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ORIGINAL ARTICLE / ÖZGÜN MAKALE

MOLECULAR ANALYSIS OF THE MCR-1 GENE IN *PSEUDOMONAS AERUGINOSA* AND *ACINETOBACTER BAUMANII* STRAINS

PSEUDOMONAS AERUGINOSA VE ACINETOBACTER BAUMANII SUŞLARINDA MCR-1 GENİNİN MOLEKÜLER ANALİZİ

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ABSTRACT

Objective: *The emergence of antibiotic resistance in Pseudomonas aeruginosa and Acinetobacter baumanii isolates poses serious risks to public health. Our study aimed to investigate the presence of colistin resistance and mcr-1 gene positivity in these isolates.*
Material and Method: *Culture, biochemical tests, antibiotic susceptibility tests, and molecular tests were used to isolate and identify P. aeruginosa and A. baumanii strains.*
Result and Discussion: *A sum of 156 clinical isolates of Pseudomonas aeruginosa (n = 89) and Acinetobacter baumannii (n = 67) were obtained using the 550 clinical samples collected in one year from the largest hospital in Van, Turkey. The results of antibiotic susceptibility tests showed that approximately 82.8% of P. aeruginosa and 94.6% of A. baumannii strains were multidrug-resistant (MDR). Colistin resistance was detected in 11.23% (10/89) of P. aeruginosa isolates and 11.94% (8/67) of A. baumannii isolates using agar dilution and microdilution methods. Out of the 18 colistin-resistant isolates, the mcr-1 gene was detected in three P. aeruginosa and two A. baumannii strains. The detection of plasmid-mediated colistin resistance in P. aeruginosa and A. baumannii is of great concern due to the high potential for colistin resistance to spread in clinical settings. Understanding the unique circumstances of worldwide colistin resistance can be facilitated by promoting the creation of quick processes for identifying colistin resistance profiles and putting them into practice in hospital laboratories. Colistin and carbapenem treatment are two effective ways to treat emerging resistant super-microbes and slow down the emergence of resistance.*
Keywords: *Acinetobacter baumannii, antibiotic resistance, mcr-1, Pseudomonas aeruginosa*

ÖZ

Amaç: *Pseudomonas aeruginosa ve Acinetobacter baumanii izolatları içerisinde birçok antibiyotiğe direnç meydana gelmesiyle birlikte halk sağlığı açısından ciddi riskler ortaya çıkmıştır. Çalışmamızın amacı bu suşlar içerisinde kolistin direnci varlığı ile mcr-1 geni pozitifliğinin araştırılmasını hedeflemektedir.*
Gereç ve Yöntem: *P. aeruginosa ve A. baumanii suşlarının izolasyonu ve identifikasyonu amacıyla farklı yöntemler kullanılmıştır. Bunlar sırasıyla kültür, biyokimyasal testler, antibiyotik duyarlılık testleri ve moleküler testlerdir.*
Sonuç ve Tartışma: *Türkiye'nin Van kentindeki en büyük hastaneden bir yılda toplanan 550 klinik örnekten tam olarak 156 klinik P. aeruginosa (n = 89) ve A. baumannii (n = 67) izolatı elde edildi. Yapılan antibiyotik duyarlılık testleri araştırma sonuçlarına göre yaklaşık %82.8 P. aeruginosa ve*

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*%94.6 A. baumannii suşlarının MDR olduğu görüldü. Agar dilüsyon ve mikro dilüsyon yöntemleri kullanılarak P. aeruginosa izolatlarının %11.23'sinde (10/89) ve A. baumannii izolatlarının %11.94'unda (8/67) kolistin direnci keşfedildi. Kolistine dirençli 18 izolat arasında mcr-1 geni, üç P. aeruginosa ve iki A. baumannii suşunda tespit edildi. P. aeruginosa ve A. baumannii'da plazmit aracılı kolistin direncinin meydana gelmesi, kolistin direncinin klinik alanlarda yayılma eğiliminin yüksek oranda olması nedeniyle oldukça önemlidir. Küresel kolistin direncinin özel durumunun anlaşılması için kolistin direnç profillerinin tespitine yönelik hızlı prosedürlerin geliştirilmesi ve bu prosedürlerin hastane laboratuvarlarında uygulanması teşvik edilmelidir. Kolistin ve karbapenemin kombinasyonu ile oluşturulacak terapinin uygulanması, direnç gelişimi sürecinin hızının azaltılmasına ve ortaya çıkan bu dirençli süper mikropların tedavisinde yardımcı ola*bilir.

