Abstract 913
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The Effect of Testing Parameter Variations on the *In Vitro* Activity of Retapamulin and Mupirocin

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Abstract

Background: Retapamulin (SB-275833) is a novel pleuromutilin antimicrobial agent being developed for the topical treatment of skin infections. Retapamulin has a unique mode of action and demonstrates no target specific cross-resistance to other antibacterials. This study was undertaken to determine the effect of various testing parameters on the *in vitro* activity of retapamulin and a comparative agent, mupirocin. **Method:** 10 *Staphylococcus aureus* (five methicillin-susceptible and five methicillin-resistant), 10 *Streptococcus pyogenes* and quality control strains *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *S. pneumoniae* ATCC 49619 were tested by three Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) recommended methods: broth microdilution, macrodilution and agar dilution. In addition, three different broths (IsoSensitest, brain heart infusion and trypticase soy) were compared with cation-adjusted Mueller–Hinton broth using broth microdilution. A total of nine testing variables (temperature, inoculum, pH, calcium, magnesium, zinc, potassium, thymidine and serum) were also studied using microdilution and agar dilution methods. **Results:** Under standard, reference test conditions, all three methods produced equivalent results. As shown in the table below, of all the variables tested, the ones that affected retapamulin and mupirocin MICs were: pH, inoculum, zinc and serum.

	Mean dilution difference (MICs compared with reference)	
Testing Variable	Retapamulin	Mupirocin
pH 6	+1–2	-2
pH 7.5	-2	+1
pH 8.5	->2	+3–5
Inoculum 10^5–10^8	+>3	+>3
Zinc 50–100 mmol/L	->3	->2
Serum 25%	+0.5	+2–2.75
Serum 50%	+1.25	+3–>3

Conclusion: When performing susceptibility testing with retapamulin and mupirocin it is important to control the pH of the media and the inoculum concentration and to be aware that the addition of serum and extremely high levels of zinc can also affect the MICs.

Introduction

Retapamulin (Figure 1, SB-275833) is a novel semi-synthetic pleuromutilin, representing a new class of antibacterial agents, which has been formulated as a topical treatment for uncomplicated skin infections. Due to the unique pleuromutilin mode of action, retapamulin shows no target specific cross-resistance to other classes of antibacterials.

This study was performed in order to determine the influence of various factors on the *in vitro* susceptibility testing of retapamulin.

Methods

Study strains

- 10 strains of *Staphylococcus aureus* (five methicillin-susceptible and five methicillin-resistant).
- 10 strains of *Streptococcus pyogenes*.
- Quality control strains: *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *S. pneumoniae* ATCC 49619.

Comparison of three (CLSI) MIC methods

Broth microdilution

- 11 concentrations of retapamulin in doubling dilutions (0.004–4 µg/mL) and mupirocin (0.008–8 µg/mL) were prepared in cation-adjusted Mueller–Hinton broth (CAMHB) and 50 µL of each antimicrobial dilution was added to the appropriate well in 96-well microtiter trays.
- A 1:100 dilution of an organism suspension (0.5 McFarland) was made in CAMHB (*S. aureus*) or CAMHB + 10% lysed horse blood (*S. pyogenes*). 50 µL was added to each well resulting in the final concentration range desired for the antimicrobial agent. Final antimicrobial agent concentrations in the inoculated plates were 0.002–2 µg/mL and 0.004–4 µg/mL for retapamulin and mupirocin, respectively.
- Plates were incubated under ambient conditions at 35°C for 18 h.
- The MIC was recorded as the lowest concentration showing no growth.
- All study strains were tested once initially and in triplicate on a subsequent day.

