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ORIGINAL

Colistin Resistance in Clinical Isolates of *Acinetobacter baumannii* by Broth Microdilution Method, Biofilm Production, and Antimicrobial Susceptibility Profiles: Experimental Study

Resistencia a la colistina en aislados clínicos de Acinetobacter baumannii por el método de microdilución en caldo, producción de biopelículas y perfiles de susceptibilidad a los antimicrobianos: Estudio experimental

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Abstract

Objective: Colistin susceptibility tests become more importance because of the need for colistin use increases especially treatment of the multidrug-resistant (MDR) bacterial infections. In addition, biofilm formation by microorganisms is an important cause of antibiotic resistance. The aim of this study was to evaluate the colistin susceptibility by broth microdilution (BMD) method, biofilm formation, and antibiotic resistance profiles in the *Acinetobacter baumannii (A. baumannii)* strains.

Materials and Methods: Fifty A. baumanni strains which were isolated from clinical specimen were included. Identification of the isolates were studied with the BD Phoenix100 automated system by using the Phoenix NMIC-400/ID panels. The antimicrobial susceptibility test for the colistin were studied by the Mueller-Hinton BMD method according to EUCAST. The biofilm production were investigated using Congo Red Agar.

Results: Of the 50 isolates, colistin resistance by BMD method was found 2% (n:1). The rates of biofilm formation and MDR were %92 (46/50) and %76 (38/50), respectively. It was determined that the biofilm formation rate increased in parallel with the age of the patients (p=0.008). Moreover, MDR positive strains were found to produce biofilms more frequently than negatives (p=0.038).

Conclusion: Unfortunately, our study is the first report for the colistin resistance in Northern Cyprus. Biofilm formation of *A. baumannii* strains examined in our study were found to be high. Therefore, we think that the application of correct diagnostic methods, full sterilization/disinfection procedures, and rational use of antibiotics may affect the morbidity and mortality rates, as they will prevent the development of MDR microorganisms.

Key words: *Acinetobacter baumannii*; colistin; biofilm; multi-drug resistance.

Resumen

Objetivo: Las pruebas de susceptibilidad a la colistina adquieren mayor importancia debido a que aumenta la necesidad de utilizar colistina, especialmente en el tratamiento de las infecciones bacterianas multirresistentes (MDR). Además, la formación de biopelículas por parte de los microorganismos es una causa importante de resistencia a los antibióticos. El objetivo de este estudio fue evaluar la susceptibilidad a la colistina por el método de microdilución en caldo (BMD), la formación de biofilms y los perfiles de resistencia a los antibióticos en las cepas de *Acinetobacter baumannii (A. baumannii).*

Materiales y métodos: Se incluyeron 50 cepas de A. baumanni aisladas de muestras clínicas. La identificación de los aislados se estudió con el sistema automatizado BD Phoenix100 utilizando los paneles Phoenix NMIC-400/ID. La prueba de susceptibilidad antimicrobiana para la colistina se estudió mediante el método Mueller-Hinton BMD según el EUCAST. La producción de biofilm se investigó mediante el uso de Agar Rojo Congo.

Resultados: De los 50 aislados, se encontró una resistencia a la colistina por el método BMD del 2% (n:1). Las tasas de formación de biofilm y de MDR fueron del 92% (46/50) y del 76% (38/50), respectivamente. Se determinó que la tasa de formación de biofilm aumentaba en paralelo a la edad de los pacientes (p=0,008). Además, se observó que las cepas MDR positivas producían biofilms con mayor frecuencia que las negativas (p=0,038).

Conclusiones: Lamentablemente, nuestro estudio es el primer informe sobre la resistencia a la colistina en el norte de Chipre. La formación de biopelículas de las cepas de A. baumannii examinadas en nuestro estudio resultó ser elevada. Por lo tanto, creemos que la aplicación de métodos de diagnóstico correctos, procedimientos de esterilización/desinfección completos y el uso racional de antibióticos pueden afectar a las tasas de morbilidad y mortalidad, ya que evitarán el desarrollo de microorganismos MDR.

Palabras clave: Acinetobacter baumannii; colistina; biofilm; multirresistencia.

