The effect of testing parameter variations on the in-vitro activity of iclaprim against Staphylococcus aureus

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ABSTRACT

Background: Iclaprim is a novel diaminopyrimidine antibiotic that exhibits potent bactericidal activity against major Gram-positive pathogens, notably including MRSA. This study aimed to determine effect of various testing parameters on the in-vitro activity of iclaprim against S. aureus.

Method: 10 S. aureus strains (4 MSSA and 6 MRSA including 2 VISA) and quality control strain S. aureus ATCC 29213 were tested. CLSI broth microdilution (BMD; using cation adjusted Mueller Hinton and Isosensitest broth), macrodilution and agar dilution (using Mueller Hinton Isosensitest agars) MICs were determined in triplicate for each strain using trimethoprim as a class comparator. A total of 10 testing variables (incubation temperature and time, incubation in CO², inoculum concentration, calcium, magnesium, pH, serum and polysorbate-80) were also studied using CLSI BMD method.

Results: There was good correlation of iclaprim MICs by microbroth and macrobroth methods. MHA dilution MICs were slightly lower than CAMHB MICs by approximately one dilution. Iclaprim MICs were only slightly impacted by the addition of plasma and broth pH of 5.5, but were most impacted by addition of serum. Incubation at 48 hours, at 30°C and 40°C, using an inoculum concentration of 107cfu/mL had a slight effect (1-2 dilutions). All other variables tested did not significantly impact S. aureus MIC results (within \pm 1 dilution).

| Method variation | Mean MIC (µg/mL) of CLSI reference and method variation (Test), n = 30 | | | |
|---------------------|--|--------|--|--|
| | Reference | Test | | |
| 30°C | 0.1436 | 0.0474 | | |
| 40°C | 0.1436 | 0.2679 | | |
| 48 hrs | 0.1340 | 0.2939 | | |
| 10 ⁷ CFU | 0.1104 | 0.2308 | | |
| pH 5.5 | 0.077 | 0.3232 | | |
| Serum, 25% | 0.1279 | 0.5 | | |
| Serum, 50% | 0.1279 | 1 | | |
| Plasma, 50% | 0.1039 | 0.3223 | | |

Conclusions: When performing susceptibility testing with iclaprim, it is important to control the pH of the media, the incubation temperature, inoculum concentration and media pH to avoid slight variations in MICs and to be aware that the addition of serum can have a greater effect on the MICs.

Iclaprim is a novel investigational drug that is being developed for serious Gram-positive bacterial infections. The compound has been granted fast-track product designation and has recently completed two pivotal Phase III clinical studies in complicated skin and skin structures infections (cSSSI). In order to assure accurate and reproducible in vitro susceptibility results for a new antimicrobial agent, the effects of various testing factors should be known. The MICs of iclaprim were compared for different reference MIC methods and media, and by broth microdilution with modifications of select variables for 10 Staphylococcus aureus strains and a quality control strain, S. aureus (ATCC 29213). Broth macrodilution, broth microdilution and agar dilution MICs were determined using both Mueller Hinton and Isosensitest media. In addition, MIC testing was performed by broth microdilution with modifications to incubation temperature, time, atmospheric conditions, inoculum concentration, calcium, magnesium, pH, and with addition of Oserum, plasma and wetting agent, polysorbate 80 (P80).

METHODS



Media:

| Media | Supplier |
|--|------------------|
| Cation Adjusted Mueller Hinton Broth (CAMHB) | Becton Dickinson |
| Mueller Hinton Broth (MHB) | Becton Dickinson |
| IsoSensitest Broth (ISB) | Oxoid |
| Mueller Hinton Agar (MHA) | Becton Dickinson |
| IsoSensitest Agar (ISA) | Oxoid |

MIC Methods:

INTRODUCTION

| tibiotic | Concentrations |
|----------|----------------|
| prim | 0.015–16 mg/L |
| | |

Microorganisms:

 10 S. aureus strains (4 MRSA, 2 VISA, 4 MSSA) • QC: S. aureus ATCC 29213 (CLSI QC Range: 0.03–0.12 mg/L)

According to CLSI procedures with exception of additional testing using ISA and ISB

- 1. Broth microdilution (CAMHB and ISB; trays were made daily)
- 2. Broth macrodilution (CAMHB)
- 3. Agar dilution (MHA and ISA; plates were made no more than 4 days prior to use and stored at 2–8°C)
- 4. Broth microdilution (modification of test variables; see Table 1)

Inoculum counts were performed for each isolate tested. Quality control strain, lower than reference MICs) S. aureus ATCC 29213, was tested on each day of testing.