Macrodilution

- Multiple concentrations of retapamulin in doubling dilutions (0.03–2 µg/mL for *S. aureus* and 0.016–1 µg/mL for *S. pyogenes*) and mupirocin (0.06–4 µg/mL for *S. aureus* and 0.03–2 µg/mL for *S. pyogenes*) were prepared in CAMHB and 1 mL of each concentration was transferred to sterile polystyrene tubes.
- A 1:100 dilution of an organism suspension (0.5 McFarland) was made in CAMHB (for *S. aureus*) or CAMHB + 10% lysed horse blood (for *S. pyogenes*) and 1 mL was added to each tube. Final antimicrobial agent concentrations in the inoculated plates were 0.016–1 µg/mL for *S. aureus* and 0.008–0.5 µg/mL for *S. pyogenes* with retapamulin and 0.03–2 µg/mL for *S. aureus* and 0.015–1 µg/mL for *S. pyogenes* with mupirocin.
- Tubes were incubated under ambient conditions at 35°C for 18 h.
- The MIC was recorded as the lowest concentration showing no visible growth.
- All study strains were tested in triplicate.

Agar dilution

- Multiple concentrations of retapamulin in doubling dilutions (0.016–1 µg/mL for *S. aureus* and 0.03–1 µg/mL for *S. pyogenes*) and mupirocin (0.03–1 µg/mL for *S. aureus* and *S. pyogenes*) were prepared in Mueller–Hinton agar (MHA) or IsoSensitest agar (ISA) for *S. aureus* and MHA + 5% lysed horse blood or ISA + 5% defrinated horse blood and 20 mg/L NAD for *S. pyogenes*. 25–30 mL of each antibiotic-containing agar was poured into 100 mm sterile Petri dishes.
- A 1:10 dilution of an organism suspension (0.5 McFarland) was made in CAMHB and 3 µL was inoculated on each agar plate.
- Plates were incubated under ambient conditions (for *S. aureus*) and at 5% CO_2 (for *S. pyogenes*) at 35°C for 24 h.
- The MIC was recorded as the lowest concentration showing no visible growth.
- All study strains were tested in triplicate.

Modification of testing variables

- The 20 strains were tested by broth microdilution and agar dilution (as described above) with the exception of variations of the following test factors: broth, temperature, inoculum, calcium, magnesium, zinc, potassium, thymidine, pH, CO_2, serum.
- The specific differences studied are shown along with a summary of the results in Table 1.

Results

Initial mean MIC results by broth microdilution, macrodilution and agar dilution methodologies are shown in Table 2. Results were all within 1 doubling dilution of one another with the exception of ISA agar dilution MICs against *S. aureus*, which were approximately 2 dilutions higher for retapamulin and 2 dilutions lower for mupirocin than their respective MHA agar dilution MICs.

Table 1 shows the percentage of essential agreement of the results within ± 1 dilution of the reference Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) method for all testing variables. Differences in MICs were considered significant if <90% of the test condition results were within ± 1 doubling dilution of the results for the reference method. The mean MIC results for microdilution and agar dilution for each study variable are shown in Figure 2.

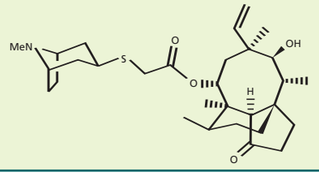

Figure 1. Chemical Structure of Retapamulin

Table 1. Essential Agreement (%) Within ± 1 Dilution for Reference and Comparative Conditions for Retapamulin and Mupirocin

