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Comparison of Microdilution and Standard Agar Dilution Method for Determining Penicillin Resistance among *S.pneumoniae*

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ABSTRACT

Background: Antibiotic resistance among different microbial organisms is increasing. No study exists from Pakistan comparing methods of determining antibiotic resistance among bacterial isolates.

Methods: Ten clinical patient sample strains of *S.pneumoniae* were used to compare agar dilution with broth microdilution method for determining Minimum Inhibitory Concentrations (MICs) of Penicillin G. Agar dilution was conducted as per National Committee for Clinical Laboratory Standards. Broth microdilution was performed in Brain Heart Infusion broth using sterile plastic microtiter trays with 96 U-shaped wells. In both methods, concentration of Penicillin G ranged from 0.03 to 16µg/mL and *S.aureus* ATCC 29213 with an MIC of 0.25µg/mL was used as a control. MIC ≤0.06µg/mL was considered sensitivity to antibiotic, MIC 0.12-1.0 intermediate resistance and MIC ≥2 full resistance.

Results: Good agreement was found between two methods. Four strains gave identical MICs in both. The other six strains agreed to within one log2 dilution step. Both methods categorized same five strains as Penicillin sensitive and four as Penicillin resistant; one strain was classified as sensitive on agar dilution but resistant on broth microdilution. Broth microdilution was more expensive, tedious and time consuming than the agar dilution with multipoint inoculator. This would limit its clinical applicability in a busy diagnostic lab with a large sample turnover.

Conclusions: We propose agar dilution for the clinical labs in developing countries that use Kirby-Bauer disk diffusion method and want to initiate MIC determination of antibiotics for patient samples.

Keywords: Minimum Inhibitory Concentration; Pakistan; penicillin; resistances; *Streptococcus pneumoniae*.

INTRODUCTION

Streptococcus pneumoniae, a frequent commensal of upper-respiratory-tract, is an important cause of pneumonia, meningitis, bacteremia, sinusitis/otitis-media[1] and morbidity/mortality at all ages globally.[2] In mid-1960s, first isolates of penicillin-resistant *S.pneumoniae* were discovered in Papua-New-Guinea,[1] Australia[3,4] and US.[4,5] Since then penicillin-resistant-strains were reported in many areas of world, including Pakistan.[6] Penicillin-resistance among *S.pneumoniae* is on rise over last two decades.[1,7] Intercontinental spread of clones expressing altered penicillin-binding-proteins contributed to this trend.[8] Penicillin-resistance among pneumococcal-isolates at our hospital during year-2002 was 3.2%.[9]

The most common method for antimicrobial-susceptibility-testing is Kirby-Bauer-disk-diffusion, which uses 1µg/ml Oxacillin-disk. Kirby-Bauer-disk-test is not an accurate predictor of penicillin-resistance (false positive in 42% of cases);[10] also does not distinguish between isolates with high - (Minimum-Inhibitory-Concentration (MIC) ≥2µg/ml) and intermediate-

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(0.12≤MIC<2µg/ml) - resistance.[11,12] We evaluated broth-microdilution and standard-agar-dilution for determining Penicillin-G MICs against *S.pneumoniae*. Our findings will assist laboratories in Pakistan choose a cost-effective, reliable method for MIC-determination of antibiotics against microbes.

METHODS

The Aga Khan University Hospital (AKUH) is a tertiary care referral center in Karachi, Pakistan. The study included ten clinical patient sample strains of *S.pneumoniae*. The isolates were stored at -70°C in 10% glycerol broth. After recovery from frozen aliquots, the strains were sub-cultured on 5% sheep blood agar and chocolate agar plates. The plates were then incubated for forty eight hours at 37°C with 3 – 5% CO_2. All isolates were confirmed as pneumococci by their α-hemolytic colonies on blood agar, by gram staining, bile solubility and susceptibility to ethylhydrocupreine hydrochloride (optochin). MICs of Penicillin G were determined using both the broth microdilution and agar dilution techniques.

Agar dilution method. MICs were determined by the agar dilution method as recommended by the National Committee for Clinical Laboratory Standards,[13] using Mueller Hinton agar supplemented with 5% defibrinated sheep blood. The concentration of Penicillin G used ranged from 0.03 to 16µg/ml in doubling (two fold) dilutions. Two Penicillin – free plates were prepared to serve as growth controls. The plates were refrigerated and used within 36 hours of preparation. The inoculum density was standardized to a turbidity equivalent to that of 0.5 McFarland, by making a direct saline suspension of colonies selected from a twenty four hour sheep blood agar plate. This resulted in a suspension containing approximately 10^8 CFU/ml. The 0.5 McFarland suspensions were further diluted 1:10 in sterile Brain Heart Infusion broth to obtain the inoculum concentration of 10^7 CFU/ml. The plates were then inoculated with the Multipoint inoculator, which deposited 1µL of the adjusted suspensions on the agar surface, thus achieving the ultimate desired inoculum size of 10^4 CFU per spot. A growth plate with no Penicillin G was inoculated first and then, starting with the lowest concentration, the plates containing different Penicillin concentrations were inoculated. A second growth control plate was inoculated last to ensure that there was no contamination or significant antimicrobial carryover during the inoculation. The plates were allowed to stand at room temperature for the spots to dry and then incubated at 35 – 37°C for 24 hours in 5% CO_2. The MIC was recorded as the lowest concentration of Penicillin G that completely inhibited growth, disregarding a single colony or a faint haze caused by the inoculum. *Staphylococcus aureus* ATCC 29213, with an MIC of 0.25µg/ml and a penicillin sensitive strain of *S.pneumoniae* were included in each plate as quality control organisms.

