	5	4	4	3	2	5	1			1	70.0
Reference Day 2*	30								1	8	8	5	6	2									100
5.5 pH	30	2									1	5	3	2	6	1	4	3	1	2			60.0
8.5 pH	30	7												2	2	5	3	3		1		7	30.0
Reference Day 4*	30										1	13	9	4	2	1							100
10^6 CFU/mL	30													1	4	6	7	7	2		1	2	36.7
10^7 CFU/mL	30								1			2	2	7	9	3	1	2	1		1	1	80.0
10^9 CFU/mL	30	1				2	3	3	4	7	5	3	2										80.0
Reference Day 5*	30								2	6	8	8	5	1									100
40°C	30		1	3		3	7	6	5	2	2						1						50.0

*Reference for each day compared to the mean of all reference results (n = days 1-7)

Table 7. Quality control results for GSK2140944 10 µg BD and MAST disks (number of reference method results at each zone)

Disk MFR.	QC Strain	20	21	22	23	24	25	26	27	28	29	30
BD	*S. aureus* 25923								3	1	1	
	S. aureus 29213								1		3	1
	S. pneumoniae 49619			3		2		2				
MAST	*S. aureus* 25923							1	2	2		
	S. aureus 29213								1	3	1	
	S. pneumoniae 49619		2	1	1	1		1	1			

Table 8. Quality control results for linezolid 10 and 30 µg disks (number of reference method results at each zone)

QC Strain	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Linezolid 30 µg disk zone (mm)															
S. aureus 25923									1	2	1	1			
S. aureus 29213									2	2	1				
S. pneumoniae 49619						1	2	2	2						
Linezolid 10 µg disk zone (mm)															
S. aureus 25923							1								
S. aureus 29213				1											
S. pneumoniae 49619				1											

Table 2. *S. pneumoniae* (n = 15) GSK2140944 10 µg disk zone differences (compared to reference result on same day) by variable

Variable	Disk Mfr.	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference Day 1*	BD									3	2	6	2	1	1								100
	MAST									2	4	6	1	2									100
BSAC	BD													2	6	5	1		1				86.7
	MAST													3	3	6	2		1				80.0
Reference Day 2*	BD								2	1	1	5	5	1									100
	MAST								1		3	4	6	1									100
5.5 pH	BD												1	2			1	4	3	2	1	1	20.0
	MAST											1	1		2	1	4	2	2			2	33.3
8.5 pH	BD							1			1	1	4	5		2		1					93.3
	MAST								1				3	3	4	1	2	1					80.0
Reference Day 5*	BD											1	4	6	2	2							100
	MAST												5	5	4	1							100
10^6 CFU/mL	BD															1	5	3	1	2	1	2	6.7
	MAST														1	1	2	6	2	2	1		13.3
10^7 CFU/mL	BD												2	4		4		3	2				66.7
	MAST											1		2	3	4	4	1					66.7
Reference Day 6*	BD					3	2	5	1	3	1												66.7
	MAST						7	1	5	1	1												53.3
10 CO_2	BD		1	1	5	2	1	2	2				1										33.3
	MAST			2	4	2	4	1	1						1								20.0
1 µg/mL Thymidine	BD			2		1	4	1	1	3	1	2											53.3
	MAST						5	2	3	1	2	1	1										66.7
5 µg/mL Thymidine	BD		1	1		2		6		2	1	1			1								73.3
	MAST						5	6	1		2				1								66.7
Reference Day 7*	BD									6	2	4	1	2									100
	MAST									3	7	3	1	1									100
40° C	BD			1	1		1	1	3	4	3		1										80.0
	MAST			1			2	1	3	3	2	1	2										80.0

*Reference for each day compared to the mean of all reference results (n = days 1-7)

Table 5. *S. pneumoniae* (n = 15) linezolid disk zone differences (compared to reference result on same day) by variable