Reference (CLSI) Condition	Comparative Condition	Retapamulin				Mupirocin			
		S. aureus		*S. pyogenes*		*S. aureus*		*S. pyogenes*	
		Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
Broth (CAMHB)	ISA	100	NT	NT	NT	100	NT	NT	NT
	BHI	100	NT	NT	NT	100	NT	NT	NT
	TSB	100	NT	NT	NT	100	NT	NT	NT
Temperature (35°C)	30°C	100	90	100	100	100	100	100	100
	40°C	100	90	100	100	100	100	40	100
Inoculum (10^5 CFU/mL)	10^2 CFU/mL	NT	100	NT	90	NT	100	NT	60
	10^3 CFU/mL	30	100	100	100	100	100	90	80
	10^4 CFU/mL	100	90	100	0	90	100	100	10
	10^6 CFU/mL	100	0	100	0	100	0	70	0
	10^7 CFU/mL	0	0	40	0	0	0	20	0
	10^8 CFU/mL	0	NT	40	NT	0	NT	60	NT
Atmospheric (Ambient)	5% CO_2	80	30	100	90	100	100	70	70
	10% CO_2	30	20	100	100	90	70	70	10
Calcium (25 µg/mL)	No calcium	100	100	100	100	100	100	100	100
	10 µg/mL	100	90	100	100	100	100	100	100
	50 µg/mL	100	100	100	100	100	100	100	100
	100 µg/mL	100	100	100	100	100	100	100	100
Magnesium (10 µg/mL)	No Magnesium	100	100	100	100	100	100	100	100
	15 µg/mL	100	100	100	100	100	100	100	100
	25 µg/mL	100	100	100	100	100	100	100	NT
	50 µg/mL	100	100	100	100	100	100	100	100
	100 µg/mL	100	100	100	100	100	100	100	100
Potassium (None)	5 mol/L	100	100	90	100	100	100	100	100
	10 mol/L	100	100	90	100	100	100	100	100
	20 mol/L	100	100	90	100	100	100	100	100
Zinc (None)	1 mol/L	100	100	100	100	100	100	100	100
	5 mol/L	100	100	100	100	100	100	100	100
	10 mol/L	100	100	100	100	100	100	100	100
	25 mol/L	90	90	90	100	100	100	100	100
	50 mol/L	90	90	10	100	80	100	100	100
	100 mol/L	0	60	0	100	0	10	0	70
Thymidine (None)	25 µg/mL	100	100	90	100	100	100	100	100
	100 µg/mL	100	100	90	100	100	100	100	100
pH (7)	5.5	10	20	30	10	0	0	0	20
	6	90	20	50	10	10	20	0	100
	6.5	100	100	100	90	100	100	100	100
	7.5	0	0	0	100	50	100	100	70
	8	0	0	0	70	0	90	0	50
	8.5	0	0	0	0	0	0	0	0
Serum (None)	25%	60	NT	70	NT	0	NT	30	NT
	50%	10	NT	0	NT	0	NT	0	NT

Grey shading indicates instances where essential agreement (± 1 dilution) was <90%
ISA, IsoSensitest; BHI, brain heart infusion, TSB, trypticase soy; NT, not tested

Table 2. Mean MICs for Retapamulin and Mupirocin Against 10 *S. aureus* and 10 *S. pyogenes* Strains as Determined in Triplicate by Broth Microdilution, Macrodilution and Agar Dilution Methodologies

	Retapamulin			
	Microdilution (n = 40)	Macrodilution (n = 30)	Agar Dilution – MHA (n = 30)	Agar Dilution – ISA (n = 30)
S. aureus	0.106	0.091[a]	0.115[b]	0.435
S. pyogenes	0.030	0.033	0.058	0.058
	Mupirocin			
	Microdilution (n = 40)	Macrodilution (n = 30)	Agar Dilution – MHA (n = 30)	Agar Dilution – ISA (n = 30)
S. aureus	0.313	0.25	0.228	0.072
S. pyogenes	0.125	0.119	0.125	0.125

[a] Mean based on n = 28, MIC for other strains: 2 = >0.25
[b] Mean based on n = 28, MIC for other strains: 2 = not available

Conclusions

- There was no impact (≤1 dilution difference) on retapamulin or mupirocin MICs with variations in:
  - broths used in the microdilution procedure
  - temperature and CO_2 concentration
  - addition of calcium, magnesium, potassium and thymidine
- There was some impact (>1 dilution difference) on retapamulin and mupirocin MICs with variations in:
  - pH, inoculum concentration, zinc and serum.
- When performing susceptibility testing with these two agents, therefore, it is important, to control for these particular variables according to standardized methods.
- These data support the fact that current routine standardized antimicrobial testing methodologies are likely to provide robust measures of the *in vitro* activity of retapamulin against clinical isolates.

