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INVESTIGATION OF VIRULENCE FACTORS AND ANTIBIOTIC RESISTANCE OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM A RANGE OF CLINICAL SAMPLES

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Abstract

Objective: *Pseudomonas aeruginosa* is an opportunistic pathogen, is one of the leading nosocomial infection-causing agents and over time has developed multidrug resistance. One of the most common patient groups affected by *P. aeruginosa* are on the intensive care unit (ICU), an optimal environment for the development of antibiotic resistance. The aim of this study was to investigate virulence factors and antibiotic resistance profiles of *P. aeruginosa* isolated from hospitalized patients in Turkey.
Methods: Samples from the general wards and ICU-hospitalized patients were included. A nutrient agar-elastin method was used for the biochemical activity of elastase. For *las B* assessment PCR was used while special production medium was used to assay pyoverdine and pyocyanin. Isolate biofilm production was tested with the crystal violet method. Standard broth microdilution was used for antibiotic susceptibility.
Results: A total of 208 samples were assessed. The virulence factor frequencies in ICU and ward isolates, were: pyocyanin 86.2% and 86.7%, pyoverdine 90.1%, and 89.6%, elastase 68.6% and 67.9%, *las B* 93.1% and 89.6%, and biofilm production 51.9% and 48.1%, respectively. Antibiotic resistance rates in ICU and ward were: meropenem 41.1% and 28.9%, colistin 11.7% and 13.2%, ceftazidime 43.1%,and 41.1%, and cefepime 52.9% and 48.5%.
Conclusion: Virulence factors were present in most of the hospitalized patient samples. However, antibiotic resistance rates were below 50%, except for cefepime. In addition, low rates of colistin resistance suggest that colistin resistance is not yet widespread in our hospital.

Keywords: *Pseudomonas aeruginosa, virulence factors, biofilm, antibiotic resistance.*

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative, non-fermentative, aerobic bacillus. *P. aeruginosa* is found in various natural environments, like soil and water, as well as in hospital settings where it can be present in equipment, including sinks, cleaning materials, catheters and respirators that come into direct contact with the patient. Thus, it is also an opportunistic pathogen that is commonly the cause of nosocomial infection. As *P. aeruginosa* has simple requirements for growth, it can be produced in many media in the laboratory. *P. aeruginosa* causes nosocomial infections in respiratory patients and especially in cystic fibrosis (CF) patients but is also common in urinary tract infection and skin infections, generally after a burn. It contributes significantly to morbidity and mortality rates through a range of virulence factors and resistance to antibiotics, especially in immunosuppressed patients.[1]

P. aeruginosa causes damage to the host organism through various factors, such as adhesion factors, secreted enzymes, and toxins. The toxins it secretes (ExoA, ExoY, ExoS) induce host cell necrosis, lung damage, disruption of the host epithelial membrane, and damage protein synthesis.[1,2] In addition to these toxins, *P. aeruginosa* may also secrete enzymes. Elastase causes host tissue damage by degrading extracellular matrix components, such as elastin, collagen, and fibronectin.[2] Therefore, elastase plays an important role in infections with *P. aeruginosa*. The enzyme activity of elastase is dependent on the genes *lasA*, *lasB*, *lasR*, and *rhlR*. The *lasB* gene is the structural gene of elastase and is the gene responsible for enzyme activity.[3] *LasA* increases the elastolytic activity of elastase, but not the proteolytic activity. Pyocyanin and pyoverdine are virulence factors that are produced as secondary metabolites and have effects including binding iron in the environment (pyoverdine) and causing DNA damage by increasing reactive oxygen species (ROS) production (pyocyanin) in the host cell. A study published in 2019 showed that pyoverdine accumulation was associated with pathogenicity and host death in *P. aeruginosa* specimens isolated from 70 pediatric patients with CF, and in their model organisms which were *Caenorhabditis elegans* and murine model of acute pneumonia.[4]