Introduction

Colistin, an antibiotic of the polymyxin group, was synthesized in the 1940s and its use continued until the 1970s. Polymyxin B and Polymyxin E (colistin), which are about thirty different polymyxin compounds, have a similar structure and are used clinically[1]. Polymyxin E, also known as colistin, weighs 1200 Da and it is synthesized by adding a hydrophobic fatty acid tail to the hydrophilic polycationic peptide chain[2]. Due to its serious nephrotoxic and neurotoxic effects, the parenteral use of colistin has gradually decreased after the 1970s. Because of the development of resistance against aminoglycoside and carbapenem for the Gram-negative bacteria, clinicians led to the re-introduction into parenteral use of colistin in 2000s for treatment[3,4].

Especially, the prevelance of the multi-drug resistant (MDR) *Acinetobacter baumannii (A.baumanii)* is being increased, the need for colistin usage increased. Therefore, colistin susceptibility tests gained importance. In order to test the antimicrobial susceptibility of colistin in both *Acinetobacter* species and other resistant bacterial species, studies were using the disk diffusion, gradient test, agar dilution and broth microdilution methods[5-7]. The studies indicated that the disk diffusion and gradient test methods, which are preferred in routine microbiology laboratories due to their case of use, are not reliable in testing the antimicrobial susceptibility of colistin, neither the CLSI (Clinical and Laboratory Standards Institute) nor the EUCAST (European Committee on Antimicrobial Susceptibility Testing) also not recommend these tests for antimicrobial susceptibility of colistin[5,8,9].

Biofilm formation by pathogenic microorganisms is an important reason for the development of antibiotic resistance. The main reason why *A. baumannii* strains can survive on medical instruments and are resistant to antibiotics is their ability to form biofilms on solid surfaces. *A. baumannii* strains that can form biofilms can escape from the immune mechanisms of the host and thus cause prolongation of infectivity. The omp A gene found in the bacteria is related to biofilm formation and is the most important virulence factor affecting the mortality rate in infections caused by *A. baumannii*[10,11].

Increasing resistance to antibiotics used in the treatment of *Acinetobacter spp.* infections continues to be a serious health problem. MDR *A. baumannii* strains are the bacteria responsible for hospital infections that create difficulties in the treatment of inpatients. It is of great importance to determine the risk factors for *Acinetobacter* infection and to take precautions against these risk factors due to the limited treatment options, the frequent outbreaks that are difficult to prevent, and the high mortality[12,13].

The aim of the study was to investigate the minimum inhibitory concentration (MIC) values of the colistin by broth microdilution method for the *A. baumannii* clinical isolates of the Near East University Hospital, Microbiology laboratory. In addition, examining the biofilm formation and resistance profiles of the strains is one of our other goals.

Material and metods

Bacterial isolates

A total of 50 non-duplicate clinical isolates of *A. baumanii* isolates in Microbiology Laboratory of our hospital in 2020 were included in our study. Various specimens were collected from different sites including aspirate, broncoalverolar lavage, and sputum. All samples were taken from inpatients. Patient data such as age, gender, hospital department and admission type were obtained from the hospital system and electronically stored. The clinical samples were cultured on eosin-methylene blue (EMB) agar and blood agar, and were incubated aerobically at 35°C for 24-48 hours. Repeating isolates from the patients were exluded from the study.

Identification and antimicribial susceptibility testing

The identification of the isolates were performed by the BD Phoenix 100 (Becton Dickinson, USA) automated system using the Phoenix NMIC-400/ID panels, and the colistin-resistant MICs were confirmed by broth microdilution test, according to EUCAST (European Antimicrobial Susceptibility Testing Committee) recommendations[9]. Intermediate antibiotic susceptibility results were considered resistant. The susceptibility categories were interpreted according to break-points of EUCAST guidelines. Colistin MICs >2 µg/mL were considered resistant[9]. Strains resistant to all penicillin antibiotics, as well as at least three of the cephalosporin, quinolone, carbapenem, and fluoroquinolone antibiotic groups, were considered MDR[14].

Biofilm formation

Modified Congo Red Agar (MCRA) consists of 0.4 grams of Congo red dye (Alfa Aesar, ThermoFisher GmbH, Erienbachweg 2, 768 70 Kandel, Germany), 10 grams of glucose (Merck, KgaA, Germany), and blood agar base (Merck, KgaA, Germany). The Congo red dye was prepared in 100 ml of distilled water and autoclave at 121°C for 15 minutes. The glucose and the blood agar were dissolved in 900 ml of distilled water and autoclave at 121°C for 15 minutes. The dye was combined with blood agar and glucose and thoroughly mixed until homogeneous before pouring into the sterile petri dishes. *A. baumanii* isolates were plated on MCRA and incubated aerobically at 35±2ºC for 24 hours. The red-coloured colony's observation was considered as biofilm negative and black-coloured colony as positive. ATCC6538 *Staphylococcus aureus* strain was used as biofilm positive control in MCRA method.