Acknowledgements

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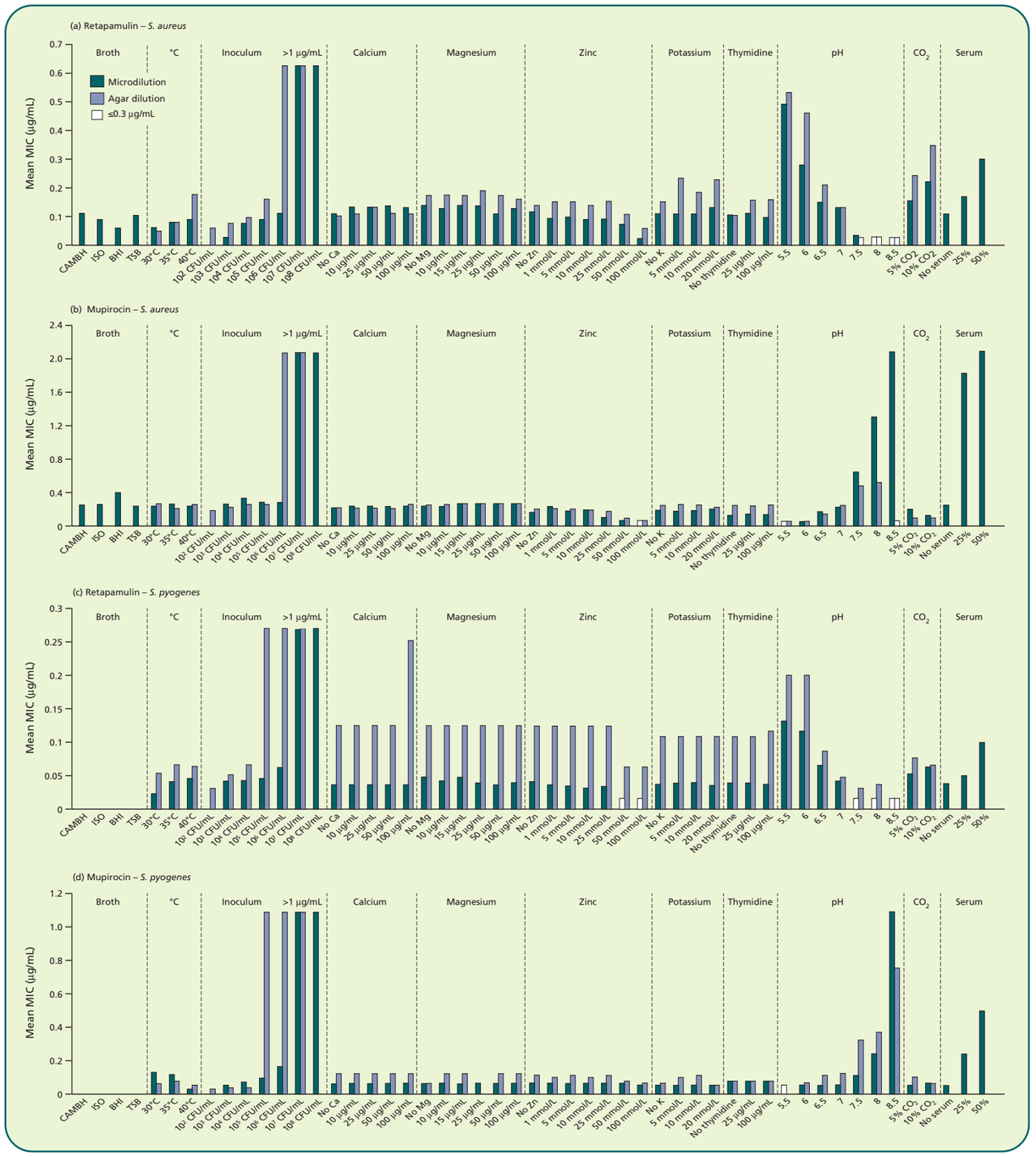

Figure 2. Mean Retapamulin and Mupirocin MICs (µg/mL) of 10 *S. aureus* and *S. pyogenes* For Each Variable Tested

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