Biofilm is a community formed by microorganisms to protect themselves under stress conditions. The microorganisms produce a matrix of extracellular polymeric substances (EPS), consisting of protein, carbohydrates, extracellular DNA (eDNA) and lipids. A typical biofilm is formed through several stages, which are adhesion of bacteria to the surface, proliferation, microcolony formation (resembling a mushroom-like structure), and finally separation from the community by planktonic movements.[5] The strong biofilm formation capability of *P. aeruginosa* plays an important role in its resistance to antibiotics and thus its survival. In some strains of *P. aeruginosa*, a mucoid form is observed in the colony morphology due to overexpression of alginate. The mucoid form is frequently observed in strains isolated from CF patients.[6] Long-term colonization of *P. aeruginosa* in the lungs of patients with CF and biofilm formation with a thick mucoid layer plays an important role in the severity of the course of the disease by increasing resistance to antibiotics used in the treatment.[7] Biofilm formation in chronic wounds leads to symptoms, such as paleness and wound bed edema, fragile granulation tissue, tissue decay, wound pain and odor.[8]

Multi-drug-resistant (MDR) and widely drug-resistant (XDR) *P. aeruginosa* are a global clinical threat.[9] Thus *P. aeruginosa* is important for improved public health and is especially important in patients with CF.[10] In general, the main mechanisms of *P. aeruginosa* antibiotic resistance can be grouped under three headings: intrinsic, acquired, and adaptive resistance. Intrinsic resistance includes decreased outer membrane permeability, expression of efflux pumps that excrete antibiotics from the cell, and production of enzymes, such as β lactamase, that inactivate antibiotics. Acquired resistance is due to mutation of the *P. aeruginosa* genes.[11] Finally, adaptive resistance occurs because of changes due to environmental conditions, such as growth status or exposure to stress.[12]

Colistin is one of the polymyxin group of antibiotics that interact with bacterial lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria and acts by disrupting the permeability of the bacteria.[13] The use of colistin has recently increased again due to the resistance of *P. aeruginosa* towards newer antibiotics. However, *P. aeruginosa* has also started to develop resistance against colistin.[14]

In the present study, antibiotic resistance, and prevalence of virulence factors (pyoverdine, pyocyanin, elastase activity, biofilm formation) were investigated and compared in *P. aeruginosa* isolates from intensive care and ward patients in a Turkish University hospital.

Methods

Sample Collection and Identification

Samples routinely sent to the Microbiology Laboratory from both the intensive care unit (ICU) and wards between 2018-2021 were included in the study. All samples were evaluated in a single laboratory. Clinical samples were identified by oxidase, hemolysis, colony morphology and gram staining.

Pyocyanin and Pyoverdine

Special isolation agars were used to identify isolates with these virulence factors. These were pseudomonas isolation agar F (Biolife, Italia) for Pyocyanin and isolation agar P (Biolife, Italia) for pyoverdine.

Bacteria were inoculated onto the isolation agars and were incubated overnight at 37 ºC. For pyocyanin, the presence of blue-green colony colors on the media was considered a positive result, while a yellow green color in the medium was considered positive for pyoverdine.

Nutrient Agar-Elastin

The nutrient agar-elastin method was used to identify isolates with elastase activity. 0.01% elastin (Sigma-Aldrich, Germany) was added to the prepared nutrient agar medium mixture. Either two or three patient samples were inoculated by streaking. The agar plates were then incubated at 37 ºC for 48 hours. The clearance around the inoculum line was evaluated.

Detection of *lasB* Gene Expression by Polymerase Chain Reaction

The *lasB* gene region in individual isolates of *P. aeruginosa* was examined by polymerase chain reaction (PCR). DNA from the isolates was extracted by the boiling method. Breifly, the colonies of *P. aeruginosa* were suspended in 200 µL sterile distilled water, heated for 10 min at 96°C and then centrifuged at 13,000 rpm for 5 minutes. DNA from the supernatant portion of the suspension was used. The primer

sequence (forward)-ACAGGTAGAACGCACGGTTG, (reverse)-GATCGACGTGTCCAAACTCC with 1220 bp was used. PCR conditions were: initial temperature 94 ºC for 5 minute denaturation, 35 one-minute cycles at 94 ºC, annealing temperature 57 ºC for one minute and elongation at 72 ºC for one minute. Final elongation cycle was 10 min at 72 ºC.[15] Distilled water was used as negative control and *P. aeruginosa* PAO1 ATCC 47085 was used as positive control.

The band images were examined by autoradiography on a 1% gel containing 0.5% ethidium bromide.

