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DETERMINATION OF THE EFFECT OF GENTAMICIN AGAINST *STAPHYLOCOCCUS AUREUS* BY USING MICROBROTH KINETIC SYSTEM*

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SUMMARY

In this study a microbroth kinetic system based on continuous monitoring of changes in the optical density of bacterial growth was used for determination of antimicrobial activity. By using this system we aimed to describe turbidimetric growth curves of Staphylococcus aureus in the presence of increasing concentrations of a model antibiotic (gentamicin) and to determine minimum inhibitory concentration (MIC). Additionally, we compared these results with conventional broth microdilution method. S.aureus (ATCC 25923) strain grown in Mueller Hinton broth and gentamicin (0.0625-64 µg/ml) were dispensed in microtiter plates for this kinetic assay. Optical densities for each well were measured for 24 hours by 15 min interval at 37°C via multi-detection microplate reader at 600 nm and were automatically recorded. Turbidimetric growth curves of S.aureus were obtained and MIC of gentamicin was determined by microbroth kinetic system. Accordingly, MIC_{50} and MIC_{90} values of gentamicin on S.aureus were determined as 0.235 and 0.488 µg/ml respectively at the first half of the incubation period. Gentamicin MIC value was also determined as 0.5 µg/ml by using conventional broth microdilution method. The results in microbroth kinetic system were similar to the conventional broth microdilution method and in vitro antimicrobial susceptibility was determined in an earlier stage. In conclusion, the microbroth kinetic system is rapid and objective method for assessing the antibacterial activity.

Keywords: *growth curves, minimum inhibitory concentration, optical density, Staphylococcus aureus*

ÖZET

Gentamisinin *Staphylococcus aureus*'a karşı Etkisinin Belirlenmesinde Mikrobuyyon Kinetik Sistemin Kullanılması

Bu çalışmada, antimikrobiyal aktivitenin belirlenmesi amacıyla bakteriyel üremenin optik dansitesindeki değişmeleri sürekli izleme temeline dayanan mikrobuyyon kinetik sistem kullanılmıştır. Bu sistemi kullanarak Staphylococcus aureus'un bir model antibiyotiğin (gentamisin) artan konsantrasyonları varlığında türbidimetrik üreme eğrilerinin oluşturulması ve minimum inhibitör konsantrasyonunun (MİK) belirlenmesi amaçlanmıştır. Ayrıca sonuçlar konvansiyonel sıvı mikrodilüsyon yöntemi ile de karşılaştırılmıştır. Bu kinetik yöntemde Mueller Hinton sıvı besiyerinde üretilen S.aureus (ATCC 25923) süspansiyonu ve gentamisin (0.0625-64 µg/ml) mikroplaklara dağıtılmıştır. Her bir kuyucuğun optik dansitesi 600 nm'de, "multi-detection" mikroplak okuyucu yardımıyla 37°C'de 24 saat süreyle ve 15 dakika aralıklarla otomatik olarak ölçülmüş ve kaydedilmiştir. S.aureus'un türbidimetrik üreme eğrileri ve gentamisin MİK değeri mikrobuyyon kinetik sistemi ile belirlenmiştir. Buna göre gentamisin $MİK_{50}$ ve $MİK_{90}$ değerleri inkübasyon periyodunun ilk yarısında sırasıyla 0.235 ve 0.488 µg/ml olarak hesaplanmıştır. Konvansiyonel sıvı mikrodilüsyon yöntemi ile gentamisin MİK değeri 0.5 µg/ml olarak belirlenmiştir. Mikrobuyyon kinetik sistem ile sıvı mikrodilüsyon yönteminin sonuçları benzer bulunmuş ve kinetik sistem ile in vitro antimikrobiyal duyarlılık daha erken bir aşamada belirlenmiştir. Sonuç olarak, mikrobuyyon kinetik sistemin antibakteriyel aktivitenin değerlendirilmesinde hızlı ve objektif sonuç veren bir yöntem olduğu anlaşılmıştır.

Anahtar sözcükler: *minimum inhibitör konsantrasyon, optik dansite, Staphylococcus aureus, üreme eğrisi*

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INTRODUCTION

Nowadays, a variety of methods are applied to evaluate the effects of various antimicrobial agents on microorganisms. The quantitative tests which Clinical and Laboratory Standards Institute (CLSI) suggests, as broth microdilution and agar dilution where the minimum inhibitory concentration (MIC, the lowest concentration of drug that prevents visible growth) values of drugs are quantified, are frequently used conventional methods in determination of antimicrobial activities of various drugs on microorganisms[2,4]. However, most of the present methods reveal the efficacy of antimicrobial agents against target microorganisms after overnight incubation. Also these procedures do not provide kinetic information on the antimicrobial activities of drugs[4]. Thus, more rapid and objective techniques have become an important investigation area, in the evaluation of the effects of frequently used antimicrobial agents[6,7].

