PmrA Gene Polymorphism and Mutation in Colistine-Resistant Acinetobacter Baumannii Strains

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Article

PmrA Gene Polymorphism and Mutation in Colistine-Resistant *Acinetobacter Baumannii* Strains

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Abstract: *Acinetobacter baumannii* has been identified as the major cause of nosocomial outbreaks associated with high morbidity and mortality worldwide. It is one of the most frequently isolated agents in nosocomial infections, especially in intensive care units. The limited number of antibiotics that can be used in the treatment of multiple-drug-resistant (MDR) *A. baumannii* infections causes serious clinical problems. The decreasing number of antibiotic options, combined with the increase in the frequency of infections with MDR Gram-negative bacteria, and the problems experienced in their treatment have necessitated the reuse of older antibiotics such as colistin and other polymyxins. Colistin-resistant *A. baumannii* strains have been reported following their use as a form of treatment. The main mechanism of colistin resistance in *A. baumannii* is the addition of a cationic group to the lipopolysaccharide (LPS) layer due to a mutation in the *PmrAB* gene region. In our study, we aimed to determine the *PmrA* gene polymorphism and mutation in colistin-resistant *A. baumannii* isolates obtained from clinical samples and to understand the phylogenetic relationship between *PmrA* genes. A total of 29 *A. baumannii* strains isolated between June 2019 and June 2020 from patients who received colistin treatment and were followed in intensive care units and services of the Research and Training Hospital of RTE University were used to perform sequence analysis. Phylogenetic analysis was performed in Clustal 2.1 Multiple Sequence Alignment using the reference strain AB030 of *A. baumannii*. The sequencing of the PCR products of the *PmrA*-positive strains revealed point mutations (SNPs) that had spread to the entire amplified region, which were higher in the first 60 bp. It was determined that there were six strains that differed from the reference strain with the SNPs examined. When our study was evaluated in terms of phylogenetic relationship, the mutations that occurred in the samples were closely related to each other. Our finding suggests that the mutations detected in the 175 bp region of the *PmrA* gene are related to the resistance of colistin, as well as the sequence analysis of the *PmrA* gene region and the determination of the resulting changes. This is one of the first studies in this field in our country, paving the way for new studies to be carried out in this line of research. New-generation genome technologies are a very good option for fully revealing the resistance mechanism of colistin in MDR *A. baumannii* isolates. However, because of the high cost of these technologies, reference centers should be established related to this subject and the identified resistant strains should be further investigated.

Keywords: *Acinetobacter baumannii*; colistin; mutation; *PmrA*; polymorphism

1. Introduction

A. baumannii is increasingly being encountered as a causative agent in various infections all over the world [1]. In the definition proposed by the Centers for Disease Control and Prevention (CDC) of the United States to characterize drug resistance, microorganisms resistant to one or more antimicrobial agent classes are defined as being multidrug-resistant (MDR) [2]. The limited number of effective antibiotics to be used in the fight against MDR A. baumannii infections causes serious clinical problems [3].

In the treatment of MDR *A. baumannii*, different forms of polymyxin group antibiotics, especially colistin, are currently used in various combinations. An understanding of the limitations of colistin

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use in treatment, as well as knowledge as to whether colistin can comfortably continue to be used, is extremely important for the use of these drugs. Therefore, it is necessary to accurately define the susceptibility to polymyxin group antibiotics and to reveal their mechanisms of resistance [1,3].

2. Materials and Methods

Urine samples sent from the clinics to our Medical Microbiology laboratory were cultured on 5% KKA and EMB double agar media, while other samples were cultured on 5% KKA, EMB agar, and chocolate agar media, before being incubated at 37°C. Blood samples were incubated in an automated culture system (BACT/ALERT® 3D) and bottles with positive signals were inoculated on 5% KKA, EMB, and chocolate agar media, before being incubated at 37°C.

