Comparison of disc diffusion & E test methods with agar dilution for antimicrobial susceptibility testing of Haemophilus influenzae

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Background & objectives: Reliable methods of detection of antimicrobial resistance are of paramount importance in the treatment and management of infections caused by Haemophilus influenzae. The objective of the present study was to compare and evaluate the performance of disc diffusion and E test (Epsilometer test) with agar dilution method for antimicrobial susceptibility testing of H. influenzae.

Methods: A total of 46 isolates of H. influenzae from various invasive sites were included as test strains. Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method for ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole (TMP-SMZ) and cefotaxime. Minimum Inhibitory Concentration (MIC) determination was performed by E test and agar dilution for the same set of antimicrobials. All tests were performed on Haemophilus test medium (HTM).

Results: Disc diffusion showed a very major (2%) and minor (4%) interpretative error with TMP-SMZ and minor interpretative errors to ampicillin (13%) and chloramphenicol (24%) when compared to agar dilution method. E test produced only minor interpretative errors to chloramphenicol (7%) and TMP-SMZ (2%) and no interpretative errors with ampicillin and cefotaxime as against agar dilution. E test showed good agreement with agar dilution for each of the antimicrobial tested.

Interpretation & conclusion: Disc diffusion test may be used as a preliminary screen for susceptibility testing of H. influenzae. E test is simple, easy to perform and a reliable method for determination of resistance in H. influenzae. However its cost and limited availability in India may limit its use. The reference agar dilution method can be used reliably in routine susceptibility testing of H. influenzae.

Key words Agar dilution - disc diffusion - E test - Haemophilus influenzae - haemophilus test medium - MIC

The increasing resistance of Haemophilus influenzae to many of the commonly used antimicrobials is a cause of concern1,2. The laboratory testing of in vitro activity of these agents besides being used for direct patient management can provide important epidemiological data to assist in the selection of appropriate antimicrobial for empiric therapy. The importance of standardization of antimicrobial susceptibility tests for its ability to detect resistance among H. influenzae is widely recognized3.

For susceptibility testing of H. influenzae initially a medium containing Muelller-Hinton base with lysed horse blood and nicotinamide adenine dinucleotide (NAD) was recommended4. Since problems were encountered with this medium5, the use of haemophilus test medium (HTM) first
developed by Jorgensen and colleagues was explored. In 1990 the NCCLS published recommendations on the use of HTM for performance of susceptibility test for *H. influenzae*. Since then various studies have shown the efficacy of HTM in susceptibility testing of *H. influenzae*, particularly for screening of resistant isolates.

Many methods for susceptibility testing of *H. influenzae* have been used worldwide. Published quality assessment studies have shown significant discrepancies in findings from different laboratories particularly when testing antibiotic resistant isolates. The interpretative criteria published by NCCLS are applicable only if the NCCLS recommended methods are precisely followed or if procedural modifications have been demonstrated to produce equivalent results. The latter appears to be rarely done or published.

Currently the NCCLS recommends disk diffusion and MIC determination by broth microdilution on HTM for the susceptibility testing of *H. influenzae*. The agar dilution method has also been proved and accepted to be an equally good and optimal technique as compared to broth microdilution and has been recommended as an alternative to broth microdilution. E test is a newer and novel *in vitro* method for quantitative antimicrobial susceptibility testing. Studies have shown that this method shows good agreement with reference dilution susceptibility testing methods.

At our center surveillance studies involving *H. influenzae* have been carried on since 1993 and until recently susceptibility testing of *H. influenzae* has been performed on chocolatized Müller Hinton agar. In India there have been no reports on the validation of disc diffusion and E test against reference agar dilution method using HTM for susceptibility testing of *H. influenzae*. To address this lacuna the present study was carried out with the objective of comparing and evaluating the performance of disc diffusion and E test against agar dilution method as reference for antimicrobial susceptibility testing of *H. influenzae*.

**Material & Methods**

**Test strains**: Forty six isolates of *H. influenzae* obtained from various invasive sites (CSF (n=28), blood (n=9), deep seated pus (n=6), soft tissue abscess (n=2) and ascitic fluid (n=1)) from patients admitted to the Christian Medical College and Hospital, Vellore during 1994 to 2000 were used in the study. These isolates were selected by random number generation from amongst the 119 invasive *H. influenzae* strains isolated during this period using SPSS version 9 (SPSS Inc., USA). The strains were identified and confirmed by standard microbiological procedures.

