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Biofilm formation and antibiotic resistance in patients with urolithiasis: assessment of phenotypic and genotypic

Ürolitiazisli hastalarda biyofilm oluşumu ve antibiyotik direnci: fenotipik ve genotipik değerlendirme

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Abstract

Aim: Urolithiasis is a common public health problem that significantly impacts the community. The objective of this study was to identify the prevalent pathogens responsible for urinary tract infections in patients with urolithiasis and investigate the biofilm forming ability of these strains phenotypically and molecularly.

Materials and Methods: A total of 100 patients who presented to Kirkuk Training and Research Hospital with symptoms of urinary tract infection and were diagnosed with kidney stones were included in our study conducted between May 2021 and November 2022. Clinically significant bacteria from urine samples were identified using routine conventional methods. Biofilm formation of the identified strains was examined by microplate method.

Results: The most frequently isolated agents were *Escherichia coli* (n:36) and *Proteus mirabilis* (n:17). Biofilm formation was detected in 89% of *E. coli* strains and 94% of *P. mirabilis* strains.

Conclusion: The results obtained are important in terms of high biofilm formation, especially in *E. coli* and *P. mirabilis* strains, and the frequent presence of genes related to this biofilm formation.

Keywords: Urolithiasis, Biofilm, Kidney stones, Resistance genes.

Öz

Amaç: Ürolitiyazis, toplumu önemli ölçüde etkileyen yaygın bir halk sağlığı sorunudur. Bu çalışmanın amacı; ürolitiyazisli hastalarda gelişen üriner sistem enfeksiyonlarının yaygın etkenlerini saptamak ve bu kökenlerin biyofilm oluşturma yeteneğinin fenotipik ve moleküler olarak araştırılmasıdır.

Gereç ve Yöntem: Mayıs 2021 ile Kasım 2022 tarihleri arasında yürüttüğümüz çalışmamıza Kerkük Eğitim ve Araştırma Hastanesi'ne idrar yolu enfeksiyonu semptomları ile başvuran ve böbrek taşı tanısı alan toplam 100 hasta dahil edildi. Alınan idrar örneklerinden klinik olarak önemli bakterilerin tanımlanması için rutin konvansiyonel yöntemler kullanıldı. Saptanan kökenlerin biyofilm oluşumu mikroplak yöntemi ile incelendi.

Bulgular: En sık izole edilen etkenler *Escherichia coli* (n:36) ve *Proteus mirabilis* (n:17) olarak saptandı. *E. coli* kökenlerinin %89'unda, *P. mirabilis* kökenlerinin ise %94'ünde biyofilm oluşumu gözlendi.

Sonuç: Elde edilen sonuçlar, özellikle *E. coli* ve *P. mirabilis* kökenlerinde yüksek biyofilm oluşumu ve bu biyofilm oluşumuna ilişkin genlerin sıklıkla bulunması açısından önemlidir.

Anahtar Kelimeler: Ürolitiyazis, Biyofilm, Böbrek taşı, Direnç genleri.

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Introduction

The history of kidney stone disease, also known as urolithiasis, begins with and parallels the history of civilisation. It is estimated that around 1 in every 15 individuals within society may experience kidney stones at some point in their life. It is anticipated that the combination of climate change effects resulting from global warming and alterations in dietary habits and lifestyle will result in a rise in the occurrence of kidney stones. Several risk factors contribute to the formation of kidney stones, including urinary system infections, diabetes, obesity, medication use, renal tubular acidosis, hyperparathyroidism, and others. Given this, a more detailed exploration of these risk factors becomes imperative to comprehend the multifaceted nature of kidney stone formation.[1-3]

Urinary tract infections caused by urease-producing bacteria play a role in the formation of kidney stones. Another aspect to consider regarding urinary tract infections is the presence of biofilm layer consisting of microorganisms. These clusters of bacteria attach to inanimate surfaces, including kidney stones, and pose a challenge in terms of treatment. Infections caused by bacteria that form a biofilm layer not only exhibit high resistance to treatment but also expedite the process of stone formation. This occurs through variations in chemical concentrations produced on adjacent surfaces.[4, 5]

This study aims to phenotypically and molecularly investigate the biofilm-forming ability of bacterial strains isolated from patients with urolithiasis. Our study also aims to identify some genes associated with biofilm formation in urolithiasis patients.