At the end of the incubation period, the bacteria grown on EMB agar with blood and/or chocolate agar were considered to be Gram negative. They were passaged onto TSI and SIM agar for further identification. At the same time, they were inoculated on Mueller–Hinton agar at a density of 0.5 McFarland; susceptibility studies were performed with antibiotic disks effective against Gram-negative bacteria. These disks are routinely applied in our laboratory in accordance with EUCAST recommendations. After incubation in citrate, urea, TSI, and SIM agar at 37°C for 18-24 hours, catalase and oxidase tests were performed on citrate-positive, non-hydrolyzing, non-fermenting, and immobile bacteria. Those with positive catalase test results and negative oxidase test results were subjected to Gram staining. Those with Gram-negative coccobacilli or diplococci morphology on Gram staining were identified on the species level using the *Acinetobacter spp*. preliminary identification (VITEK® 2 Compact BioMérieux, France) automated identification system. The antibiotic susceptibilities of A. baumannii were determined and the strains were tested for colistin susceptibility.

2.1. Determination of the Colistin Resistance of Isolates

The Mıcronaut™ Mıc-Strip™ (Merlin Diagnostika Gmbh®, Bornheim, Germany) system is a commercial liquid microdilution method. Lyophilized, pre-prepared antibiotics are incubated and evaluated via the rehydration of Kamhb. The Mıcronaut MIC-Strip™ is run on a single 12-well strip. On this strip, the first well is the positive control, while the subsequent wells are composed of increasing doses of colistin (0.0625-64 µg/mL).

To investigate the molecular mechanism of resistance, 29 colistin-resistant and 1 colistin-susceptible *A. baumannii* strains (ATCC 19606) were included in the study. At this stage, we examined the expression of the chromosomal PmrA gene, which has been reported in the literature to be responsible for colistin resistance.

DNA isolation *A. baumannii* strains stored at -80°C were resuspended for DNA isolation. One colony obtained using the single-colony dropping technique was inoculated into 1 ml of Luria–Bertani broth (LB) in a microcentrifuge tube under aseptic conditions, before being incubated at 37 ± 2 ºC for 18 ± 2 hours. After incubation, the following steps were adhered to, respectively, as shown in Tables 1 and 2.

Table 1. *PmrA* primers.

Gene	Primer Sequence
***PmrA* (Forward)**	5'-ATGACAAAAATCTTGATGATTGAAGAT-3'
***PmrA* (Reverse)**	5'-TTATGATTGCCCCAAACGGTAG -3'

The reaction was carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystem, USA). The cycles are shown in Table 2.

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Table 2. PmrA PCR cycles.

	Temperature	Time	
Initial denaturation	96 ºC	2 minutes	1 cycle
Denaturation	95 ºC	30 minutes	25 cycles
(Annealing)	52 ºC	30 minutes	
(Extension)	72 ºC	80 seconds	
(Final extension)	72 ºC	5 minutes	
	4 ºC	∞	

The PCR products of 29 strains with a PmrA (+) PCR result were sent to a company for sequencing (Atlas Biotechnology LTD, Ankara) within the scope of the scientific research project TTU-2020-1102, as coordinated by the Scientific Research Projects Coordination Unit of Recep Tayyip Erdoğan University.

The phylogenetic analysis of the sequencing results was performed in Clustal 2.1 Multiple Sequence Alignment using the reference strain *A. baumannii* (GenBank: CP009257.1).

This study was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Recep Tayyip Erdoğan University, Non-Interventional Clinical Research Ethics Committee (40465587-050.01.04-31 number 2020/20).

All analyses were performed using SPSS 25 (IBM SPSS Statistics, Chicago, USA). Numerical variables were expressed as mean ± standard deviation (SD) or median (min–max), and categorical variables were expressed as frequencies (n) and percentages (%). The Mann–Whitney U test was used to compare independent variables. A value of $p < 0.05$ was considered statistically significant for all data.

3. Results

In our study, 29 *A. baumannii* strains that were grown in different clinical samples that were sent to the microbiology laboratory of Recep Tayyip Erdoğan University Training and Research Hospital between June 2019 and June 2020 from patients who received colistin treatment and were followed up in intensive care units and wards were included. Figure 1 shows the agarose gel image after PmrA PCR.