Variable	Disk Conc.	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference Day 1*	30								1	1	8	3	1	1									100
BSAC	30													2	1	7	2	1	1	1			66.7
BSAC	10													2	2	7	2	1	1				85.7
Reference Day 2*	30								2	4	2	5	1		1								100
5.5 pH	30														1	1		1			1	11	13.3
Reference Day 4*	30											3	8	1	3								100
10 mmol/L Zinc	30												2	4	5	2	1	1					86.7
Reference Day 5*	30										1	3	5	4	2								100
10^6 CFU/mL	30															1	2	3	5	3	1		6.7
10^7 CFU/mL	30													2	3	2	4	3	1				46.7
10^9 CFU/mL	30				1		1	2	4	5	2												86.7
Reference Day 6*	30							2	1	4	4	3	1										100
10% CO_2	30				2				5	1	3	1	2	1									86.7
5 µg/mL Thymidine	30						2	3	1	4	3	1	1										86.7
Reference Day 7*	30								1	3	6	1	2	2									100
30°C	30											1	1	1		3	4	2	1	1	1		40.0
40°C	30				1		1	2	5	2	2	1		1									86.7

*Reference for each day compared to the mean of all reference results (n = days 1-7)

Table 3. *S. pyogenes* (n = 15) GSK2140944 10 µg disk zone differences (compared to reference result on same day) by variable

Variable	Disk Mfr.	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference Day 1*	BD									2	6	4	3										100
	MAST									3	3	8	1										100
BSAC	BD															5	3	3	3	1			33.3
	MAST															4	3	7	1				26.7
Reference Day 2*	BD								2	2	7	4											100
	MAST									2	7	5	1										100
5.5 pH	BD										1	1	2	2	3	5	1						93.3
	MAST											1	3	2	2	3	4						73.3
Reference Day 5*	BD									1	2	2	7	2	1								100
	MAST											4	7	4									100
10^6 CFU/mL	BD														1	4	3	3	2	2			33.3
	MAST															3	4	6	1		1		20.0
10^7 CFU/mL	BD												2	1	1	8	1	1	1				80.0
	MAST													2	4	5	3	1					73.3
Reference Day 6*	BD								1	3	8	2	1										100
	MAST								1	1	8	5											100
10 CO_2	BD					1	1	4	6	3													86.7
	MAST						1	2	5	5	2												93.3
Reference Day 7*	BD									1	4	7	3										100
	MAST									3	4	6	2										100
14 hours	BD													1	1	7	5			1			60.0
	MAST												1	1	4	5	2	1	1				73.3
30°C	BD													1	3	6	4			1			66.7
	MAST												1	1	6	4	2		1				80.0
40°C	BD													1		7	3	1	1	1		1	53.3
	MAST													3	3	2	4		1	1		1	53.3

*Reference for each day compared to the mean of all reference results (n = days 1-7)

Table 6. *S. pyogenes* (n = 15) linezolid disk zone differences (compared to reference result on same day) by variable

Variable	Disk Conc.	≤ -10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	≥ 10	% ±4mm
Reference Day 1*	30									3	5	5	2										100
BSAC	30															1	2	3	1	5	2	1	6.7
BSAC	10															2	5	3	3	2			13.3
Reference Day 2*	30									7	5	3											100
5.5 pH	30											3	4	3	1	2	2						86.7
Reference Day 5*	30										2	2	6	3	2								100
10^6 CFU/mL	30																2	3	6	3	1		0
10^7 CFU/mL	30														2	3	6	3	1				33.3
Reference Day 7*	30								1		5	6	2	1									100
14 hours	30													4	2	4	4	1					66.7
30°C	30													4	4	3	4						73.3

*Reference for each day compared to the mean of all reference results (n = days 1-7)

Conclusions

- *S. aureus* GSK2140944 disk zones were affected by inoculum concentration, pH, and 10% CO_2 incubation. Linezolid zones were similarly affected except there was no affect with 10% CO_2 incubation, there was an affect with 40°C incubation temperature and linezolid zones were larger with 5.5 pH in comparison to smaller zones with GSK2140944.
- *S. pneumoniae* GSK2140944 disk zones were affected by inoculum concentration, pH, 10% CO_2 incubation, 40°C incubation temperature and higher thymidine concentrations. Linezolid zones were similarly affected except there was also an affect with 30°C incubation temperature and with addition of zinc.
- *S. pyogenes* GSK2140944 disk zones were affected by inoculum concentrations, 10% CO_2 incubation, 30°C and 40°C incubation temperature, 5.5 pH and 14-hour incubation time. Linezolid zones were similarly affected except there was no affect with 10% CO_2 incubation or 40°C incubation temperature
- When performing susceptibility testing with these agents, therefore, it is important to be aware of and control these particular variables according to standardized methods.

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