Collaborative Evaluation of an Erythromycin-Clindamycin Combination Well for Detection of Inducible Clindamycin Resistance in Beta-Hemolytic Streptococci Using the CLSI Broth Microdilution Method

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Constitutive or inducible clindamycin resistance can occur in the beta-hemolytic group of streptococci due to an \textit{erm} gene. The Clinical and Laboratory Standards Institute (CLSI) has recommended a disk approximation (D-zone test) with erythromycin and clindamycin disks and a single well broth test combining erythromycin and clindamycin for detection of inducible clindamycin resistance in staphylococci, but only a disk approximation test for the beta-hemolytic streptococci. This collaborative study assessed two different erythromycin and clindamycin concentration wells (1 + 0.25 and 1 + 0.5 µg/ml, respectively) in three different brands of Mueller-Hinton broth supplemented with 3% lysed horse blood in frozen panels prepared for this study. All labs performed the disk approximation D-zone test as described by CLSI. A total of 155 non-duplicate streptococcal isolates (50 Group A, 48 Group B, 28 Group C and 29 Group G) were tested; 99 isolates showed inducible resistance by D-zone. There were some differences noted based upon the test medium. The sensitivity of the erythromycin plus clindamycin combination of 1 + 0.25 µg/ml was 91-100% while the sensitivity of the combination of 1 + 0.5 µg/ml was 95-100%. Specificity overall was 98%. The slightly higher sensitivity of the 1 + 0.5 µg/ml is recommended. This study has demonstrated that a single microdilution well test incorporating erythromycin and clindamycin in combination would be a sensitive and specific indicator of inducible clindamycin resistance and could be included in routine test panels.
INTRODUCTION

There are two principle mechanisms of macrolide resistance in the beta-hemolytic group of streptococci. One is an active efflux mechanism encoded by the *mef* genes that affects only macrolides (6). The second mechanism is methylation of a single adenine at the 50 S ribosomal binding site used by the macrolides, lincosamides and streptogramin B drugs mediated by an *erm* gene (6). Known as the MLSB resistance phenotype, expression of MLSB resistance in staphylococci and streptococci can either be constitutive or inducible (6,10). Fourteen- and fifteen-member macrolides are good inducers of the ribosomal conformational change, but lincosamides such as clindamycin are poor inducers (6). Thus, inducible resistance is not detected by standard clindamycin MIC or disk testing. The CLSI has described a disk diffusion D-zone test for detection of inducible clindamycin resistance in staphylococci and beta-hemolytic streptococci (3). If an alternative test were available, most clinical laboratories would prefer not to have to perform a separate D-zone disk test, and would rather have an inducible resistance test included in their routine broth dilution commercial test systems. A single erythromycin-clindamycin combination well broth microdilution test for detection of inducible clindamycin resistance in staphylococci (but not streptococci) has been described by the CLSI (2,3). The goal of this study was to assess a single well broth microdilution test for detection of inducible clindamycin resistance in beta-hemolytic streptococci through a five-center collaborative study.

MATERIALS AND METHODS
Test isolates. Each of the five laboratories was asked to test at least 20 non-duplicate macrolide-resistant clinical isolates of beta-hemolytic streptococci of Groups A, B, C, or G, but not small colony S. anginosus group isolates with Groups A, C or G antigens (12).

Broth microdilution tests. Frozen broth microdilution panels were prepared in one laboratory according to CLSI guidelines using three different Mueller-Hinton broth preparations (BBL, BD Microbiology, Difco, BD Microbiology, and Oxoid) all supplemented with 3% lysed horse blood (2). The frozen panels were prepared to include erythromycin and clindamycin tested separately to define minimum inhibitory concentrations (MICs) and combinations of erythromycin and clindamycin of 1 + 0.25µg/ml, and 1 + 0.5 µg/ml in separate wells based upon the results of a prior study (1). Each panel included a growth control well and a negative (medium only) control well. Panels were shipped frozen to the four collaborating laboratories for testing. Panels were inoculated with the standard 5 x 10^5 CFU/ml density and incubated for 20-24 hr at 35°C prior to visual determination of MICs.

Disk diffusion D-zone tests. Standard disk diffusion D-zone testing was performed using erythromycin (15 µg) and clindamycin (2 µg) disks placed 12 mm apart on Mueller-Hinton 5% sheep blood agar plates incubated at 35°C in 5% CO₂ for 20 to 24 hours (3). Each laboratory provided its own plates, four labs used BBL brand (BD Microbiology Systems, Sparks, MD) and one lab used Remel (Lenexa, KS). A positive D-zone test was noted by flattening of the clindamycin zone adjacent to the erythromycin disk with erythromycin-resistant isolates.

Control strains. S. pneumoniae ATCC 49619 was used as the control strain on each day of testing and S. aureus ATCC BAA977 was used for quality assessment of the erythromycin + clindamycin combinations (3).
Data analysis. Growth or no growth in the erythromycin-clindamycin combination wells was compared to the disk diffusion D-zone test results. A combination well was considered to have accurately detected inducible clindamycin resistance if growth was present with an isolate that was determined to have a positive D-zone test (1).