Biofilm

Biofilm formation was measured using the dye crystal violet method using microtiter plates. Based on the optical density of the positive control (ODi) using the *P.aeruginosa* ATCC 27853 strain and on the average of the optical density of the negative control (ODc), the samples were classified as biofilm producer (2xODc< ODi) or biofilm non-producer (ODi < ODc).[16]

Antibiotic Susceptibility

The broth microdilution method was used for testing antibiotic susceptibility according to EUCAST.[17] Turbidity in the wells was evaluated visually and the Minimum Inhibition Concentration (MIC) results were recorded. For this test the positive control was the same as for the test for biofilm production.

This study was approved by the Ethics Committee approval KÜ GOKAEK 2022/01.11. It was performed in accordance with the principles of the Declaration of Helsinki. Since our study was a retrospective, informed consent was not required from individual patients.

Statistical Analysis

Statistical analysis was done with IBM SPSS, version 20.0 (IBM Corp., Armonk, NY, USA). Numerical variables are given as number and percentile. Fisher's exact chi-square test, Yates' chi-square test and Monte Carlo chi-square test were used to evaluate the differences between groups. A $p<0.05$ was considered sufficient for statistical significance in two-way tests.

Results

Patient Samples

A total of 208 *P. aeruginosa* samples collected from hospitalized patients between 2018 and 2021 were included. Of these, 102 (49%) were from the ICU and 106 (51%) from general wards. Among the ICU patients, 42 (41.2%) were female and amongst the ward patients 42 (39.6%) were female. The most frequently evaluated sample was aspiration samples from ICU and while the most frequently received samples from the wards were wound and sputum samples (Table 1).

Table 1. Source and type of *P. aeruginosa* isolates in clinical samples.

Samples	Intensive care unit	Ward unit
Sputum	4	29
Wound	11	29
Urine	6	21
Aspiration	55	2
BAL*	3	2
Blood	11	1
Other	11	22

*Bronchial Alveolar Lavage

Pyocyanin and Pyoverdine

The prevalence of pyocyanin and pyoverdine positivity were similar in samples from ward patients (86.7% vs. 89.6%) and ICU patients (86.2% vs. 90.1%). The distributions according to patient samples is shown in Table 2. Positivity for pyocyanin and pyoverdine were detected in the majority of both groups.

Nutrient Agar-Elastin

The clearance around the inoculation line is considered positive (Figure 1). Elastase activity was positive in 67.9% of ward patients and 68.6% of ICU patient samples. Furthermore, elastase activity was present in 39 of 55 (70.9%) aspiration samples and 8 of 11 (72.7%) wound samples, most commonly isolated in ICU. The sputum samples were the most common samples sent for analysis. In ward patients, elastase activity was observed in 19 of 29 (65.5%) sputum samples and in 26 of 29 (89.65%) wound samples (Table 2).

Table 2. Distribution of virulence factors among 208 *P. aeruginosa* clinical samples

	Intensive Care Unit					Wards				
	Pyocyanin	Pyoverdine	*las B*	Elastase	Biofilm	Pyocyanin	Pyoverdine	*las B*	Elastase	Biofilm
Sputum	2	2	3	3	3	23	27	26	19	17
Wound	10	10	11	8	6	26	25	26	26	12
Urine	4	6	4	3	6	18	17	18	14	11
Aspiration	50	50	55	39	28	2	2	2	1	0
BAL*	3	3	3	2	1	2	2	2	2	1
Blood	8	10	9	8	6	1	1	1	1	0
Other	11	11	11	7	3	20	21	20	15	10

*Bronchial Alveolar Lavage

Detection of las*B* Gene Expression

In the studied isolates, the *las B* gene region was detected in 89.6% of the ward patients and 93.1% of the ICU patient samples. The gel image of the *las B* gene (1220 bp) region is shown in Figure 2.

Biofilm Formation

Biofilm formation was found in 48.1% of ward patients and 51.9% of ICU patients. While 28 were positive for biofilm formation from ICU aspiration samples, 17 were positive from ward sputum samples. After sputum samples from

ward patients, the next most common sample type for biofilm formation was wound samples (Table 2).