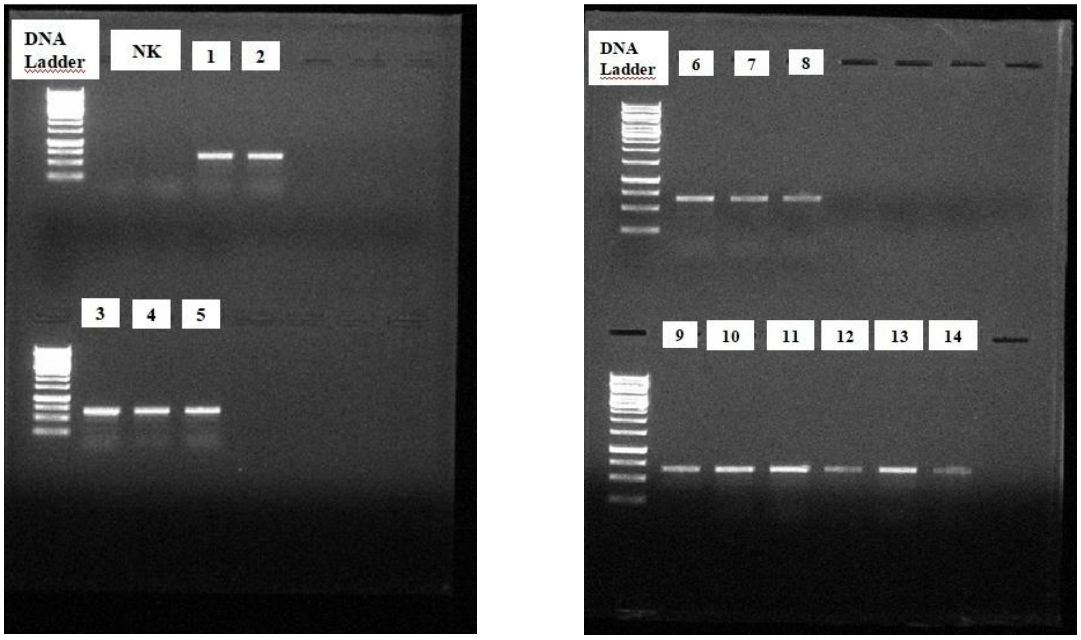

Figure 1. Agarose gel image from our study (NK: negative control).

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When the distribution of isolates was analyzed according to body site, the following isolates were found: 2 (6%) from the sputum, 1 (3%) from urine, 2 (6%) from blood/catheter, 10 (38%) from blood, 2 (3%) from a wound, and 12 (42%) from tracheal aspirate. The distribution of specimens sent to our laboratory is shown in Figure 2.

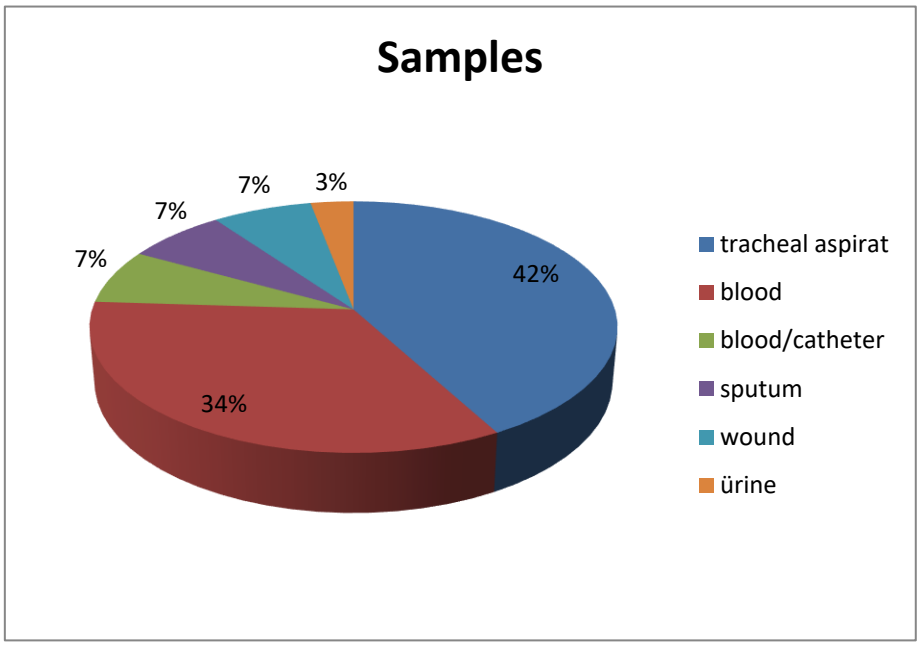

Figure 2. Distribution of samples. The distribution of samples according to clinics is shown in Table 3.

Table 3. Distribution of isolates according to clinics.