RESULTS

A total of 155 macrolide-resistant beta-hemolytic streptococcal isolates were tested in the five laboratories including 50 Group A, 48 Group B, 28 Group C, and 29 Group G streptococci (Table 1). The number of isolates tested varied from 20 to 67 in the five laboratories (Table 1). From 69% (Group C) to 90 to 93% of isolates (other groups) tested as susceptible to clindamycin when tested alone (without clindamycin resistance induction; data not further depicted). There was a slight difference in clindamycin susceptibility among the Group G streptococci based upon one strain testing as resistant in only one of the three Mueller-Hinton broth bases. Among the test isolates, 99 demonstrated inducible clindamycin resistance by the disk D-zone test and nine showed constitutive clindamycin resistance. A total of 47 isolates were resistant to erythromycin but clindamycin susceptible and D-zone-negative (data not further depicted).

In this study, combined broth concentrations of 1 µg/ml erythromycin + 0.25 µg/ml clindamycin detected 91-100% of the inducible clindamycin resistant strains as evidenced by positive agar-based D-zone tests, and 1 µg/ml erythromycin + 0.5 µg/ml of clindamycin detected 95-100% of strains (Table 2). The ranges indicate some differences by the medium used, with lysed horse blood-supplemented Mueller-Hinton broth prepared using BBL Mueller-Hinton base providing the greatest sensitivity, and Difco and Oxoid broth bases providing slightly lower sensitivities for detection of inducible resistance. Only one strain (a Group B Streptococcus) of
the 47 clindamycin susceptible, D-zone-negative isolates grew in any of the drug combination wells (Table 2). Thus, the specificity of the combined drug single well tests was 98% with all media (Table 2). Table 3 details the small number of false-positive and false-negative errors noted in the study according to the four streptococcal groups, the three brands of media, and the two drug combinations tested. The combination of 1 µg/ml erythromycin + 0.5 µg/ml of clindamycin performed slightly better than the lower clindamycin concentration of 0.25 µg/ml, especially in the Difco and Oxoid media.

As noted in a prior single center study (1), the CLSI recommends the *S. aureus* control strain ATCC BAA977 with inducible clindamycin resistance as a quality control/quality assessment strain. In this study, the *S. aureus* control strain performed well, (8 of 8 positive in the combined drug wells) despite the fact that the two drug combination concentrations tested with the streptococci contained a lower erythromycin concentration (1 µg/ml erythromycin) than the combination of 4 µg/ml erythromycin + 0.5 µg/ml of clindamycin recommended for the staphylococci (1,3).

**DISCUSSION**

This five center collaborative study has demonstrated that a single broth microdilution test well incorporating 1 µg/ml erythromycin + 0.5 µg/ml clindamycin accurately detected inducible clindamycin resistance in a collection of Groups A, B, C, and G streptococci weighted toward macrolide-resistant strains. The lower clindamycin concentration in the combination of 1 µg/ml erythromycin + 0.25 µg/ml clindamycin provided a slightly lower rate of sensitivity. The single well broth test was equivalent to performing a separate agar disk approximation test with erythromycin and clindamycin disks, and would provide a more convenient test format that could be incorporated into broth microdilution panels for routine testing of streptococci. There were
some medium differences noted in the study that slightly affected the sensitivity of the single well test.

Some evidence exists, especially with *Staphylococcus aureus*, that there is a risk of spontaneous conversion from inducible to a constitutive resistance phenotype during clindamycin therapy as a result of a single mutation in a promoter region that controls expression of the *erm* genes (7). Thus patients could be at risk of clinical failure if inducible clindamycin resistance as well as constitutive resistance were not detected during routine antimicrobial susceptibility testing of individual patient’s isolates (4,6,7). Clindamycin monotherapy or combinations of penicillin plus clindamycin are sometimes used to treat severe streptococcal soft tissue infections such as necrotizing fasciitis, in which a therapeutic failure due to emergence of resistance could be limb- or life-threatening. Routine detection and reporting of inducible clindamycin resistance in beta-hemolytic streptococcal skin and soft tissue infections or bacteremia would provide an alert to clinicians that clinical failures due to emergence of resistance could occur during therapy. Both constitutive and inducible clindamycin resistance has increased in recent years, especially in Groups A and B streptococci (5,8,11). Indeed, the 2010 guidelines from the CDC on prevention of neonatal Group B streptococcal infections now indicate that testing to detect inducible clindamycin resistance should be performed on all screening isolates from highly penicillin allergic pregnant women (9). Broth microdilution testing with a single erythromycin-clindamycin combination well would provide a convenient option to clinical laboratories to facilitate routine detection of inducible clindamycin resistance.

Group F beta-hemolytic streptococci were not included in this study, and likewise small colony variants of Groups A, C, and G isolates of *S. anginosus* group were not included (12).

