



Comparison of susceptibility test methods to detect penicillin susceptibility in *Streptococcus pneumoniae* isolates

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Abstract

The increasing prevalence of penicillin-resistant *Streptococcus pneumoniae* urges for fast and accurate susceptibility testing methods. This study evaluated the comparability of three commonly used techniques; disk diffusion, E-test and agar dilution, to detect penicillin susceptibility in clinical isolates of *S. pneumoniae*. Fifty pneumococcal isolates, obtained from patients at the University of Malaya Medical Centre, were selected to include both penicillin-susceptible strains and those that had decreased susceptibility (resistant and intermediate) to penicillin. The minimum inhibitory concentration (MIC) values of penicillin to serve as the reference was determined by the agar dilution method in which, based on the MIC breakpoints recommended by the National Committee for Clinical Laboratory Standards (NCCLS), 27 strains had decreased susceptibility to penicillin with 17 strains resistant and 10 intermediate. Comparing to the agar dilution method, oxacillin disk diffusion test detected all strains with decreased penicillin susceptibility as such while E-test showed a close agreement of susceptibility (92%) of the isolates to penicillin. This confirmed that oxacillin is a good screening test for *S. pneumoniae* isolates with decreased susceptibility to penicillin while E-test is very reliable for rapid and accurate detection of penicillin susceptibility.

Key words: Agar dilution, E-test, MIC, Oxacillin disk diffusion, Penicillin, *Streptococcus pneumoniae*

INTRODUCTION

Bacterial infections caused by *Streptococcus pneumoniae* strains with decreased susceptibility to penicillin (DSP) are increasing worldwide. DSP tends to be multidrug-resistant resulting in failure of antibiotic treatments. In response to this alarming situation, clinical microbiology laboratories are now faced with the challenge to reliably detect penicillin resistance. Accurate information on susceptibility is often important for the sake of patient care and monitoring epidemiological trend. Among the commonly used techniques are disk diffusion, E-test and agar dilution. Disk diffusion is a cheap and easy method to perform and is being widely applied for antimicrobial susceptibility testing. However, this method offers only a qualitative result and thus, the predictive value of resistant isolates by this method was reported to be low.¹ In the case of *S. pneumoniae*, oxacillin disk diffusion has been used to separate strains with decreased susceptibility to penicillin from those that are penicillin-susceptible. The prediction of penicillin susceptibility is based on the susceptibility zone

diameter breakpoint for oxacillin disk as recommended by the National Committee for Clinical Laboratory Standards (NCCLS), in which penicillin-susceptible *S. pneumoniae* isolates tend to produce an oxacillin zone diameter of ≥ 20 mm (oxacillin-susceptible) whereas those with decreased susceptibility to penicillin produce a zone diameter of ≤ 19 mm (oxacillin-resistant).² The agar dilution method may then be applied in order to determine the Minimum Inhibitory Concentration (MIC) values, which are regarded as the reference criterion for defining the susceptibility of a microorganism. However, agar dilution is time consuming and labor intensive and is not effective enough during an outbreak where large numbers of strains have to be rapidly tested.³ A commercial method, E-test, is now available, which is based on the principle of both the disk diffusion and agar dilution. It is applied in exactly the same manner as the disk diffusion method with the advantage of determining the MIC values at the same time. Due to this dual function, it has been used worldwide.

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Due to the increasing emphasis on the importance of accuracy in determining the local rates of pneumococcal resistance to penicillin, we undertook this study to assess the E-test method, as well as the predictability power of the oxacillin disk method on the local clinical isolates of *S. pneumoniae*. The results will be compared to the agar dilution method, which serves as the reference in this study.

MATERIALS AND METHODS

Pneumococcal isolates: A total of 50 isolates of *S. pneumoniae* were used for this study. These isolates were obtained from various body sites such as blood, cerebrospinal fluid and respiratory tract from patients of various ages at the University of Malaya Medical Centre (UMMC). All isolates were identified as *S. pneumoniae* by conventional method including Gram stain, colonial morphology, alpha-haemolysis, inhibition by optochin susceptibility and bile solubility.⁴ The strains studied included both penicillin-susceptible strains and those with decreased susceptibility to penicillin (resistant and intermediate) using the oxacillin disk to screen for penicillin susceptibilities. Strains were stored at -70° and subcultured onto blood agar plates prior to testing. The pneumococcal cells were harvested in saline and the cell suspensions were adjusted to a concentration equivalent to the 0.5 McFarlands standard using a spectrophotometer.