**Antimicrobial agents**: The following antimicrobials were tested by disc diffusion, agar dilution and E test methods; ampicillin (10µg), chloramphenicol (30µg), trimethoprim – sulphamethoxazole (TMP-SMZ; TMP-1.25/SMZ-23.75µg) and cefotaxime (30µg) (Becton Dickinson, Maryland, USA). Antimicrobials used for performing agar dilution were obtained from Sigma Chemicals, St. Louis, USA. E test strips were procured from AB Biodisk, Solna, Sweden.

**Media**: Haemophilus test medium (HTM) and supplement conforming to NCCLS formulation for HTM were procured from Oxoid, UK.

**Quality control strains**: The control strains were obtained from American Type Culture Collection (ATCC), Virginia, USA. These included *H. influenzae* ATCC 49247, 10211, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922. Control strains apart from *H. influenzae* ATCC 49247 were included to double check the expected NCCLS
recommended values for each antimicrobial tested. *H. influenzae* ATCC 10211 was used to quality check the growth supporting ability of HTM.

**Susceptibility test methods:**

(i) Kirby-Bauer disc diffusion (DD) — Well-isolated colonies of *H. influenzae* from a 16-18 h chocolate agar plate were taken. A suspension of the colonies with 0.85 per cent normal saline was made, opacity adjusted to 0.5 McFarland and used for performance of the procedure as described previously. After the inoculated plates had dried sufficiently the discs were placed on the medium, gently pressed and plates incubated at 35°C in 5 per cent CO\(_2\) atmosphere for 16-18 h. Each zone size was interpreted with reference to the NCCLS standards as susceptible, intermediate and resistant.

Interpretative errors were classified as; very major (a resistant strain misinterpreted as susceptible); major (a susceptible strain misinterpreted as a resistant strain) and minor errors (susceptible or resistant strain misinterpreted as intermediately resistant).

(ii) Agar dilution — For agar dilution the antimicrobials were diluted on log\(_2\) dilution intervals where each plate contained 50 per cent of the concentration of the antimicrobial in the previous dilution. The antimicrobial agents thus diluted were incorporated into the molten agar medium mixed by gentle rotation and poured into petri plates. A control plate without any antimicrobial agent incorporated into the medium was used along with each antimicrobial tested to check for growth of the test and control strains. The range of antimicrobials tested were ampicillin, 0.125-16µg/ml; chloramphenicol, 0.125-16µg/ml; TMP-0.125/SMZ-2.375-TMP-8/SMZ-152 µg/ml and cefotaxime, 0.007-2µg/ml. The inoculum preparation and inoculation procedure was as described previously. Appropriate ATCC quality control organism(s) was included along with each test. Readings were recorded after the plates had been incubated at 35°C for 16 to 18 h in a 5 per cent CO\(_2\) atmosphere.

The results were taken only if the control plate (without the antimicrobial) showed good growth of the quality control as well as test organisms and the MIC value of the quality control strain was in the expected quality control range as recommended by NCCLS. The end point MIC is the lowest concentration of antibiotic that completely inhibits growth. A barely visible haziness or single colony was disregarded. MIC values were recorded as micrograms/ml (µg/ml). Interpretation was made in accordance with NCCLS guidelines.

(iii) E test — The manufacturer’s directions were followed while performing the test. Direct colony suspension from a 16-18 h culture from chocolate agar was used. The colonies were suspended in 0.5ml of 0.85 per cent normal saline and the opacity adjusted to 0.5 McFarland standard. The suspension was inoculated on to HTM in plates of 90 mm diameter and 4 mm depth. E test strips were placed on the plate using sterile forceps and then incubated under suitable conditions as outlined above for agar dilution. On incubation an elliptical zone of inhibition was produced, and the MIC read directly from the graduated E test strip at the point of intersection of the zone of inhibition of growth with the strip. Readings were interpreted as per NCCLS recommendations.

The concentration range of the antimicrobial on the E test strip corresponds to two-fold dilutions in a conventional MIC method. In other words the antimicrobial dilution follows the log\(_2\) dilution intervals as in the conventional MIC method of agar dilution and the zone of
inhibition is proportional to the log of the concentration of the antimicrobial. Hence when E test results were compared with the agar dilution results the agreement was calculated comparing log₂ concentration of E test value against reference agar dilution method\textsuperscript{16,17}.

**Statistical analysis:** The geometric mean and their confidence intervals were computed to determine the titre levels of the three different test methods used in the study. The different test titres were classified into three interpretative subgroups of sensitive, intermediate and resistance and tested for agreement using chance corrected weighted kappa with squared weight.