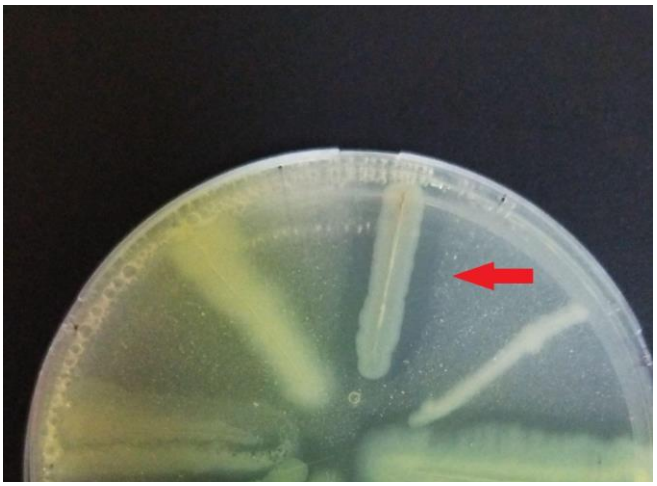

Figure 1. Appearance of elastase (+) isolates on agar plates (indicated by the arrow).

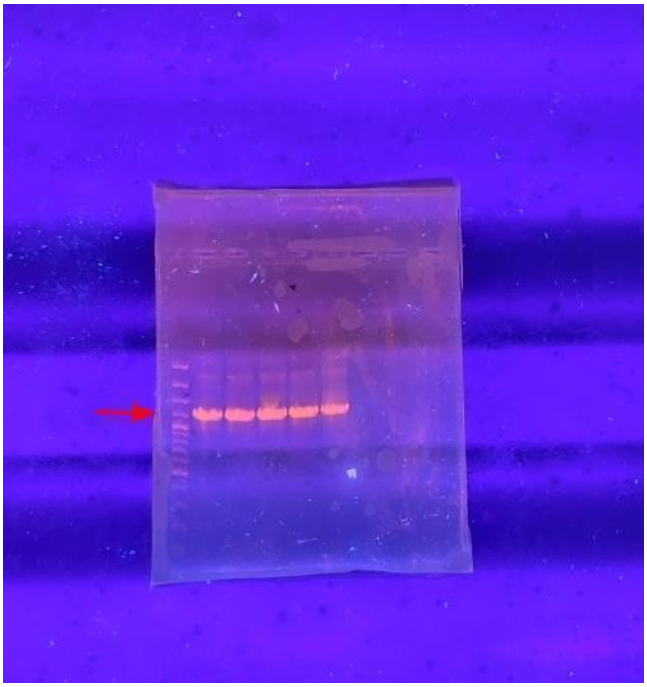

Figure 2. PCR amplification of *las B* (1220bp) among *P. aeruginosa* isolates.

Antibiotic Susceptibility

The breakpoints of antibiotics for *P. aeruginosa* meropenem (8 mg/L), colistin (4 mg/L), ceftazidime (8 mg/L), and cefepime (8 mg/L) were evaluated, as recommended by EUCAST. Results are shown in Table 3. Fourth generation cefepime resistance was found to be higher than other antibiotics in both groups. This was followed by ceftazidime and meropenem, respectively. The prevalence of resistance to the antibiotic colistin was the lowest in both groups and colistin resistance rates did not differ significantly between groups (Table 3).

The role of biofilm formation in antibiotic resistance was evaluated by analysis of the resistance profiles of antibiotics in biofilm-positive samples. This showed meropenem resistance at 45.2%, colistin at 15.0%, ceftazidime at 39.6% and cefepime at 52.8% in 53 ICU patient samples. In ward patient samples with biofilm positivity, these rates were 25.4% for meropenem, 9.8% for colistin 39.2% for ceftazidime and 45.0% for cefepime (Table 4).

Table 3. Antibiotic resistance of *P. aeruginosa* isolates

Antibiotics	Intensive Care N (%)	Ward N (%)	*p* value
Meropenem	42 (41.1)	31 (28.9)	0.079
Colistin	12 (11.7)	14 (13.2)	0.733
Ceftazidime	44 (43.1)	44 (41.1)	0.860
Cefepime	54 (52.9)	52 (48.5)	0.626

Table 4. Association between antibiotic resistance and biofilm

Antibiotics	Intensive Care R (%)	*p* value	Ward R (%)	*p* value
Meropenem	24(45.2)	0.500	13(25.4)	0.545
Colistin	8(15.0)	0.164	5(9.8)	0.478
Ceftazidime	21(39.6)	0.586	20(39.2)	0.792
Cefepime	28(52.8)	0.861	23(45.0)	0.432

R: Number of resistant isolates in biofilm positive samples

Discussion

The aim of this study was to investigate if *P. aeruginosa* isolates found in hospitalized patients in a Turkish University hospital differed in terms of virulence characteristics and antibiotic resistance between patients in general wards and in the ICU. A further aim was to contribute to the understanding of the resistance profile in Turkey by evaluating *P. aeruginosa* resistance to the promising antibiotic, colistin for use against *P. aeruginosa*.