Oxacillin disk diffusion and E-test: Pneumococcal cell suspensions were inoculated onto Mueller-Hinton agar plates supplemented with 5% sheep blood, which were then allowed to air dry for 15 minutes. Oxacillin disks (1 μ g) (Oxoid, UK) and E-test penicillin strips (AB Biodisk, Sweden) with gradient concentrations ranging from 0.016 to 256 μ g/ml were placed onto the inoculated plates and incubated overnight at 37°C in CO_2 . Interpretation of the susceptibility to penicillin was based on the guidelines recommended by NCCLS.⁵ Pneumococcal isolates with oxacillin zone diameter of ≥ 20 mm were reported as susceptible, whereas those with oxacillin zone diameter of ≤ 19 mm were considered as having decreased susceptibility. E-test MICs were read at the point of intersection between the inhibition ellipse edge and the test strip. For comparative purposes, MICs by the E-test that fell between two marks on the E-test strip were rounded up to the next higher twofold dilution.⁶ Strains were

categorized as penicillin-susceptible if the MIC was ≤ 0.06 μ g/ml, intermediate if the MIC was between 0.12 and 1 μ g/ml and resistant if the MIC was ≥ 2 μ g/ml.

Agar dilution: Plates of Mueller Hinton agar containing 5% sheep blood and various concentrations of penicillin were prepared as described by the NCCLS.⁵ The concentrations of penicillin tested were doubling dilutions from 64 to 0.008 μ g/ml. The test pneumococcal suspensions were inoculated onto the agar plates using the multipoint inoculator (AM80 Automatic Inoculator, Denley-Tech. Limited, Switzerland) with 3 mm pins delivering inocula of 1 μ l [10^4 -colony-forming units (CFU)].⁵ Following an overnight incubation at 37°C in CO_2 , the MIC was read as the lowest penicillin concentration that inhibited growth of the test isolate. Categorization of penicillin susceptibility was similar that of the E-test.

Quality control: *S. pneumoniae* ATCC 49619 was included as a control in each test run for all methods as recommended by the NCCLS.⁵ Results were accepted only if the quality control results were within the NCCLS-specified ranges.

Analysis: Results for penicillin susceptibility as determined by the agar dilution method were used as the reference for the other methods. Results produced by the E-test were considered in agreement with the reference when they happened to be in the same category of susceptibility. Discrepancies of errors were characterized by 'very major', 'major' and 'minor'. In errors classified as 'very major', the result is resistant by the reference method but susceptible by the other methods. 'Major' means the result is susceptible by the reference method and resistant by the others, while 'minor' refers to one-category difference between the methods (e.g. intermediate by the reference but susceptible or resistant by the others or vice versa).²

RESULTS

Results generated by the disk diffusion, E-test and agar dilution methods are summarized in Table 1. Overall, 27 isolates were detected by agar dilution to have decreased susceptibility to penicillin with 17 strains resistant and 10 intermediate. The E-test detected 26 strains having decreased susceptibility to penicillin out of which 18 strains were resistant and 8 were intermediate. All strains detected as having



PENICILLIN SUSCEPTIBILITY TEST METHODS

decreased susceptibility to penicillin by the E-test and agar dilution were identified as such by the oxacillin disk test. Of note were 2 strains identified as having decreased susceptibility to penicillin by the oxacillin screening test as shown by their inhibition zone diameter of ≤ 19 mm, but found to be susceptible by both E-test and agar dilution methods.

Table 2 showed the number and percentage of susceptibility agreement as well as the error of discrepancies for the results by the E-test methods as compared to that of agar dilution as the reference. Overall, results from the E-test showed a close agreement to those obtained from agar dilution with 46 strains (92%) out of the 50 strains had the same category of susceptibility while the other 4 strains had only minor errors. Two of the strains with the minor errors were reported as intermediate by agar dilution but resistant by E-test. The other two

strains were reported as resistant and intermediate by the agar dilution but intermediate and susceptible by the E-test respectively. Oxacillin disk test does not differentiate between intermediate and resistance and thus the percentage of agreement and discrepancy were not determined. However, in terms of identifying the strains with decreased susceptibility to penicillin, oxacillin disk diffusion has detected all such isolates as detected by agar dilution method.

For the strains that had definitive MICs (e.g. 0.016 $\mu\text{g/ml}$ as compared to < 0.016 $\mu\text{g/ml}$), the agreement of MIC values by the E-test to the indicated \log_2 dilution of those obtained by agar dilution, was also examined as shown in Table 3. Forty strains were analyzed and 29 of them showed identical E-test MIC values when compared to those obtained by the agar dilution method. MIC values onefold and twofold dilution

Table 1: Penicillin susceptibility detected by oxacillin disk diffusion, E-test and agar dilution methods

Methods	No. of strains detected as DSP ^b	Category of susceptibility to penicillin		
		Susceptible	Intermediate	Resistant
Disk diffusion ^a	29	21	-	-
E-test	26	24	8	18
Agar dilution	27	23	10	17

^aOxacillin disk test is unable to distinguish between strains that are resistant or intermediate.