**Results**

The geometric mean zone sizes for ampicillin, TMP-SMZ and chloramphenicol were found towards their resistance zone size values (Table I). E test was found to show lower geometric mean value for ampicillin, while for chloramphenicol and cefotaxime, the E test values were marginally higher than the agar dilution. For TMP-SMZ the geometric mean MIC values by E test were much higher than agar dilution values (Table I).

**Table I.** Geometric mean values obtained by agar dilution, E test and mean zone diameter by disc diffusion amongst *H. influenzae* (n=46)

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Agar dilution (µg/ml)</th>
<th>E test (µl/ml)</th>
<th>Zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>1.64 (0.98-2.74)</td>
<td>1.25 (0.91-3.19)</td>
<td>17.66 (15.72-19.83)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2.03 (1.29-3.18)</td>
<td>2.43 (1.40-4.23)</td>
<td>21.51 (19.46-23.80)</td>
</tr>
<tr>
<td>Trimethoprim-Sulphamethoxazole</td>
<td>2.25 (1.34-3.79)</td>
<td>3.69 (2.05-6.49)</td>
<td>10.75 (8.92-12.95)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.016 (0.01-0.02)</td>
<td>0.017 (0.01-0.02)</td>
<td>30.02 (29.27-30.79)</td>
</tr>
</tbody>
</table>

Values are mean (95% CI)

**Table II.** Comparison of disc diffusion test results with MIC determined by agar dilution method

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Disc diffusion method no. of isolates (%)</th>
<th>MIC by agar dilution no. of isolates (%)</th>
<th>Overall agreement*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>19(41)</td>
<td>7(15)</td>
<td>20(44)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>17(37)</td>
<td>4(9)</td>
<td>25(54)</td>
</tr>
<tr>
<td>Trimethoprim-Sulphamethoxazole</td>
<td>17(37)</td>
<td>4(9)</td>
<td>25(54)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>46(100)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*overall agreement calculated by the weighted kappa method
S, susceptible; I, intermediately resistant; R, resistant

By disc diffusion the overall agreement of results was the highest for cefotaxime (100%) and the least for chloramphenicol (86%) when compared to agar dilution (Table II). A very major
interpretative error occurred with TMP-SMZ (2%) by disc diffusion. Minor interpretative errors were found to occur with respect to ampicillin (13%), chloramphenicol (24%) and TMP-SMZ (4%) by disc diffusion method when compared with agar dilution. Five strains resistant by agar dilution to ampicillin were categorized as intermediately resistant by disc diffusion; these strains were β-lactamase positive and hence were considered as truly resistant to ampicillin. The highest minor interpretative error rates were observed with respect to chloramphenicol where the disc diffusion test categorized four strains as intermediately resistant while by agar dilution they were completely resistant. Further seven strains intermediately resistant by agar dilution were either categorized as susceptible or resistant by disc diffusion.

Table III. Comparison of E test results with MIC determined by agar dilution method

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>E test method no. of isolates (%)</th>
<th>MIC by agar dilution no. of isolates (%)</th>
<th>Overall agreement*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S(4) I(2) R(25)</td>
<td>S(20) I(1) R(25)</td>
<td>100%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>20(44) 1(2) 25(54)</td>
<td>20(44) 1(2) 25(54)</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>22(48) 5(11) 19(41)</td>
<td>19(41) 7(15) 20(44)</td>
<td>92%</td>
</tr>
<tr>
<td>Trimethoprim-Sulphamethoxazole</td>
<td>15(33) 2(4) 29(63)</td>
<td>15(33) 3(7) 28(60)</td>
<td>95%</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>46(100) - -</td>
<td>46(100) - -</td>
<td>100%</td>
</tr>
</tbody>
</table>

*overall agreement calculated by the weighted kappa method
S, susceptible; I, intermediately resistant; R, resistant

Table IV. Comparison of log₁₀ concentration agreement of E test with agar dilution method

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>No. of E test MICs within indicated conc (log₁₀) of HTM agar dilution MICs</th>
<th>% *agreement within 1 log₁₀ conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;-2 -2 -1 0 +1 +2 &gt;+2</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>- 3 15 24 3 1 -</td>
<td>91</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>- 2 17 17 9 1 -</td>
<td>87</td>
</tr>
<tr>
<td>Trimethoprim-Sulphamethoxazole</td>
<td>- - 3 17 14 11 1</td>
<td>74</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>- 1 12 23 5 5 -</td>
<td>87</td>
</tr>
<tr>
<td>Overall agreement</td>
<td></td>
<td>84.7</td>
</tr>
</tbody>
</table>