Clinics	n	%
Internal Medicine Intensive Care	26	89.6
Anesthesia Intensive Care	1	3.4
Infectious Diseases Service	1	3.4
Internal Medicine Service	1	3.4

When the MIC results were reviewed, 29 strains were resistant to colistin according to the VITEK® 2 Compact automated system (BioMérieux, France). VITEK® 2 Compact colistin results were 100% compatible with the liquid microdilution results in terms of susceptibility. A 0.5 McFarland standard turbidity suspension was prepared from all colistin-resistant isolates (MIC >2 mg/L) using liquid microdilution and the automated system. The suspension was broadly inoculated on the surface of Mueller–Hinton agar plates. After the plates were dried, commercial colistin-impregnated strips (Liofilchem SRL, Italy) were incubated in an oven at 35°C for 18-20 hours. The MIC values obtained for colistin were evaluated according to the recommendations of EUCAST (v12). Accordingly, if the MIC value at the points where the E-test strips intersected the inhibition ellipses was >2 mg/L, the isolate was considered resistant to colistin; if ≤ 2 mg/L, the isolate was considered susceptible. The results are presented in Table 4.

Table 4. Obtained MIC results (mg/L).

Strain Number	Broth Microdilution	VITEK 2	Colistin gradient test
1	>64	>=16	16
2	32	>=16	4
3	>64	>=16	4
4	4	>=16	4
5	32	>=16	1

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6	16	>=16	2
7	16	8	2
8	>64	>=16	16
9	8	>=16	4
10	32	4	4
11	64	>=16	8
12	>64	>=16	16
13	16	4	4
14	16	4	1
15	>64	>=16	8
16	>64	>=16	4
17	>64	>=16	4
18	64	>=16	2
19	64	>=16	2
20	>64	>=16	4
21	>64	>=16	4
22	32	4	2
23	>64	>=16	4
24	16	4	2
25	16	4	2
26	>64	>=16	8
27	64	>=16	4
28	>64	>=16	8
29	>64	>=16	8
30 ATCC 19606	1	0,5	0,5
31 NCTC 13846	4	>=16	4

After PmrA PCR, the PCR products were sent to Atlas Biyoteknoloji LTD, Ankara, for sequencing. The phylogenetic analysis of the sequence results was performed in Clustal 2.1 Multiple Sequnce Alignment, whereby a 175 bp section of a 675 bp gene region (PmrA) was amplified. Although point mutations were observed throughout the amplified region, they were more common in the first 60 bp (Figure 3).

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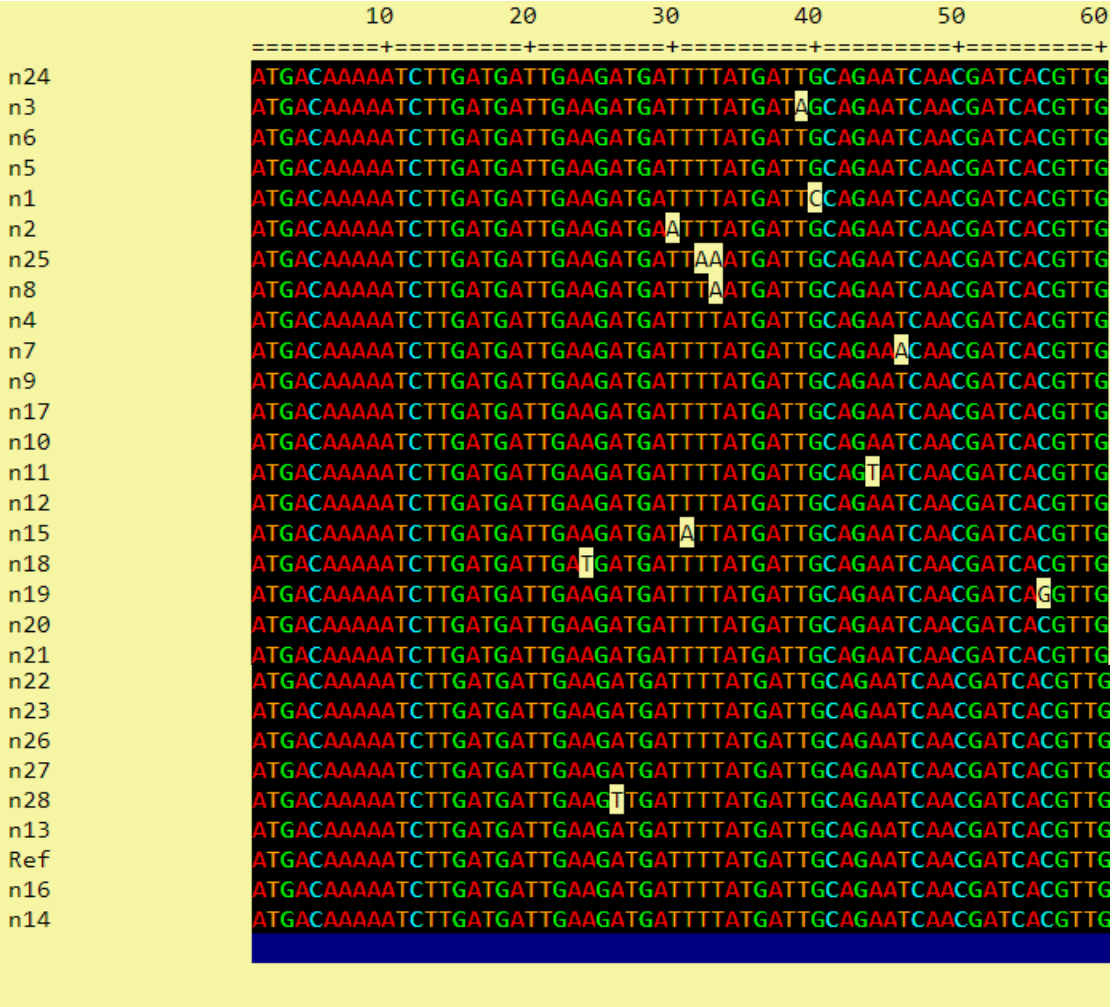