^bDSP = Decreased susceptibility to penicillin.

Table 2: Agreement and discrepancies in determining susceptibility by E-test method compared to that by agar dilution method

Methods	No. (%) of Agreement	No. (%) of discrepancies		
		Minor	Major	Very major
E-test	46(92)	4(8)	-	-

Table 3: Distribution of isolates with E-test determined MIC values falling within the indicated \log_2 dilution of penicillin MIC values by agar dilution

No. of Strains	Log ₂ dilution of penicillin concentration				
	-2	-1	Same	+1	+2
40 ^a	1	2	29	7	1

^a Analysis was based on 40 isolates that had definitive MIC values.

higher were seen in 7 strains and 1 strain respectively while 2 strains had MIC values onefold lower and 1 strain had a value that was twofold lower. However, despite the MIC differences, many of these strains were still classified within the same category of susceptibility. There were only 4 strains having MIC values that differed by only onefold dilution but clustered at the breakpoint separating two different categories (e.g. 1 µg/ml (intermediate) to 2 µg/ml (resistant) or 0.064 µg/ml (susceptible) to 0.12 µg/ml (intermediate) resulting in minor errors.

DISCUSSION

The accurate detection of penicillin-resistant *S. pneumoniae* is of paramount importance in the clinical microbiology laboratory. In this study, we compared the techniques of oxacillin disk test, E-test and agar dilution.

Various reports have indicated that the oxacillin disk susceptibility test is very useful for predicting penicillin susceptibility.^{7,8} In this study, this test had accurately detected all the strains with decreased susceptibility to penicillin. Although it does not differentiate strains that are penicillin-resistant and -intermediate, strains with decreased susceptibility to penicillin would not go undetected because susceptible strains of *S. pneumoniae* reliably produced zone diameter of ≥ 20 mm.^{2,9} There were two strains, which were determined to be susceptible by the agar dilution test but exhibited an inhibition zone diameter of ≤ 19 mm by the oxacillin-screening test. Such cases have been observed in other studies, usually among strains with penicillin MIC at the upper limit of the susceptible category (0.06 µg/ml).^{7,10,11} In this study the MIC values for both of these strains were 0.032 µg/ml by agar dilution, which was one dilution less than the upper limit of the susceptible category. This confirms oxacillin disk to select well strains that need to be given attention for confirmation of susceptibility by an MIC method.

In comparing the E-test with that of agar dilution, a good agreement was observed in this study. No very major or major interpretive category errors were encountered while the minor errors were observed only with four strains. The differences of the MIC values for each of the strain were also mostly within onefold dilution. Those that had minor errors had MIC values which was also within onefold dilution that bordered two susceptibility categories leading to minor errors. This is consistent with many

other studies in which the E-test was observed to show a very good agreement in identifying accurately the susceptibility categories of *S. pneumoniae* to penicillin.¹² It has been reported that the accuracy of determination of penicillin MIC by the E-test to be within one doubling dilution to the reference method was 90% while that within two doubling dilution was 99%.¹³ In the E-test method, MIC values that fall within any two doubling dilution, were rounded up to the next higher dilution so that the values could be compared with that of the reference method. Thus, no values that were within the doubling dilutions were reported although it is possible to do so with the E-test since it has a continuous antibiotic concentration gradient along the strip. This suggests that the E-test could be more precise in reporting the MIC values and could give a lower MIC as compared to agar dilution. Thus, although the E-test was only a commercial method, not a 'gold standard' for determining the MIC, its accuracy provided an alternative for a simple method that was easy to interpret and reliable in determining susceptibility to penicillin.¹⁴⁻¹⁶

Due to the simplicity and accuracy of the oxacillin and E-test, it is recommended that both methods are good options to detect penicillin resistance in *S. pneumoniae* isolates. The former can be used as a screening test to separate strains with decreased susceptibility to penicillin from the susceptible ones. MICs may then be determined by the E-test or agar dilution. In case of a situation where the MICs need to be determined quickly, such as in an outbreak, or when the reference method is not readily available, the E-test may be then applied since it is easy to perform in less time and at the time giving an accurate and reliable results.

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PENICILLIN SUSCEPTIBILITY TEST METHODS

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