Table 1. Variables studied by broth microdilution.

| Variable description | Supplementation (stock) | Specific variables tested | |
|------------------------|--------------------------------|--|--|
| Temperature | | 30, 35 and 40°C | |
| Incubation time | | 16, 18, 20, 24 and 48 hrs | |
| Atmospheric conditions | | Ambient, 5% and 10% CO_2 | |
| Inoculum | | 10 ⁴ , 10 ⁵ , 10 ⁶ and 10 ⁷ CFU/mL | |
| Calcium | 0.1 M CaCl ₂ | 4.9, 26.9, 54.2 and 98.1 mg/L | |
| Magnesium | 10,000 meq/L MgCl ₂ | 3.95, 10.2 and 23.4 mg/L | |
| рН | 10% Acetic acid, 2.5M NaOH | 5.5, 6.5, 7.4 and 8.5 | |
| Serum | Pooled normal human serum | 25% and 50% | |
| Plasma | Pooled human plasma | 25% and 50% | |
| Polysorbate 80 | | 0.002% | |

RESULTS

Broth microdilution, broth macrodilution and agar dilution (Table 2 & Figure 1)

- Broth macrodilution and broth microdilution (ISA) mean MICs were within one doubling dilution to broth microdilution (MHB) reference mean MICs.
- Iclaprim agar dilution (MHA) MICs were slightly lower than reference microdilution MICs but within one doubling dilution.
- Iclaprim agar dilution (ISA) were lower than reference microdilution MICs by 1–2 doubling dilutions.

Broth microdilution: effect of testing variables (Tables 3 & 4 and Figure 1)

The majority of all MICs were within one doubling dilution compared to the reference MICs. The variables that slightly impacted the MICs were incubation temperature, 48 hr incubation, inoculum concentration of 107 CFU/mL, broth pH of 5.5, and plasma. MICs were more affected by the addition of 50% serum.

Table 2. Broth microdilution variables.

| Broth microdilution variables | | | | |
|-------------------------------------|------------------------|----------------------|--|--|
| Temperature | 30°C | 1.5 dilutions lower | | |
| | 40°C | 1 dilution higher | | |
| Incubation Time | 48 hrs | 1 dilution higher | | |
| Inoculum | 10 ⁷ CFU/mL | 1 dilution higher | | |
| • pH | 5.5 | 2 dilutions higher | | |
| Plasma | 25% | 1 dilution higher | | |
| | 50% | 1.5 dilutions higher | | |
| Serum | 25% | 2 dilutions higher | | |
| | 50% | 3 dilutions higher | | |

An initial single MIC determination by broth microdilution using CAMHB was Based on a subset of eight S. aureus strains, there was no significant impact Figure1.MeaniclaprimMICs(mg/L)against10S.aureusstrains(3replicateseach)for performed for each strain. Then triplicate testing was performed by of P80 at a concentration of 0.002% on the *in vitro* activity of iclaprim (MICs each method variation. all methods/media, utilizing the same initial inoculum for all methods. for four strains were similar and MICs for four strains were one dilution

> **Table 3.** In-vitro activity of iclaprim against 10 S. aureus strains as determined by broth microdilution, macrodilution and agar dilution methodologies.

| Reference | method | Comparative method | | Comparative method – CLSI reference method | | |
|--|-------------|---------------------------|-------------|---|------------------------|-----------------------|
| Method (media) | Mean MIC | Method (media) | Mean MIC | Mean MIC difference* | n (%) ±1 dilution** | n (%) ±2 dilution* |
| Broth microdilution 0.122 ⁻ (CAMHB) | | Macrodilution (CAMHB) | 0.0926 | -0.0296 | 28 (93.3) | 30 (100) |
| | 0.1221 | Agar dilution (MHA) | 0.0769 | -0.0452 | 30 (100) | 30 (100) |
| | | Broth microdilution (ISB) | 0.0825 | -0.0397 | 30 (100) | 30 (100) |
| Agar dilution (MHA) | 0.0769 | Agar dilution (ISA) | 0.0474 | -0.0296 | 30 (100) | 30 (100) |