Anahtar Kelimeler: *Acinetobacter baumannii, antibiyotik direnci, mcr-1, Pseudomonas aeruginosa*

INTRODUCTION

Due to the absence of new antibiotic discoveries, drug-resistant (MDR) Gram-negative bacteria have become more common. These bacteria include members of the Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. It is critical that this matter be handled as soon as possible [1]. Following the addition of carbapenem-resistant *P. aeruginosa* and *A. baumannii* to the list of major killer infections in urgent need of antibiotics and novel modes of action, the World Health Organization (WHO) recognized growing concerns around MDR in February 2017 [2,3].

P. aeruginosa and *A. baumannii* are classified as ESKAPE pathogens, along with *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Enterobacter* spp. These pathogens are responsible for nosocomial infections, including bacteremia, skin infections, urinary tract infections, soft tissue infections, and ventilator-associated infections [4,5]. It can also lead to severe health issues in individuals who are already ill or have weakened immune systems [4,6,7]. The mentioned pathogens are found to be resistant to most significant antibiotic types, such as carbapenems, beta-lactams, fluroquinolones, and aminoglycosides, making appropriate treatment difficult [8]. This presents significant challenges for physicians treating infections caused by MDR *P. aeruginosa* and *A. baumannii* strains. As a result, the WHO has included colistin in the group of "antibiotics of last resort" that can be used to treat these newly discovered super-microbes [9].

Colistin, a member of the polymyxin family of antibiotics, is often referred to as polymyxin E. Although it was developed in the 1950s, its usage was outlawed in a number of nations because of its detrimental effects on human health, especially renal function. Nevertheless, after a considerable amount of time, colistin was once again utilized in clinical practice to treat infections brought on by the carbapenem-resistant strains of *A. baumannii*, *P. aeruginosa*, and Enterobacteriaceae [10,11].

Many mechanisms contribute to colonistin resistance in Gram-negative bacteria. The most frequent way that Gram-negative bacteria become resistant to colistin is by genetic mutations [12-14]. Resistance resulting from differentiation in the lipo-polysaccharide layer and phospho-ethanolamine transferase binding to the phosphate group in the lipid A structure, produced by the two-component regulatory systems PhoP-PhoQ and PmrA-PmrB, is a major issue [15-17]. Although PhoP/PhoQ is responsible for colistin resistance in *K. pneumoniae* and *P. aeruginosa*, in *A. baumannii*, the PmrA/PmrB component mediates the mechanism of colistin resistance [18,19].

Following the first report by Lui et al. [23] on the plasmid-mediated *mcr-1* gene, numerous bacterial species have been the subject of research [20-27]. These species include *K. pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Salmonella*, *Cronobacter sakazakaii*, *Moraxella*, *Kluyvera*, *Shigella sonnei*, and *Citrobacter*. The plasmid-mediated *mcr-1* gene in MDR *P. aeruginosa* and *A. baumannii* is currently the subject of scant global data. Since our region lacks information on these two MDR bacterial species, our goal was to find the *mcr-1* gene in them.

MATERIAL AND METHOD

Sample Collection and Microbiological Analysis

From January to December 2022, the microbiology laboratories of Van Training and Research

Hospital obtained 156 clinical isolates of *A. baumannii* (n = 67) and *P. aeruginosa* (n = 89) from various samples, including blood, urine, ulcer swabs, and respiratory secretions. The samples were then transferred to the Microbiology laboratory at Van Yüzüncü Yıl University's Faculty of Pharmacy in a cold chain environment for further analysis. Prior to inoculation, the media underwent incubation in an autoclave to ensure sterility. The sterility of the media was confirmed by transparent plates without any microbial contamination. The samples were inoculated on MacConkey agar, Cystein Lactose Electrolyte Deficient (CLED) agar, chocolate agar, and blood agar media. The plates were then incubated at 37°C for 24 hours. All isolates were identified as *P. aeruginosa* and *A. baumannii* based on colony morphology, Gram staining results, and the API-10S system (bioMérieux, France). The bacterial isolates were preserved in Luria-Bertani broth medium (Oxoid, UK) supplemented with 30% glycerol and stored at -20°C until further analysis.