Figure 3. Sequence array between 10 and 60 bp (11 polymorphic regions).

The phylogenetic tree showing the relationship between 29 colistin-resistant A. baumannii strains and the reference strain [*A. baumannii* strain AB030 (GenBank: CP009257.1)] is shown in Figure 4.

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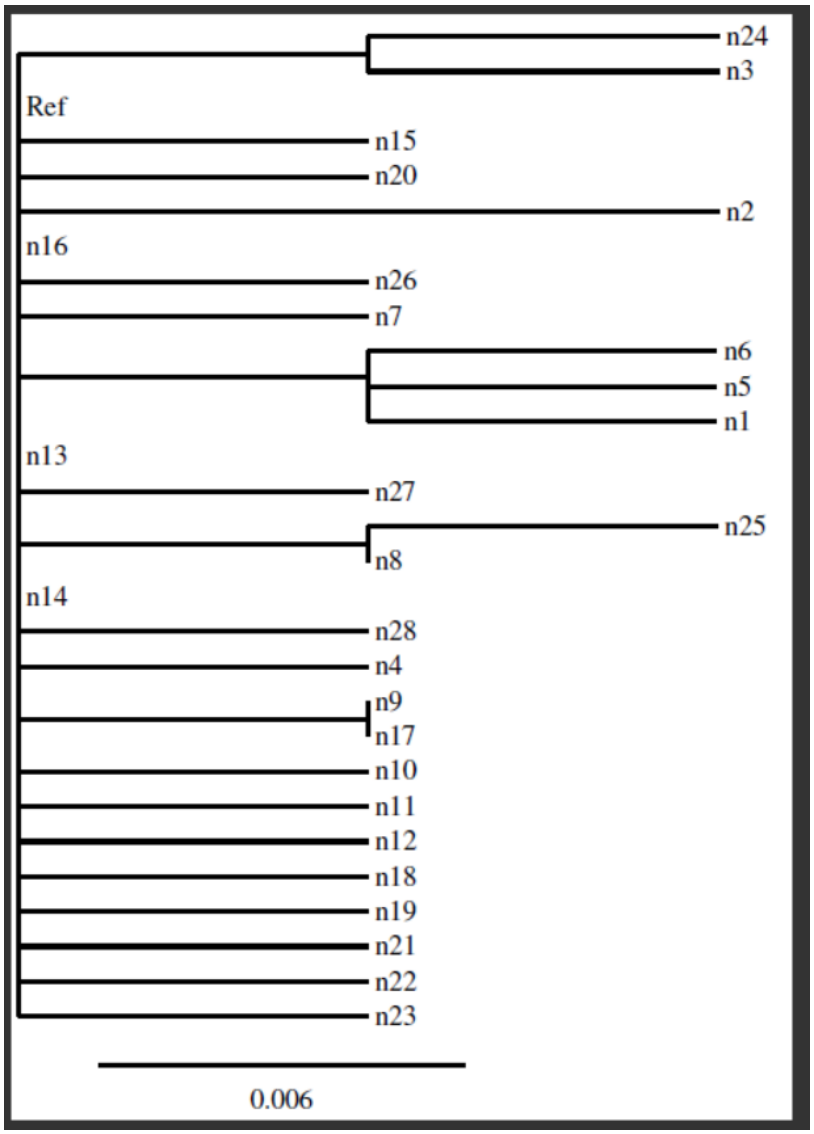