Statistical analysis

Statistical analyses were performed using SPSS Demo Ver 22 (SPSS Inc., Chicago, IL, USA). Pearson chi-square or Fisher's exact tests measured the differences between two proportions. A one-way ANOVA test was used to determine the statistical differences in the groups' mean. A p-value <0.05 was accepted to be statistically significant.

Ethical approval

Ethics committee approval was obtained with the project number NEU/2021/95-1402 at the meeting held by the NEU Scientific Research Ethics Committee on 30.09.2021. In addition, the study was carried out in accordance with the principles of the Declaration of Helsinki.

Results

In our study, 50 patients hospitalized in our hospital in 2020 and *A. baumanii* isolated in any culture sample were included. Of these patients %74 (37/50) were male, %26 (13/50) were female, and ve their mean age was 65.16±15.44, 67.15±20.78 (general 65.68±16.78, between 24-91 years), respectively. There was no significant relationship between gender and the mean age of the patient (p=0.717).

Of the examined samples, 62% (31/50) were aspirate, 16% (8/50) were sputum, and 10% were (5/50) broncho alveolar lavage (BAL). Fifteen aspirate samples (48.4%) were isolated from the intensive care unit (ICU). According to the EUCAST guidelines for *A. baumannii* isolate sensitivity testing of colistin, we found 1 strain (2%) was resistant and 49 strains (98%) were sensitive.

The resistance rates given by the automated system to amikacin, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, and trimethoprim/sulfametoxazole (SXT) antibiotics are 72% (36/50), 80% (40/50), 74% (37/50), 80% (40/50), 80% (40/50), 82% (41/50), and 74% (37/50) idi. All susceptibility results are shown in **table I**.

It was determined that 92% (46/50) of the strains isolated from the patients produced biofilm. Of these patients, 78.3% (36/46) were male and 21.7% (10/46) were female. The relationship between gender and biofilm was found to be significant (p=0.049). Accordingly, *A. baumannii* strains were more likely to produce biofilms in males than in females. The mean age of patients with biofilm-producing strains was found to be significantly higher than those with non-biofilm-producing strains (p=0.008). It was determined that %76 (38/50) of *A. baumannii* strains were MDR. There was no relationship between the gender and mean age of the patients and MDR (p=0.146, p=0.119, respectively) (**Table II**). However, a statistical significancewas found between biofilm formation and being MDR. MDR positive strains appear to be more capable of forming biofilms than MDR negative strains (p=0.038).

Table I: Antibiotic susceptibility profiles of *A. baumannii* strains.

Antibiotic	Sensitive (n, %)	Resistant (n, %)
Amikacin	14, 28%	36, 72%
Ciprofloxacin	10, 20%	40, 80%
Gentamicin	13, 26%	37, 74%
Imipenem	10, 20%	40, 80%
Levofloxacin	10, 20%	40, 80%
Meropenem	9, 18%	42, 82%
SXT	13, 26%	37, 74%
Colistin*	49, 98%	1, 2%

SXT: Trimethoprim/sulfamethoxazole
*Colistin resistance by broth microdilution method

Discussion

Colistin is an antibiotic used especially in the treatment of carbapenem-resistant gram-negative bacteria, despite its side effects such as nephrotoxicity. However, with the increase in colistin resistance in recent years, the treatment options of clinicians are gradually decreasing[15].

Many studies have been focused on the comparing broth microdilution, disc diffusion, agar dilution, gradient tests and automated systems to determine the antimicrobial sensitivity of colistin. Antimicrobial susceptibility method of colistin has changed over the years and is still a subject of ongoing debate. The gold standard methods used in comparative studies of antimicrobial sensitivity of colistin are not always the same, which poses a problem for determine the resistance rate of the colistin. Therefore, the results of the antimicrobial sensitivity of colistin are contradictory and misleading. However, EUCAST recommends the broth microdilution method as the reference method for the determination of the Minimum Inhibitory Concentration (MIC) for colistin for *Acinetobacter spp*.[16].