Antibiotic Susceptibility Tests

The Kirby-Bauer disk diffusion method was used to test the antibiotic susceptibility of all isolates. This was performed on Müller-Hinton agar (MHA) plates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [28]. To determine the MDR status of *P. aeruginosa* and *A. baumannii* isolates, Aztreonam, amikacin, ciprofloxacin, levofloxacin, cefepime, cefotaxime, imipenem, and piperacillin/tazobactam were used. The results were compared with CLSI practice guidelines. Colistin resistance was demonstrated through agar dilution and liquid medium micro-dilution methods using colistin sulfate powder (Sigma-Aldrich) [29]. The MIC results were interpreted according to the European Guidelines for Antimicrobial Susceptibility Testing (EUCAST) [30]. *E. coli* NCTC 13846 (*mcr-1* positive) strain was used as a positive control for sensitivity analysis of the tests.

DNA Extraction and Molecular Analysis

For molecular analysis, all colistin-resistant strains confirmed by phenotypic analysis were subjected to standard alkaline lysis method for plasmid DNA extraction [31]. The quantity of extracted DNA was measured using a micro volume spectrometer (Colibri, Titertek Berthold) and its quality was evaluated on a 1.5% agarose gel stained with ethidium bromide.

The *mcr-1* gene was amplified using gene-specific primers (MCR1-F: 5'-CGGTCAGTCCGTTTGTTC-3'; MCR1-R: 5'-CTTGGTCGGTCGGTCTGTAGGG-3') through conventional PCR, resulting in a 309 bp target gene region [32]. The protocol for DNA amplification of bacteria was carried out using the May Taq™ DNA Polymerase (Bioline, Bio-21105) kit. The Polymerase Chain Reaction (PCR) was performed using a set of chemical solutions and substances. The reaction mixture contained 10µl of 5x MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl2), 5µl of template DNA, 1µl of each primer (20uM), 1µl of MyTaq DNA polymerase, and 8µl of nuclease-free water, respectively, calculated as 25µl of the final solution. The PCR cycling conditions were as follows: denaturation at 94°C for 3 minutes (1 cycle), denaturation at 94°C for 30 minutes (25 cycles), annealing at 52°C for 30 seconds, initial extension at 72°C for 1 minute, and final extension cycle at 72°C for 10 minutes. The amplicon sizes were accurately analyzed using the HyperLadderTM marker (50 Base Pair, Bioline, USA). To visualize the bacterial amplicon products accurately, we operated a Thermo EC300XL2 electrophoresis device at 100 Volts on a 1.5% agarose gel for 1 hour. The Bio-Print-ST4 (Vilber Lourmant, France) was used to visualize the amplicons.

Our study was authorized by the Van Training and Research Hospital's clinical research ethics committee (decision dated 25/01/2018 and numbered 2018/02) to evaluate the accuracy of blood collection in patients.

RESULT AND DISCUSSION

Clinicians throughout the world are experiencing a great deal of comfort with the reintroduction of colistin as the newest treatment option for illnesses brought on by Gram-negative bacteria that is resistant to drugs. Colistin effectively treats illnesses brought on by MDR bacteria when taken either by alone or in conjunction with other antibiotics [33]. Colistin is not only used in humans but also in animals

for conditioning and in agriculture to increase yields. This usage has led to a significant rise in the incidence and prevalence of Gram-negative bacteria that are resistant to colistin [34].