Materials and Methods

Type of the study

The study was done as a cross-sectional study.

Study design and patients

A total of 100 patients (61 male, 39 female), aged between 20 and 60, who suffered from urinary tract infections associated with or suspected to be caused by kidney stones, were included in our cross-sectional and clinically conducted study. The study was undertaken during the period from May 2021 to November 2022. The definitive diagnosis of kidney stones was established through a comprehensive approach, involving imaging studies such as ultrasound and/or CT scans, along with a physical examination and a detailed medical history review. Among the initially suspected 112 patients, the definitive presence of kidney stones was confirmed in 100 cases through these diagnostic methods. The diagnostic process allowed us to focus our study on a subset of patients with a confirmed association between urinary tract infections and kidney stones. In our prospective study, we obtained informed consent from our patients who were chosen based on their symptoms of urinary tract infection and the likelihood of kidney stones.

Bacteriological analysis

Urine samples for bacteriological culture were inoculated on Blood agar (BIOMARK® Laboratories, India), Chocolate agar (BIOMARK® Laboratories, India) and Mac Conkey agar (Neogen®, USA) media and evaluated after incubation at 37°C for 18-36 hours. The identification of clinically important bacteria and determination of antibiotic resistance was carried out using the VITEK® 2 system (bioMérieux, France) in addition to routine conventional microbiological techniques.

Detection of biofilm formation

Biofilm formation was detected by spectrophotometric microplate method.[6] The biofilm-forming ability of the bacteria isolated in the microplate method was quantitatively determined by measuring at 630 nm in a microplate reader device with spectrophotometric measurement.

Investigation of virulence genes associated with biofilm formation

The presence of virulence genes (*sfa* and *foc* for *E. coli*[7], *pmfA* and *mrpA* for *P. mirabilis*[8]) that are considered to be associated with biofilm formation in isolated strains of *E. coli* and *P. mirabilis* were investigated by PCR (Table 1). DNA extraction kit (Alliance Bio,

USA) was used to extract bacterial DNA. Virulence genes were amplified using a one-step PCR assay. PTC-200 (Peltier Thermal Cycler, MJ Research, USA) was used as a thermal cycler in the experiment.

Table 1. Primers for target genes.

Target gene	Primer name	Primer sequences 5`--3`	Amplicon size	References
pmfA	*pmfA*-F	GGATCATCTATAATGAAACTG	564 bp	33
	pmfA-R	CTGATAATCAACTTGGAAGTT		
mrpA	*mrpA*-F	TTCTTACTGATAAGACATTG	512 bp	33
	mrpA-R	ATTTCAGGAAACAAAAGATG		
sfa	*sfa*-F	CCGTAAGATGTCTGCGAG	100 bp	7
	sfa-R	AGCAAGTCTGGCAACGAG		
foc	*foc*-F	GGTGGAACCGCAGAAAATA	388 bp	7
	foc-R	GAACTGTTG GGGAAAGAGTG		

Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows, Version 22 (IBM Corp. Armonk, NY: USA. Released 2013). Categorical variables were represented using frequency and percentage.

Ethics committee approval

Ethical committee approval was received from the Ethics Committee of Kirkuk Health Office (Date: March 1, 2022, Number: 149). The study was conducted under the principles of the Declaration of Helsinki.

Results

Sixty bacterial strains were isolated from urine samples of 100 patients who were suspected to have kidney stones and had urinary tract infections. The most frequently isolated agents were *E. coli* (n:36), *P. mirabilis* (n:17), *Staphylococcus aureus* (n:3), *Klebsiella pneumoniae* (n:2) and *Pseudomonas aeruginosa* (n:2).

E. coli strains showed a high rate of resistance against piperacillin/ticarcillin at 78%, ciprofloxacin at 59%, and trimethoprim-sulfamethoxazole at 58%. Imipenem was detected as the most effective antibiotic for *E. coli* strains (Figure 1). On the other hand, *P. mirabilis* strains had a high resistance rate against minocycline at 59%, and the lowest resistance rate against imipenem at 18% (Figure 2).