Recently, a microbroth kinetic system based on continuous monitoring of changes in the optical density (OD) of microbial growth has been used. Through this assay, the turbidimetric growth curves of the microorganisms in the presence of increasing concentrations of antimicrobial agents are obtained and MIC values of drugs can be determined in a shorter period. The results can be detected during exposure. Thus, using kinetic procedures, the effects of antibiotics on target microorganisms can be determined at any desired time-point of the incubation period[4-6]. We aimed both to describe turbidimetric growth curves of *Staphylococcus aureus* in the presence of increasing concentrations of gentamicin and to determine the MIC by using this system. We also aimed to compare these results with conventional broth microdilution method.

MATERIALS AND METHODS

Bacterial strain and preparation of inoculum: *S.aureus* (ATCC 25923) strain was used in this study. The bacterial suspension equivalent to the turbidity of 0.5 McFarland (10^8 CFU/ml) standard was prepared by comparing density standard (Phoenix Spec Nephelometer, Becton Dickinson, USA) from a fresh subculture of *S. aureus* in Mueller Hinton Broth (MHB) (Fluka, BioChemica, Germany) and then this suspension was diluted to 10^6 CFU/ml using MHB.

Antimicrobial agent: The antibacterial agent gentamicin (BioChemica, Germany) was dissolved in distilled water. Further dilutions were made using the same solvent according to CLSI document M100-S18. Gentamicin was used in the concentrations range of 0.0625-64 µg/ml.

Growth curves: The adjusted bacterial inoculum (100 µl) were added to each well of sterile 96-well flat-bottomed microtiter plate containing the test concentrations of gentamicin (100 µl/well). As a result, last inoculum concentration of 5×10^5 CFU/ml was obtained in each well. Two trials were performed for each concentration of gentamicin. Two wells containing bacterial suspension with no drug (growth control) and two wells containing only media (background control) were included in this plate. Optical densities were measured for 24 hours at 37°C using a multi-detection microplate reader (Bio-Tek-Synergy HT Microplate Reader, Bio-Tek Instruments, Winooski, Vt, USA) at 600 nm and automatically recorded for each well every 15 min. Turbidimetric growth curves were obtained depending on the changes in the optical density of bacterial growth for each drug concentration and the drug-free growth control.

For the determination of MIC of gentamicin by the microbroth kinetic assay, the percentage of growth at each drug concentration was calculated with the following equation: % growth = [(OD_{600} of wells containing the drug/ OD_{600} of the drug-free well) x 100] after substraction of background ODs (ODs of microorganism-free wells).

Using the Microplate Data Collection & Analysis Software (Bio-Tek Instruments, Gen5, Winooski, USA), highest OD (ODmax), t at Mean Max OD, the changes in ODs (Delta OD = OD_{final} - $OD_{initial}$) for each well, and % inhibition

values were calculated. MIC Curve, MIC Curves Fitting Results, and MIC Curve Interpolations were obtained using these values.

Broth microdilution method: The MIC of gentamicin was also determined by using conventional broth microdilution method according to the CLSI guidelines[2]. The adjusted bacterial inoculum (50 µl/well, 10^6 CFU/ml) were added to each well of sterile U based microtitre plate containing the test concentrations of gentamicin (50 µl/well). Consequently, last inoculum concentration of 5×10^5 CFU/ml was obtained in each well and this plate was incubated for 24 hours at 37ºC. The lowest concentration of antibiotic which inhibited the visible bacterial growth was determined as the gentamicin MIC of the isolate.

RESULTS

Growth curves of *S.aureus* during the incubation period in the presence of increasing concentrations of gentamicin are presented in Figure 1. A_1-B_1 wells include only media for background control and C_1-D_1 wells include bacterial suspension with no drug for growth control in this plate. E_1-B_4 wells include bacterial suspension and concentrations range of 0.0625-64 µg/ml of gentamicin. The growth curves of *S.aureus* in each well could be monitored for each minute from the beginning of incubation period. With the increase of the concentration of gentamicin inside the wells, an inhibition was observed at the growth curves.

Gentamicin MIC_{50} and MIC_{90} values on *S.aureus* were determined as 0.235 and 0.488 µg/ml respectively by microbroth kinetic system at the first half of the incubation period (12th hours). Gentamicin MIC value was determined as 0.5 µg/ml by conventional broth microdilution method at the end of overnight incubation period. The results in microbroth kinetic system were similar to the conventional broth microdilution method.