Figure 4. Phylogenetic (neighbor-joining) tree. The branch length is proportional to the number of SNPs among strains. The scale bar represents the average number of nucleotide substitutions per base.

Within the examined SNPs, six strains differed from the reference strain; these are shown in the circular phylogenetic tree (Figure 5).

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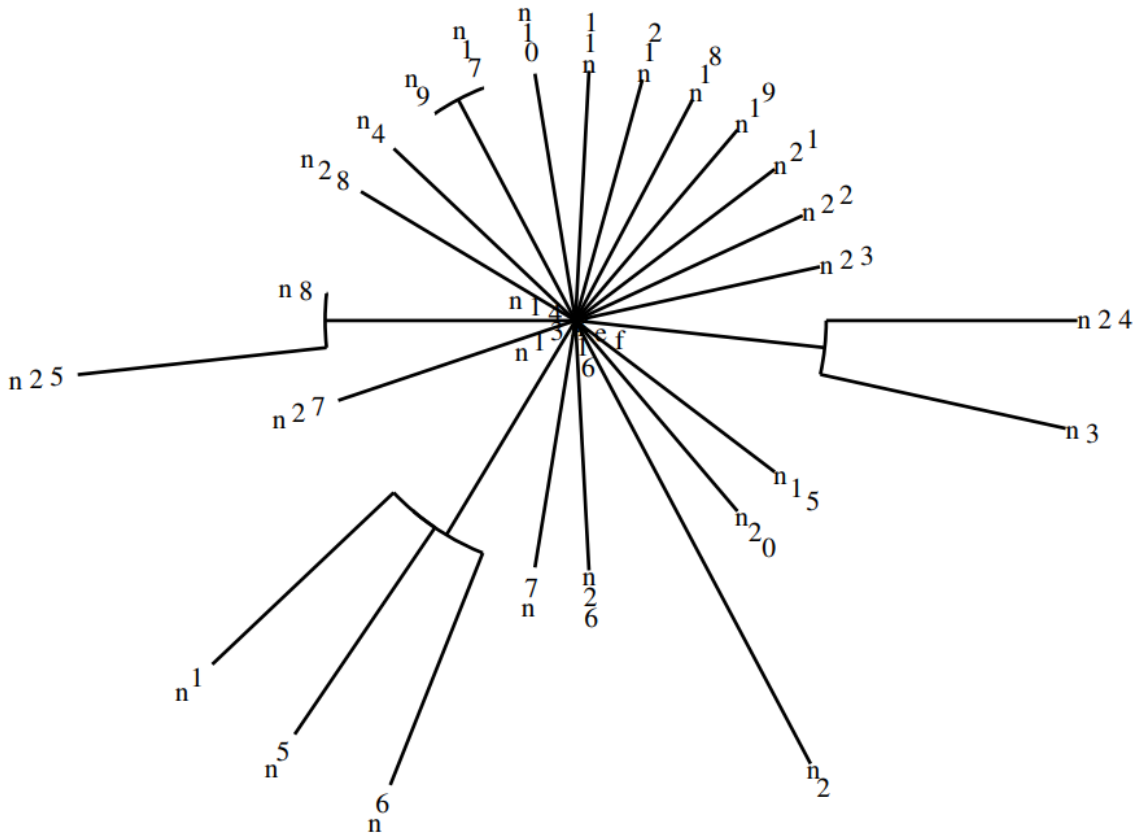

Figure 5. Circular phylogenetic tree of six strains differing from the reference strain according to SNPs.