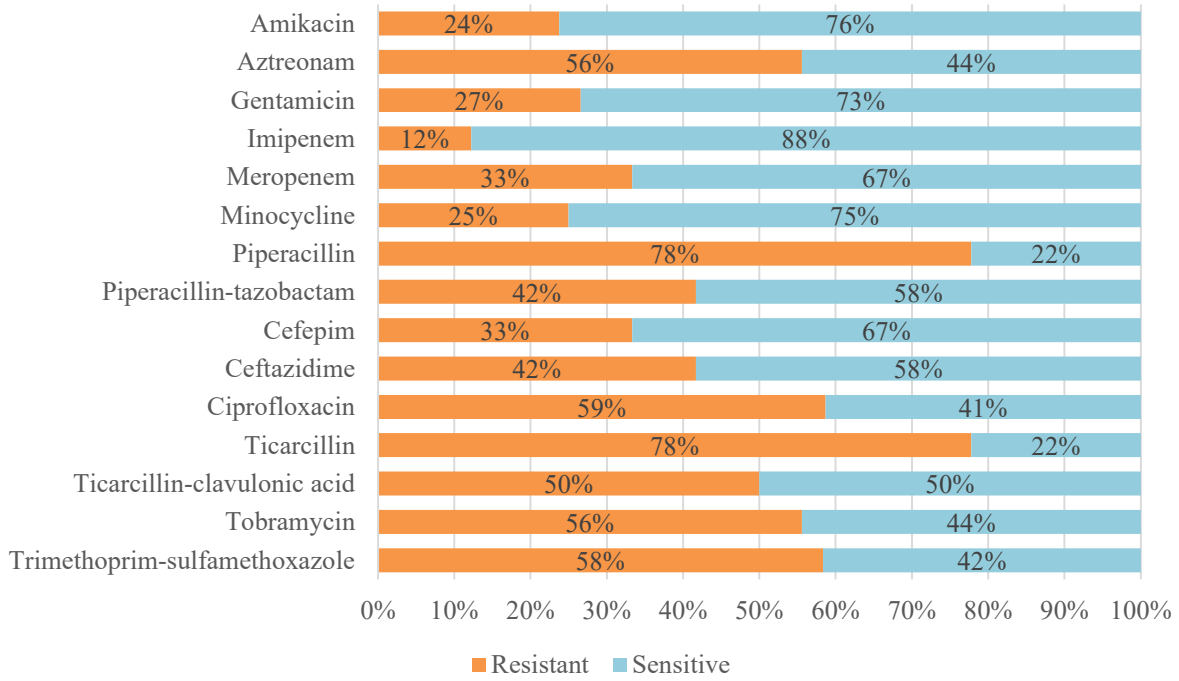

Figure 1. Antibiotic resistance pattern of *E. coli* isolates.