Table II: Biyofilm and MDR properties of *A. baumannii* strains.

	Biofilm negative	Biofilm positive	p-value	MDR negative	MDR positive	p-value
Toplam	4 (8%)	46 (92%)		12 (24%)	38 (76%)	
Average age	44.74±22.99	67.50±15.14	0.008*	59.08±18.12	67.76±16.02	0.119
Male	1 (2.7%)	36 (97.3%)	0.049*	7 (18.9%)	30 81.1%)	0.149
Female	3 (23.1%)	10 (76.9%)		5 (38.5%)	8 (61.5%)	

MDR: Multi-drug resistant *Statistically significant.

Many surveillance programs have been initiated to monitor antimicrobial resistance in all countries, including the Global Antimicrobial Resistance and Use Surveillance System (GLASS), the European Antimicrobial Resistance Surveillance Network (EARS-Net), and Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR). A report of colistin resistance in bloodstream infections (BSIs) from the SENTRY Antimicrobial Surveillance Program from 2009 to 2016 showed a resistance rate 3.1% in *A. baumannii*[17]. The CANWARD surveillance study indicated that *A. baumannii* colistin resistance rates of approximately 2.5% between 2007 and 2016 for Canada[18]. The National Antimicrobial Resistance Surveillance Center, Thailand (NARST) also study colistin resistance in clinically important microorganisms and reported that the resistance rates to colistin for *A. baumannii* were less than 5% in 2019[19]. In our study, the resistance rate of the colistin was 2%. Unfortunately, our study is the first report for the colistin resistance in Northern Cyprus. Therefore, we were not compare our results.

The most important difficulty in colistin susceptibility tests is the problems in determining resistance[20]. There are different test methods to detect colistin susceptibility such as automated systems, broth microdilution, and agar dilution, which are phenotypic method[21]. Since polymyxins are big cationic peptide molecules, their distribution on the agar surface is not good, and therefore, disk diffusion and gradient tests are not recommended for susceptibility tests[14,22].

While biofilm positivity was 92% in all *A. baumannii* strains examined in our study, this rate was 97.4% in MDR A. baumannii strains. This provides that there is a parallel relationship between biofilm formation and antibiotic resistance. When similar studies in the literature are exained, it is reported that *A. baumannii* strains produce high levels of biofilm. Amin et al. reported that biofilm formation was observed in 55 (85.9%) of 64 *A. baumannii* isolates[11]. In another study, the rate of biofilm formation in MDR *A. baumannii* strains was reported as %75.8[23]. In the study of Yang et al., biofilm genes were investigated by molecular methods 154 *A. baumannii* strains and a total of 93.5% (15.6% weak, 32.4% moderate, 45.4% strong) biofilm positivity was detected[24]. The rate of biofilm formation in our study was found to be relatively high, especially in MDR A. baumannii strains.

A. baumannii strains isolated from patients hospitalized in the ICU of a university hospital in Northern Cyprus were examined and carbapenem resistance was found at arate of 70.7%. The main point that the study wanted to emphasize was the resistance gradually increased between 2016 and 2018[12]. The MDR rate was 76% in our study, shows that antibiotic resistance of *A. baumannii* strains continues to increase over the years in our region.

Conclusion

EUCAST and CLSI recommendation reference method is broth microdilution method for colistin susceptibility test. Since the broth microdilution method is usually a laborious and expensive method, it is difficult to use in routine laboratories. In addition, we think that necessary precautions should be taken considering the high rate of biofilm formationdetected in our study. The relationship between biofilm and MDR shows that inappropriate and incorrect antibiotic use and the lack of necessary sterilization and disinfection procedures in hospital environments can increase the effect on mortality rates, especially in hospitalized patients.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Meryem Güvenir, Anas M.J. Jamal Masalmeh; Design: Meryem Güvenir; Control/ Supervision: Meryem Güvenir, Kaya Süer; Data Collection and/or Processing: Emrah Güler, Anas M.J. Jamal Masalmeh; Analysis and/or Interpretation: Meryem Güvenir, Emrah Güler; Literature Review: Meryem Güvenir, Emrah Güler, Anas M.J. Jamal Masalmeh; Writing the Article: Meryem Güvenir, Emrah Güler, Anas M.J. Jamal Masalmeh; Critical Review: Meryem Güvenir, Kaya Süer.

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