4. Discussion

A. baumannii has emerged as being the causative agent of various infections worldwide [1]. In recent years, this species has been widely identified as the major cause of nosocomial outbreaks associated with high morbidity and mortality rates worldwide. Although *A. baumannii*, which is a Gram-negative opportunistic pathogen, possesses only a limited number of "traditional" virulence factors, the mechanisms underlying its pathogenicity remain of great interest due to its increasing prevalence [4]. *A. baumannii* is naturally resistant to many antimicrobials but has developed resistance to a variety of antibiotics used in its treatment (such as β-lactams, fluoroquinolones, tetracyclines, and aminoglycosides) due to its capacity to readily acquire new antimicrobial resistance determinants [5]. Polymyxin group antibiotics, especially colistin (with various combinations), are currently used in the treatment of MDR *A. baumannii*. The accurate identification of susceptibility to polymyxin group antibiotics, as well as their mechanisms of resistance, is crucial for the use of these drugs [6]. It is thought that without serious intervention, hospital-acquired *A. baumannii* infections may soon become untreatable [5].

The colistin resistance mechanism of *A. baumannii* has not yet been fully elucidated. As in the colistin resistance mechanism of other Gram-negative bacteria, LPS modification, as a result of a mutation in the PmrAB two-component regulatory system, has an important place in the literature [7].

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In this study, we aimed to understand from which species and units the colistin resistant *A. baumannii* strains isolated from the samples sent to our laboratory were isolated the most, as well as which strains had PmrA gene polymorphism/mutation and a phylogenetic relationship between PmrA genes. The purpose of examining the PmrA gene in this study is that colistin resistance in *A. baumannii* is frequently caused by mutations in the two-component PmrAB gene.

In a study investigating healthcare-associated infections in our country, it was reported that A. baumanii was the most common infectious agent in the ICU, causing respiratory tract infections. It was most frequently detected in tracheal aspirate samples [8]. In many studies conducted in our country, it has been reported that *A. baumannii* strains were mostly isolated from patients hospitalized in intensive care units [9]. In many studies conducted globally, *A. baumannii* has been the most common opportunistic pathogen in ICUs, causing respiratory tract infections and being the most frequently produced from samples such as tracheal aspirate due to its long residence time on surfaces [10]. Similarly, in our study, tracheal aspirate (42%) was the most common sample type in which *A. baumannii* was detected. It was isolated most frequently in the ICU, especially in the internal medicine ICU (89.6%). These data emphasize the necessity of infection control measures, especially in the ICU, for this agent, which is strongly associated with healthcare-associated infections.

Colistin was first discovered in 1947; however, its use was limited in the 1980s due to the renal toxicity manifested by acute tubular necrosis and neurotoxic side effects such as vertigo, visual disturbances, confusion, ataxia, and neuromuscular blockade [11]. In recent years, it has been observed that colistin has a high therapeutic success against serious infections caused by MDR Gram-negative bacteria [12]. The mechanism of resistance of MDR *A. baumannii* to colistin should be clarified to prevent the spread of this microorganism. Although different hypotheses have been put forward regarding the mechanism of action of colistin, the most widely accepted mechanism is that it binds to LPS and disrupts the phospholipid bilayer, causing osmotic imbalance, which leads to bacterial death [13].

The complete loss of the lipopolysaccharide layer or mutations in LPS can lead to colistin resistance by blocking the effect of colistin on the cell membrane [14]. Mutations in the lpxA, lpxC, and lpxD genes associated with lipid A synthesis result in the complete loss of LPS, causing colistin resistance [15]. Another resistance mechanism is the decrease in the negative charge of LPS due to the addition of phosphoethanolamine to lipid A as a result of increased PmrCAB expression due to mutations in the PmrAB regulatory system [16]. In the literature, there are cases in which colistin treatment led to PmrAB mutation and paved the way for resistance [17]. Another colistin resistance mechanism that leads to lipopolysaccharide modification occurs with the addition of galactosamine to lipid A by increasing naxD expression as a result of PmrB mutation [18]. In addition to the colistin resistance mechanisms caused by chromosomal mutations, colistin resistance caused by plasmid-mediated mcr-1 gene transfer has been detected in some Gram-negative bacteria; however, this has not yet been detected in *A. baumannii* [19].