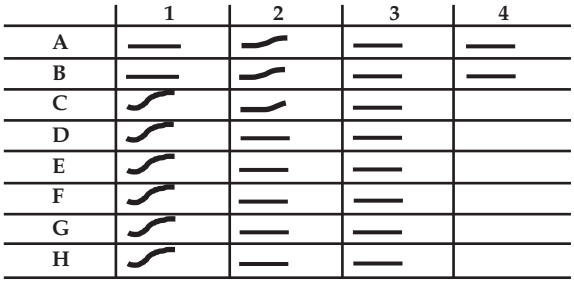

Figure 1. *Growth curves of S.aureus. A_1-B_1, bacteria-free medium; C_1-D_1, drug-free wells for growth control; E_1-F_1, 0.0625 µg/ml; G_1-H_1, 0.125 µg/ml; A_2-B_2, 0.25 µg/ml; C_2-D_2, 0.5 µg/ml; E_2-F_2, 1 µg/ml; G_2-H_2, 2 µg/ml; A_3-B_3, 4 µg/ml; C_3-D_3, 8 µg/ml; E_3-F_3, 16 µg/ml; G_3-H_3, 32 µg/ml; A_4-B_4, 64 µg/ml concentrations of gentamicin.*

MIC curve, MIC curves fitting results, and MIC curve interpolations are given in Figure 2 and Table 1-2, respectively.

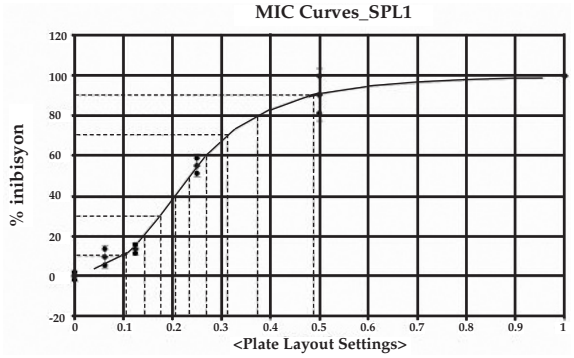

Figure 2. *MIC curve of gentamicin against S.aureus. (Y, % inhibition; X, concentrations of gentamicin)*

Table 1. *MIC curves fitting results of gentamicin against S.aureus.*

Curve name	Curve formula	A	B	C	D	R^2
MIC Curves_SPL1	Y = (A-D)/ (1+(X/C)^B) + D	3.37	3.05	0.24	100	0.998

Table 2. *MIC curves interpolations of gentamicin against S. aureus.*

Y	X
10	0.102
20	0.144
30	0.175
40	0.205
50	0.235
60	0.269
70	0.312
80	0.373
90	0.488

(Y, % inhibition; X, concentrations of gentamicin)

DISCUSSION

Automated antimicrobial susceptibility test systems have been used to obtain rapid MIC results in clinical microbiology laboratories for a long time. Reproducibility, having faster results and less labor are the advantages of these systems[3]. In this study, a recently described microbroth kinetic system which provides kinetic data throughout the incubation period was used for determination of antimicrobial activity[6].

Kinetic measurements of the bacteriostatic, bactericidal and bacteriolytic activities of antimicrobial agents can be performed by bioluminescence, fluorescence, and optical density based on real-time assay with the multi-detection microplate reader[4]. Some investigators reported that this assay is more rapid and reliable than conventional methods for determination of antimicrobial susceptibility[1,4,6].

Lehtinen et al.[4] have performed the kinetic measurements of the bacteriostatic, bactericidal, and bacteriolytic activities of six antibiotics against *Escherichia coli* by bioluminescence, fluorescence, and optical density based on real-time assay. The 10 hours exposure of the bacteria to the antibiotics has been adequate to determine their activities. Inhibitory concentration of 50 % (IC_{50}), minimum bactericidal concentration (MBC), and bactericidal concentration of 50 % (BC_{50}) of each antibiotic were calculated by using these procedures. As a result, they have shown that bacteriostatic, bactericidal or bacteriolytic activities of each antibiotic can be readily determined in the time interval from exposure to visible effect, from kinetic data.

In this study we have shown the utility of microbroth kinetic system which is based on continuous monitoring of the OD of bacterial growth for determination of the effect of a model antibiotic (gentamicin) against *S.aureus*. The results in microbroth kinetic system were similar to the conventional broth microdilution method and MIC value of antibiotic was determined in shorter incubation period than conventional method.

Meletiadis et al.[5] have shown that the antifungal drug resistance of various filamentous fungi can be determined earlier using a microbroth kinetic system which is based on continuous monitoring of the OD of fungal growth over time. They have determined itraconazole, terbinafine, and amphotericin B resistance using this system, within incubation periods of 5.0 to 7.7 h for *Rhizopus oryzae*, 8.8 to 11.4 h for *Aspergillus fumigatus*, 6.7 to 8.5 h for *Aspergillus flavus*, and 13 to 15.6 h for *Scedosporium prolificans*, while awaiting formal MIC determination by the NCCLS reference method at 18 to 24 h. Supportingly, in this study, 12 hours incubation period was adequate to determine the MIC of gentamicin on *S.aureus* with microbroth kinetic assay while broth microdilution method requires 24 hours for the same detection.

Using the kinetic measurements, the growth characteristics of bacteria can be monitored even in the time interval from exposure to visible effect for antimicrobial agents in real-time. By this way, the effects of antimicrobials on target microorganisms can be observed at any desired time-point of the incubation period[3]. Therefore the treatment regimen can easily be designed in an earlier stage.

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