*calculated taking 1 log₁₀ concentration of E test dilution above (+1) and below (-1) zero to reference agar dilution method
HTM, haemophilus test medium; MIC, minimum inhibitory concentration

With the E test method the overall agreement of results was the highest for cefotaxime (100%) and ampicillin (100%) and the least for chloramphenicol (92%) when compared with agar dilution method (Table III). Comparison of 1 log₁₀ concentration agreement of E test with agar dilution values (Table IV) showed that the highest agreement was for ampicillin (91%) and the least for TMP-SMZ (74%), the overall agreement was found to be 84.7 per cent between both these methods for all the antimicrobials tested. Minor interpretative errors with E test were observed with chloramphenicol (7%) and TMP-SMZ (2%). Ampicillin E test interpretations
correlated well with agar dilution with one intermediate strain being correctly categorized by E

test.

**Discussion**

A contemporary concern with *H. influenzae* infections is the emergence of antimicrobial

resistance\(^1,2,9\). Standardization of methods for detection of antimicrobial resistance has been

hampered due to the fastidious nature of the organism. Also variations in methods, media and use

of different interpretative criteria have resulted in significant discrepancies in test results\(^8,11,12\). In

the present study we evaluated disc diffusion and E test method and compared their performance

with reference agar dilution method.

Disc diffusion using HTM is one of the NCCLS recommended method for susceptibility
testing of *H. influenzae* isolates\(^13,14\). Previous studies\(^8,15,21\) have shown that problems with disc
diffusion arise when predicting ampicillin resistance in *H. influenzae* however, in the present

study disc diffusion produced only minor interpretative errors with ampicillin and

chloramphenicol. Disc diffusion also tends to wrongly classify \(\beta\)-lactamase negative ampicillin

susceptible (BLNAS) strains as resistant\(^15\), no such misclassification occurred in the present study

since no BLNAR strains were encountered. Studies\(^8,15\) have shown that the best method to test

ampicillin resistance is the broth microdilution or the agar dilution method using HTM.

The geometric means of the zone sizes obtained by disc diffusion for all the antimicrobials

were reflective of the susceptibility pattern of the study isolates. More than 50 per cent of the

strains tested were resistant to ampicillin, chloramphenicol and TMP-SMZ resulting in resistant

mean zone sizes to these three antimicrobials.

E test has been shown to be a good alternative to the agar and broth dilution tests for

susceptibility testing of *H. influenzae*\(^16,17\). In this study E test MICs compared favorably with agar
dilution for all the antimicrobials tested especially for ampicillin. Similar results were obtained in

a study by Giger *et al*\(^17\). The least agreement was found for TMP-SMZ (74%), similar findings

were reported by Jorgensen *et al*\(^16\). The higher geometric mean values of MICs for

TMP-SMZ with E test as compared to agar dilution may be due to the E test strips having an inequitable

concentration increment for TMP-SMZ as compared to the agar dilution\(^16\). This may have

contributed to the lower agreement between E test and agar dilution values. Studies\(^8,17\) have

shown that E test tends to give lower values than the agar or broth dilution method and tends to

falsely categorize intermediately resistant strains as susceptible and resistant strains as

intermediately resistant thus producing minor interpretative errors. Although such errors

occurred in this study, no very major or major interpretative errors were observed with E test as

compared to agar dilution. Further MIC determination by E test correlated well with existing

NCCLS interpretative criteria thereby proving to be a good alternative to reference agar dilution

method. However drawbacks of E test such as cost and availability should be taken into

consideration\(^20\).

In this study agar dilution was used as the reference method since it has been shown to be

equally good when compared to microbroth dilution in previous studies\(^8,10\). The difficulties in

obtaining commercial microbroth dilution trays, the logistical complexities of preparing the

media in-house and the cost factor prompted us to adopt agar dilution as the reference method.

In summary disc diffusion is useful as a preliminary screen, however intermediately resistant

and resistant isolates by disc diffusion must be confirmed by an MIC method. E test is simple,
easy to perform and a reliable method for determination of resistance in *H. influenzae*. However its cost and limited availability in India may limit its use. The reference agar dilution method can be used reliably in routine laboratory susceptibility testing of *H. influenzae*.

References


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