In a study based on whole-genome sequencing, 21 colistin-resistant *A. baumannii* strains were examined; PmrAB mutation was detected in 71.4% of them [20]. In a study conducted with 29 patients in Türkiye, the PmrCAB region was examined, and a total of 14 non-synonymous mutations were detected, i.e., one in PmrA, nine in PmrB, and four in PmrC [21]. Oikonomou et al. [22] associated PmrA and PmrC mutations with colistin resistance; they observed that these mutations did not increase resistance to other antimicrobials. In an American study, PmrA and PmrB gene mutations were found in all 14 colistin-resistant strains isolated from patients, and colistin resistance was associated with PmrAB mutations [17]. In our study, PmrA mutation was found in colistin-resistant strains in parallel with these studies. Although PmrAB mutations have been reported to cause heterodistance in addition to colistin resistance, the number of studies in the literature is quite small since heterodistance cannot be determined using routine tests [16].

Not all mutations occurring in PmrAB cause colistin resistance. Lean et al. [23] found 3 point mutations in the PmrA gene, 11 in the PmrB gene, and 8 in the PmrC gene; however, there was no significant difference between colistin-sensitive and -resistant isolates. Similarly, in a study

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conducted in China in which different clinical isolates were examined, several mutations were found in this gene region, but these mutations were not associated with colistin resistance [24]. This difference may be due to the fact that different gene regions of PmrAB were examined in the studies.

In a study conducted in the UK, no mutations were found in the PmrA and PmrC gene regions in colistin-resistant isolates, while mutations were found in the PmrB gene region. In addition to the absence of PmrA mutations, PmrA expression was found to be increased up to 4-13-fold in colistin-resistant isolates [25]. This reveals the importance of a holistic view not only of PmrA but also of the PmrCAB complex.

In another study conducted in the USA, a model for the N-terminal domain and full-length PmrA structure was created using X-ray crystallography to solve the structure of PmrA in MDR A. baumannii. Through biochemical and computational approaches, detailed information on two biologically relevant PmrA mutants and their potential structural disruptions was obtained [26], providing information on the complexity and diversity of colistin resistance in *A. baumannii* and highlighting the need for further studies.

Sequence analysis of the PmrA gene region and determination of the resulting changes is one of the first studies conducted in our country related to this field. When our study was evaluated in terms of phylogenetic relationship, the mutations in the samples were found to be closely related to each other. In contrast to some studies that failed to associate PmrA mutation with colistin resistance, we found that mutations in the 175 bp region of the PmrA gene were associated with colistin resistance, potentially paving the way for future studies in this field.

This study shows the necessity of conducting more detailed studies with larger budgets and larger sample pools for a microorganism such as *A. baumannii*, which is undoubtedly one of the most dangerous opportunistic pathogens globally; it continues to be on our agenda due to its resistance to the last-resort drug colistin. Our study is of great importance as a reference for both the epidemiologic and phylogenetic diversity of colistin resistance in colistin-resistant *A. baumannii* strains to be isolated from our region.

Multidrug-resistant *A. baumannii* isolates are an important problem in intensive care units; the morbidity and mortality of infections caused by these isolates are high. Increasing resistance to colistin decreases the treatment options for infections caused by these isolates. In any case, the increase in resistance to colistin has reached alarming proportions. The irregular, incorrect, and unconscious use of colistin, which is preferred to treat infections caused by multidrug-resistant *A. baumannii*, has drastically increased the resistance to this molecule. As such, specific colistin use guidelines should be established for each patient.

Resistance to colistin and antibiotics is an indication of the need for new antibiotic regimens. New molecules should be developed and resources should be allocated to prevent antibiotic resistance, which is a common problem of humanity and is on the agenda of the United Nations.

In addition, it is clear that it is very important to monitor the resistance rates of these isolates, especially in the ICU, and to take the necessary infection control measures to prevent the colonization of resistant isolates in hospitals.

Next-generation genomic technologies are a very good option to determine the exact mechanism of colistin resistance in multidrug-resistant *A. baumannii* isolates. However, since these technologies are costly, reference centers should be established and identified resistant strains should be subjected to further research.

In order to prevent resistance globally, serious policies should be developed, preventive services should be increased, and traditional and natural methods should be used more frequently to increase treatment options.

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