The CLSI recommends that all *S. anginosus* group isolates, whether alpha-, beta-, or non-
hemolytic should be considered as members of the viridans group of streptococci (3). The CLSI testing recommendations and certain drug interpretive breakpoints differ based upon isolates belonging to the “beta-hemolytic group” as opposed to the “viridans group” (3).

This multicenter study has confirmed the findings of an earlier single center study (1) that a single well broth screening test successfully detects inducible clindamycin resistance, and is suitable for inclusion in broth panels used for routine testing of beta-hemolytic streptococci without need for additional agar-based D-zone testing. Based upon a review of these data, the CLSI Subcommittee on Antimicrobial Susceptibility Testing has included the recommendation that the 1 µg/ml erythromycin + 0.5 µg/ml clindamycin single well test can be used for detection of inducible clindamycin resistance in the beta-hemolytic streptococci in M100-S21, 2011 (3).

References


Table 1. Numbers of Beta-hemolytic streptococcal isolates tested in each laboratory.

<table>
<thead>
<tr>
<th>Group</th>
<th>UTHSC</th>
<th>CDC</th>
<th>MDH</th>
<th>U Iowa</th>
<th>MGH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>20</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td>Group B</td>
<td>17</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>Group C</td>
<td>19</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>Group G</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>33</td>
<td>18</td>
<td>14</td>
<td>16</td>
<td>155</td>
</tr>
</tbody>
</table>

UTHSC – University of Texas Health Science Center
CDC – Centers for Disease Control and Prevention
MDH – Minnesota Department of Health
U Iowa – University of Iowa
MGH – Massachusetts General Hospital
Table 2. Overall sensitivity and specificity of single well combined erythromycin + clindamycin broth tests determined in three media in five laboratories as compared to agar D-zone tests.

<table>
<thead>
<tr>
<th>Brand of Mueller-Hinton broth base and the two tested drug combinations&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BBL</th>
<th>BBL</th>
<th>Difco</th>
<th>Difco</th>
<th>Oxoid</th>
<th>Oxoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 + 0.25</td>
<td>1 + 0.5</td>
<td>1 + 0.25</td>
<td>1 + 0.5</td>
<td>1 + 0.25</td>
<td>1 + 0.5</td>
</tr>
<tr>
<td>% Sensitivity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100</td>
<td>100</td>
<td>91</td>
<td>95</td>
<td>99</td>
<td>97</td>
</tr>
<tr>
<td>% Specificity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
</tr>
</tbody>
</table>

<sup>a</sup>Erythromycin 1 + 0.25 µg/ml clindamycin and erythromycin 1 + 0.5 µg/ml clindamycin

<sup>b</sup>Sensitivity based upon 99 strains with inducible clindamycin resistance based on D-zone testing; strains with constitutive clindamycin resistance were not included

<sup>c</sup>Specificity based upon 47 strains without inducible clindamycin resistance based upon D-zone testing
Table 3. Single well broth dilution errors as compared to the D-zone test (expressed as number of errors/number of tests)  

<table>
<thead>
<tr>
<th>Brand of Mueller-Hinton broth base and the two tested drug combinations</th>
<th>BBL</th>
<th>BBL</th>
<th>Difco</th>
<th>Difco</th>
<th>Oxoid</th>
<th>Oxoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A Strep</td>
<td>1 + 0.25</td>
<td>1 + 0.5</td>
<td>1 + 0.25</td>
<td>1 + 0.5</td>
<td>1 + 0.25</td>
<td>1 + 0.5</td>
</tr>
<tr>
<td>D-zone positive</td>
<td>0/32</td>
<td>0/32</td>
<td>2/32</td>
<td>1/32</td>
<td>2/32</td>
<td>2/32</td>
</tr>
<tr>
<td>D-zone negative</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
<td>Group B Strep</td>
<td>0/28</td>
<td>0/28</td>
<td>1/28</td>
<td>1/28</td>
<td>0/28</td>
<td>0/28</td>
</tr>
<tr>
<td>D-zone positive</td>
<td>0/17</td>
<td>0/17</td>
<td>1/17</td>
<td>1/17</td>
<td>1/17</td>
<td>1/17</td>
</tr>
<tr>
<td>Group C Strep</td>
<td>0/11</td>
<td>0/11</td>
<td>5/11</td>
<td>2/11</td>
<td>0/11</td>
<td>1/11</td>
</tr>
<tr>
<td>D-zone positive</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
</tr>
<tr>
<td>D-zone positive</td>
<td>0/28</td>
<td>0/28</td>
<td>0/28</td>
<td>0/28</td>
<td>0/28</td>
<td>0/28</td>
</tr>
<tr>
<td>D-zone negative</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

The nine constitutively clindamycin resistant isolates were excluded from the error calculations.

Erythromycin 1 + 0.25 µg/ml clindamycin and erythromycin 1 + 0.5 µg/ml clindamycin