A total of 156 clinical isolates were obtained from 550 clinical samples collected in one year from the largest hospital in Van, Turkey. Of these, 89 were identified as *P. aeruginosa* and 67 were identified as *A. baumannii* using API-10S test strips for microbiological identification of bacteria. Blood (43.6%), urine (27.1%), respiratory secretions (21.8%), and ulcer swab specimens (7.5%) were the sources of several *A. baumannii* isolates. In a similar manner, isolates of *P. aeruginosa* were obtained from blood samples (7.9%), urine (55%), wounds (25.8%), and stools (11.3%). The findings of the antibiotic susceptibility test showed that the strains of *P. aeruginosa* and *A. baumannii* exhibited a significant degree of multidrug resistance. Antibiotic susceptibility test results revealed that 94.6% of *A. baumannii* strains and 82.8% of *P. aeruginosa* strains were multidrug-resistant (MDR). Imipenem (41.2%) and cefotaxime (44.8%) showed the lowest resistance among *A. baumannii* isolates, whereas aztreonam (80.5%) and piperacillin/tazobactam (73.4%) showed the highest resistance. Antibiotic resistance to amikacin (92.3%) and aztreonam (85.8%) was highest in *P. aeruginosa* isolates, while antibiotic resistance to cefepime (27.8%) and imipenem (29.4%) was lowest. Table 1 provides a detailed list of all tested antibiotic percentages.

Table 1. Analysis of antibiotic resistance of *P. aeruginosa* and *A. baumanii strains*

Antibiotics	***P. aeruginosa***		***A. baumanii***	
	S (%)	**R (%)**	**S (%)**	**R (%)**
Aztreonam	14.2	85.8	19.5	80.5
Amikacin	7.7	92.3	43.3	56.7
Ciprofloxacin	23.9	76.1	27.8	72.2
Levofloxacin	43.2	56.8	29	71
Cefepime	72.2	27.8	33.8	66.2
Cefotaxime	20.6	79.4	55.2	44.8
Imipenem	70.6	29.4	58.8	41.2
Piperaciline/Tazobactam	20.6	79.4	26.6	73.4

Eighteen of the 67 (11.94%) *A. baumannii* isolates and ten (11.23%) *P. aeruginosa* isolates were among the eighteen colistin-resistant isolates discovered by the investigation. For isolates of *A. baumannii*, the MIC values varied between 8 and 16 μg/ml, while for isolates of *P. aeruginosa*, they ranged from 8 to 64 μg/ml. 43.6% of the isolates of *A. baumannii* were isolated from blood samples. India accounted for 45% of the reported *A. baumanniii* isolates, the bulk of which were recovered from wound swabs [35]. Our results are in line with the analysis of the same study, which found that 7% (7/100) of the isolates of *A. baumannii* were resistant to colistin. According to Oikonomou et al. [36], the MIC of colistin-resistant *A. baumannii* strains varied from 16 to 64 μg/ml, and the rate of colistin-resistant *A. baumanii* was 7% (86/1228). A different investigation discovered that 57% (12/21) of the isolates of *A. baumannii* were resistant to colistin, with MIC values ranging from 4 to >128 μg/ml [37]. The colistin-resistant *P. aeruginosa* isolates in our investigation had MIC values ranging from 8 to 64 μg/ml. This is not the same as the rate that Snesrud et al. [38] reported. Lescat et al. [37] reported 41.1% (7/17) of colistin-resistant *P. aeruginosa* isolates with MICs ranging from 4 to 128 μg/ml, which is in contradiction to our findings regarding colistin resistance and MIC.

Out of the eighteen isolates that were resistant to colistin, we found the *mcr-1* gene in three strains of *P. aeruginosa* and two strains of *A. baumannii*. The PCR picture of the *mcr-1* gene region is displayed in Figure 1. The *mcr-1* gene test results for the remaining 13 colistin-resistant isolates, however, were negative. This is the first report of *mcr-1* presence in *A. baumannii* and *P. aeruginosa*, as far as we are aware. However, prior research has demonstrated that these two clinically significant pathogenic bacteria have distinct mechanisms behind their colistin resistance. Chromosome mutations are the main cause of colistin resistance in *A. baumannii*. These mutations can be linked to changes in the outer membrane (such as those in pmr, lpx, lpsB, lptD, and vacJ) or unrelated to them (such as increased cell osmotic sensitivity and efflux pump activation) [39]. In the meantime, the main mechanisms linked to

the emergence of colistin resistance in *P. aeruginosa* are alterations in two-component regulatory systems [18]. It was recently discovered that *P. aeruginosa* harbors the *mcr-5* gene, which is encoded chromosomally. On the other hand, no cases of colistin resistance resulting from *mcr-1* gene mediated by plasmids have been recorded [38].