Pyocyanin and pyoverdine are important virulence factors in *P. aeruginosa* infection. Pyocyanin increases production of intracellular ROS, damages cell cycle components and some enzymes and causes damage to host cell DNA.[18] Pyoverdine is a siderophore with a peptide structure. The accumulation of pyoverdine, especially in CF patients, is an important virulence factor that increases the mortality of the disease.[3,19] In addition to absorbing iron, pyoverdine acts as signaling molecules for the production of two further virulence factors, endo-proteinase and exotoxin A.[1,19] There are many studies on the prevalence of these two factors and their relationship with clinical outcomes. El-Mahdy et al. found the prevalence of pyocyanin in 80 clinical *P. aeruginosa* isolates to be 58.8%.[19] In contrast, in the present study, the prevalence of pyocyanin production was 86.7% in general ward samples and 86.2% in ICU samples. El-Mahdy et al. explained their lower prevalence figures because some of the isolates they studied were MDR. In another study published in 2018, pyocyanin was found in 87% of *P. aeruginosa* isolates from 61 respiratory samples, which is in keeping with our findings.[20]

In a study of ten *P. aeruginosa* isolates with MDR, pyoverdine production was observed in all of the isolates.[21] This same study reported that there was a positive relationship between biofilm production and pyoverdine, which also affected the pathogenicity of *P. aeruginosa*. In the present study, pyocyanin and pyoverdine factors were found in more than 85% of all samples, similar to previous reports.[20,21] These results suggest that these factors are currently common virulence factors in clinical isolates of *P. aeruginosa* and may thus play an important role in pathogenicity.

In a study conducted at Ege University Hospital, Izmir, Turkey, elastase enzyme activity was evaluated in 83 samples of *P. aeruginosa* isolates from different clinics, and enzyme activity was observed in 100%.[22] When elastase activity in *P. aeruginosa* strains isolated from ICU patients at the University of Pittsburgh Medical Center was examined, it was found in 75% of the isolates and there was an association with higher mortality.[23] Although elastase enzyme activity were not as frequent in samples from the present study as in these earlier reports, elastase activity was present in nearly 70% of samples from ICU and ward patients. In another study examining *las B* by PCR, in addition to elastase activity, the *las B* gene was detected in 98% of *P. aeruginosa* isolated from clinical samples.[20] In another study, *las B* was detected in 82% of 54 *P. aeruginosa* isolates.[15] Our study results were consistent with these previous studies. In addition, there seems to be a difference in frequency between the presence of the *las B* gene and enzyme activity in our isolates. We speculate that the reason may be a suppression of *las B* gene expression or a down-regulation of the *las A* gene which plays a role in increasing enzyme activity.

In a study from Turkey, 60 *P. aeruginosa* isolates from patients with CF were examined and it was reported that 33% of them formed a biofilm.[24] In another study with *P. aeruginosa* isolated from different clinical samples, biofilm formation was detected in 58% of 104 isolates.[25] In the present study biofilm formation was present in 48.1% of ward patients and 51.9% of ICU patients. The relationship between antibiotic resistance and the presence of biofilm was investigated but no significant relationship was determined.

Infections caused by MDR *P. aeruginosa* are becoming more common globally.[26] *P. aeruginosa* causes increased morbidity and mortality due to its ability to develop rapid resistance to various antibiotics.[26,27] In a study from Iran published in 2018, meropenem resistance was 53.6% and ceftazidime resistance was 63.7% in 138 *P. aeruginosa* clinical isolates.[27] In a different study conducted with *P. aeruginosa* isolated from various clinical samples, 63% of 175 isolates were found to be resistant to ceftazidime and 5.3% to meropenem.[28] In a study of 120 *P. aeruginosa* isolates from Turkey, meropenem resistance was present in 90.1% and ceftazidime in 7.5%.[29] The authors reported that meropenem resistance was associated with a history of cerebrovascular attack, and ceftazidime was associated with a history of stay in the neurology ICU. In comparison, we found higher ceftazidime (43.1%) and lower meropenem (41.1%) resistance. This may be due to different sources of the clinical samples, which were a general ICU in our study and the neurology ICU in the earlier study from Turkey.