* Mean difference in log, MICs, comparative method – CLSI reference method

Table 4. In vitro activity of iclaprim against 10 S. aureus strains – comparison of the CLSI MIC microbroth dilution reference condition with other testing conditions.

| Test (CLSI reference condition) | Comparative condition | Mean difference* | n (%) ±1 dilution** | n (%) ±2 dilution** |
|---|------------------------|---------------------|------------------------|------------------------|
| Temperature: 35°C | 30°C | -0.0962 | 10 (33.3) | 30 (100) |
| VIEan IVIIC = 0.1436 | 40°C | 0.1244 | 26 (86.7) | 30 (100) |
| | 16 hrs | -0.0324 | 30 (100) | 30 (100) |
| Incubation time: 24 hrs | 18 hrs | 0.0000 | 30 (100) | 30 (100) |
| Mean MIC = 0.1340 | 20 hrs | 0.0063 | 30 (100) | 30 (100) |
| | 48 hrs | 0.1599 | 24 (80) | 30 (100) |
| Atmospheric: Ambient | 5% CO ₂ | 0.0226 | 30 (100) | 30 (100) |
| VIEan VIIC = 0.1114 | 10% CO ₂ | 0.0390 | 30 (100) | 30 (100) |
| | 10 ⁴ cfu/mL | -0.0096 | 27 (90) | 27 (90) |
| Inoculum: 10⁵ cfu/mL Mean MIC = 0.1104 | 10 ⁶ cfu/mL | 0.0115 | 26 (86.7) | 26 (86.7) |
| | 10 ⁷ cfu/mL | 0.1203 | 24 (80) | 24 (80) |
| | 4.93 mg/L | 0.0464 | 30 (100) | 30 (100) |
| Calcium: 25 mg/L Mean MIC = 0.1114 | 54.19 mg/L | 0.0464 | 30 (100) | 30 (100) |
| | 98.15 mg/L | 0.0321 | 30 (100) | 30 (100) |
| Magnesium: 10.2 mg/L | 3.95 mg/L | 0.0320 | 30 (100) | 30 (100) |
| Mean MIC $= 0.1575$ | 23.4 mg/L | -0.0235 | 30 (100) | 30 (100) |
| | 5.5 | 0.2454 | 1 (3.3) | 26 (86.6) |
| pH: 7.4 Mean MIC = 0.0770 | 6.5 | 0.0424 | 29 (96.6) | 30 (100) |
| | 8.5 | -0.0052 | 30 (100) | 30 (100) |
| Serum: none | 25% | 0.3721 | 4 (13.3) | 23 (76.6) |
| Mean MIC = 0.1279 | 50% | 0.8721 | 0 (0) | 4 (13.3) |
| Plasma: none | 25% | 0.0945 | 28 (93.3) | 30 (100) |
| Mean MIC = 0.1039 | 50% | 0.2184 | 13 (43.3) | 28 (93.3) |

Mean difference in log, MICs, comparative condition - CLSI reference

* Number and percentage of MICs by the two methods within ± 1 (± 2) doubling dilutions of each other Mean difference is $> \pm 1$ dilution from reference mean

90% of MICs are within ± 1 dilution from reference



CONCLUSIONS

- → There was good correlation of iclaprim MICs by microbroth and macrobroth methods.
- Iclaprim agar dilution MICs were slightly lower (1–2 dilutions) compared with broth MICs.
- Iclaprim MICs were most affected by the addition of 50% serum, but notably less with the addition of plasma.
- Incubation for 48 hrs, at 30°C and 40°C, using an inoculum concentration of 10⁷ CFU/mL, broth pH of 5.5, addition of plasma and of 25% serum had a slight impact (1–2 dilutions) on iclaprim MICs.
- → Other variables tested did not have a significant
 → effect on iclaprim MICs.