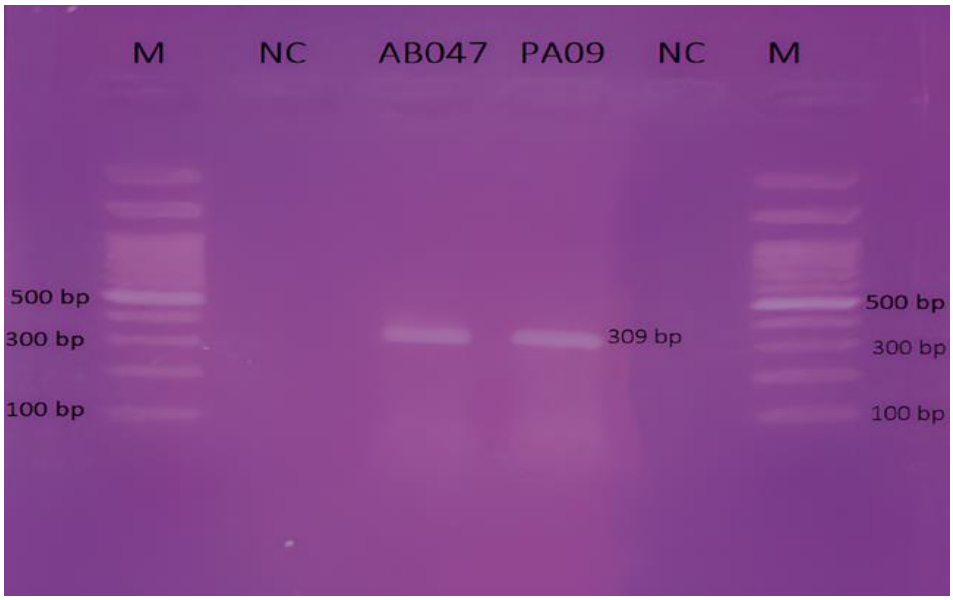

Figure 1. 309bp amplicon image of *P. aeruginosa* and *A. baumanii* strains obtained by PCR. M: 100bp marker; NC: Negative control; AB047: A. *baumanii* isolate; PA09: *P. aeruginosa* isolate

This is the first study to document the existence and frequency of the plasmid-mediated *mcr-1* gene in *A. baumannii* and *P. aeruginosa* isolated from different clinical samples in the Eastern Turkish province of Van. The only thing we looked at in these two infections was the presence of the *mcr-1* gene. As a result, we were unable to look into the processes underlying the remaining resistant strains' colistin resistance. Our results point to the necessity of additional experimental methods to identify plasmid-mediated colistin resistance in these two newly discovered pathogenic bacteria. Because of the great potential for resistance to spread in clinical settings, the presence of plasmid-mediated colistin resistance in *P. aeruginosa* and *A. baumannii* is noteworthy. To stop the spread of resistance, it is essential to carefully assess and set criteria for the usage of this medication used as a last-resort treatment. Understanding the unique circumstances of worldwide colistin resistance can be facilitated by promoting the creation of quick processes for identifying colistin resistance profiles and putting them into practice in hospital laboratories. Colistin and carbapenem treatment are two effective ways to treat emerging resistant super-microbes and slow down the emergence of resistance.

AUTHOR CONTRIBUTIONS

Conception: Ö.A.; Design: Ö.A.; Control: Ö.A.; Sources: Ö.A.; Materials: Ö.A.; Data Collection and/or Processing: Ö.A.; Analysis and/or Interpretation: Ö.A.; Literature Review: Ö.A.; Manuscript Writing: Ö.A.; Critical Review: Ö.A.; Other: -

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

Our study was authorized by the Van Training and Research Hospital's clinical research ethics committee (decision dated 25/01/2018 and numbered 2018/02) to evaluate the accuracy of blood

collection in patients.

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