In another retrospective study from Turkey, meropenem, ceftazidime and cefepime resistance were found to be 23%, 26%, and 28%, respectively, from 631 *P. aeruginosa* isolates.[30] Dursun et al. found the resistance rate for ceftazidime to be 33.8% and for cefepime 19.1% for *P. aeruginosa* isolates in the pediatric ICU.[31]

In a study from Balikesir University, Turkey, the antibiogram results of *P. aeruginosa* strains isolated from the ICU showed resistance rates to ceftazidime of 29.4% and cefepime of 28.1%.[32] Again, the sample type in which *P. aeruginosa* was mostly isolated was an aspiration sample, which was consistent with our findings. In the present study these resistance rates were found to be higher as 43.1% to ceftazidime and 52.9% to cefepime.

Especially in recent years, the emergence of resistance to colistin, which has been used in *P. aeruginosa* infections, makes treatment more challenging. Therefore, colistin resistance has become an important issue, as shown by the many recent studies.[29,33,35] In a surveillance study conducted with data from many regions between 1997 and 2016, *P. aeruginosa* was one of the most common pathogenic bacterial species. It ranked first in Europe, the Asia Pacific region, and Latin America. However, in 2015-16 colistin susceptibility was 99.8%, 99.7%, 100.0% in Europe, the Asia Pacific region, and Latin America, respectively.[36] In contrast, colistin resistance was present in 100% of clinical *P. aeruginosa* isolates evaluated in Ankara Research Hospital in Turkey in 2016.[29] In a more recent study, colistin resistance was reported at a very low rate of 5% in 420 *P. aeruginosa* isolated from ICUs.[33] In the present study, the colistin resistance rate was 11.7% in isolates from ICU patients. Among the reasons why colistin resistance was found to be higher in our sample group may be the inclusion of recently collected samples (2018-2021). In another study published in 2018, Şafak et al. reported that amikacin and colistin were the most effective antibiotics in inpatients.[34] Santos et al. found colistin resistance of 3.89% in 62 clinical *P. aeruginosa* isolates.[35]

Colistin resistance has come to the fore with the addition of polymyxin group antibiotics to the treatment list, as a result of the development of *P. aeruginosa* resistance to many other antibiotics in recent years. The limited treatment options for *P. aeruginosa* makes the issue of colistin resistance important. It is reassuring that colistin resistance was not high in *P. aeruginosa* strains isolated from our hospitalized patients, in both ward and ICU patients, and that there was no significant difference between the groups, suggests that colistin can still be used as an option in *P. aeruginosa* infections.

Conclusion

The prevalence of the evaluated virulence factors differed between ICU and ward patients. Virulence factors were found in high proportions of isolates from both ICU and ward patients. This highlights the importance of developing treatments for these factors.

In addition, colistin resistance was only found in a low proportion of isolates from both ICU and ward patients. This suggests that there is not widespread resistance to colistin in isolates from our region and thus colistin is a viable option for the treatment of *P. aeruginosa* infection in hospitalized patients in our region.

Limitations

The isolates used in our study are isolates collected between the years 2018 and 2021. Conducting studies evaluating more antibiotic types with an even larger sample group, including current samples, may provide more robust results. In addition, more comprehensive studies on the clinical features of the evaluated isolates and the patients from whom the samples were taken may be important to understand the effect of virulence factors.

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Conflict of Interest
There is no conflict of interest between the authors.

Compliance with Ethical Statement
This study was approved by the Ethics Committee approval KÜ GOKAEK 2022/01.11. It was evaluated in accordance with the principles of the Declaration of Helsinki. Since our study was a retrospective, an informed consent form was not used.

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Author Contributions
All of the authors declare that they have all participated in the study design, data preparation, analysis, literature reviewing, manuscript writing and critical review of the paper, and that they have approved the final version.

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