Broth microdilution. The MICs were performed in sterile plastic microtiter trays with 96 U- shaped wells. The organism suspensions were prepared in saline from overnight growth on blood agar plates to a turbidity of 0.5 McFarland standard. A dilution of 1:100 was performed in Brain Heart Infusion broth, supplemented with 5% lysed sheep blood to obtain a concentration of 10^6 CFU/ml.100µL of the diluted organisms were added to 100µL of Penicillin G, thus having a total volume of 200µL in each well. Penicillin was tested at doubling dilutions and its final concentration was from 0.03 to 16µg/ml. The final inoculum density was $5x10^5$CFU/ml. The strains were tested in triplicate and in each line, a positive and a negative control were included. The positive controls were broth plus organisms with no antibiotic, and the negative control wells had broth only. *Staphylococcus aureus* ATCC 29213 with an MIC of 0.25µg/ml was used as a control in all the series of tests. The trays were incubated overnight at 35 - 37°C in the CO_2 incubator. The MIC was defined as the lowest concentration of Penicillin G that inhibited growth of the organisms, as detected by lack of visual turbidity, matching the negative growth control.

RESULTS

The Minimum Inhibitory Concentrations obtained are shown in Table 1. Good agreement was found between broth microdilution and agar dilution methods. Four strains gave identical MICs with the two methods. The other six strains agreed to within one $\log_2$ dilution step. Both the methods categorized same five strains as penicillin sensitive and four as penicillin resistant; only one strain was classified differentially, as sensitive on agar dilution but resistant on broth microdilution method. The two techniques were further compared in terms of time and cost incurred. The time spent per isolate in agar dilution was half of that spent in broth microdilution. Similarly, financial cost of broth microdilution in our laboratory was fifteen times more than that of agar dilution using multipoint inoculator. This cost differential was marked because the multipoint inoculator technique in agar dilution allows for simultaneous testing of eighteen isolates (in addition to controls).

Table 1. Minimum Inhibitory Concentrations (MICs) of Penicillin G against *S.pneumoniae* using the methods of agar dilution and broth microdilution.

Strain #	Degree of penicillin resistance	Agar dilution method MIC in µg/mL	Broth microdilution MIC in µg/mL
1	Sensitive	0.03	0.03
2	Sensitive/ Intermediate	0.06	0.125
3	Intermediate	0.50	0.50
4	Intermediate	1.00	0.50
5	Intermediate	0.50	0.25
6	Intermediate	0.50	0.25
7	Sensitive	0.015	0.03
8	Sensitive	0.015	0.015
9	Sensitive	0.015	0.03
10	Sensitive	0.03	0.03

DISCUSSION

Resistance to penicillin among strains of *S.pneumoniae* is now a global phenomenon. The rate of resistance in our hospital during the year 2002 was 3.2%.[9] Higher figures of 9%[6] and 19.8%[14] have been reported in other studies of Pakistan. In a study from Bangladesh, 42 of 362 (11.6%) strains were intermediate and 4(1.1%) were completely penicillin resistant.[15] Rates in studies from Sri Lanka,[16] Saudi Arabia[17] and Korea[18] were 41.2%, 48.8% and 70% respectively. There is variation in the prevalence of penicillin resistance in different geographical regions and medical centers of Asia. The presence of penicillin resistance among our isolates has necessitated establishing a method for MIC determination which is cost effective and also reliable.

At the moment, in laboratories of most developing countries, determination of MIC by broth microdilution for every strain of *S.pneumoniae* is not economically feasible. The broth microdilution method is tedious, cumbersome and time consuming. These factors, therefore, limit its use for large-scale antimicrobial susceptibility testing. The challenge facing such laboratories is to reliably determine bacterial resistance to antimicrobial agents at minimum possible cost, with a technically robust assay adaptable for a busy diagnostic lab. Using a multipoint inoculator, the cost incurred per strain by agar dilution method was fifteen times less than that for the broth microdilution method. This method, moreover, allows eighteen strains to be tested at a time. Furthermore, microbial contamination is more easily detected in the agar dilution method. Each agar plate can be subjected to strict quality control, using two or more test/control organisms with known reactivity. In situations where few strains need to be tested, small Petri plates or a quadrant plate may be used.

CONCLUSIONS

The determination of MICs is important to accurately know the degree of antimicrobial resistance. The broth microdilution technique is expensive, cumbersome and requires trained manpower. Given good agreement between the two methods, we therefore propose agar dilution method for all the clinical laboratories in developing countries that still use Kirby-Bauer disk diffusion method and want to initiate the process of MIC determination of antibiotics.

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