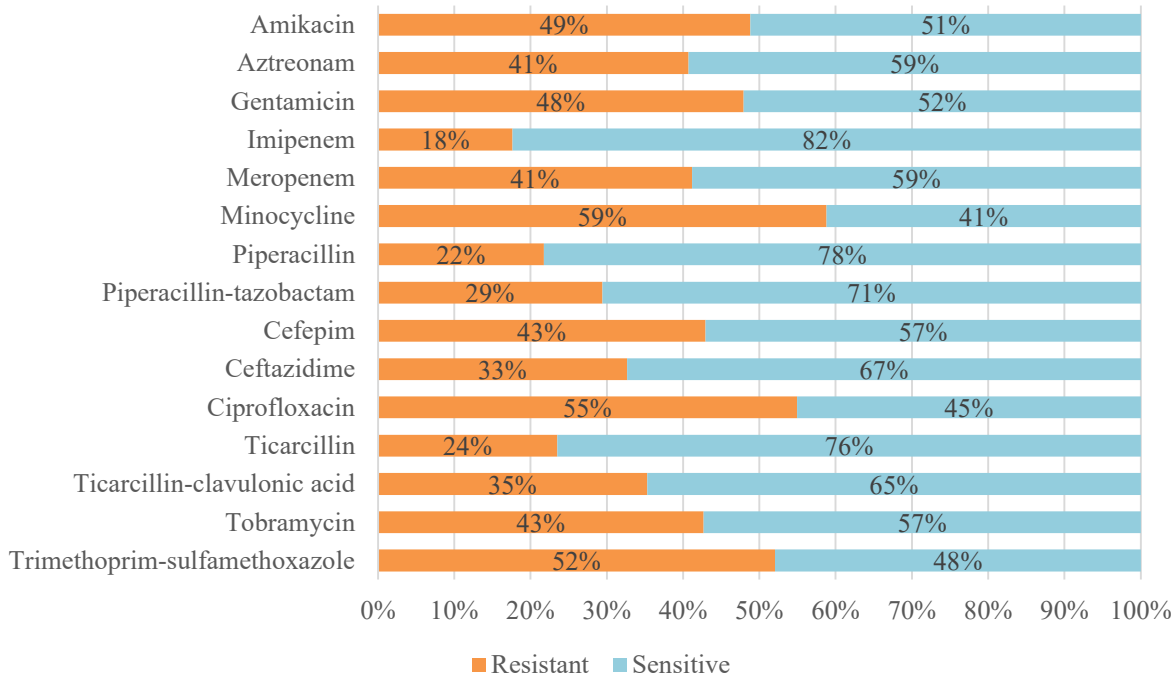

Figure 2. Antibiotic resistance pattern of *P. mirabilis* isolates.

Based on the biofilm formation abilities of *E. coli* and *P. mirabilis* isolates, the most commonly isolated bacterial species from urine samples, strong biofilm capacity was observed in 16 (44.4%), moderate biofilm capacity in 11 (30.6%) and weak biofilm capacity in 5 (13.9%) of the 36 *E. coli* isolates. Biofilm formation was not detected in 4 (11.1%) of these isolates. Out of the 17 isolates of *P. mirabilis*, 10 (58.8%) had strong biofilm ability, 4 (23.5%) had moderate biofilm ability and 2 (11.8%) had weak biofilm ability. Biofilm formation was not detected in 1 (5.9%) isolate of *P. mirabilis*.

Twenty four out of 36 *E. coli* isolates were found to have the *foc* gene, while 20 had the *sfa* gene. Additionally, the *pmfA* gene was detected in 12 out of 17 *P. mirabilis* isolates, and 10 isolates had the *mrpA* gene. The prevalence rates of virulence genes in *E. coli* and *P. mirabilis* isolates from patient samples are shown in Table 2.

Table 2. Virulence genes detected in *E. coli* and *P. mirabilis* isolates.

Bacteria	**n**	**Virulence genes**			
		foc	***sfa***	***pmfA***	***mrpA***
E. coli	36	24 (66.7%)	20 (55.6%)	-	-
P. mirabilis	17	-	-	12 (70.6%)	10 (58.8%)

The *sfa* gene was detected in 10 isolates of *E. coli* strains that formed strong biofilms, while the *foc* gene was detected in 14 isolates. On the other hand, *pmfA* and *mrpA* genes were detected in 6 isolates of *P. mirabilis* strains that formed strong biofilms. The biofilm formation capacity of *E. coli* and *P. mirabilis* isolates and the association of virulence genes are given in Table 3.

Table 3. Biofilm formation capacity of virulence genes in *E. coli* and *P. mirabilis* isolates.

E. coli	**Biofilm formation**			
	Strong (n:16)	**Moderate (n:11)**	**Weak (n:5)**	**Negative (n:4)**
sfa	10 (62.5%)	7 (63,6%)	3 (60%)	0
foc	14 (87.5%)	8 (72.7%)	2 (40%)	0
P. mirabilis	**Biofilm formation**			
	Strong (n:10)	**Moderate (n:4)**	**Weak (n:2)**	**Negative (n:1)**
pmfA	6 (60%)	4 (100%)	1 (50%)	1 (100%)
mrpA	6 (60%)	3 (75%)	1 (50%)	0

Discussion

Several studies conducted on patients with urolithiasis have indicated that the incidence of bacterial infection may vary. In our study, a total of 60 bacteria were isolated from urine samples obtained from 100 patients with urinary tract infections and symptoms consistent with kidney stone formation. This is significantly higher than the rates of a recent study conducted in Egypt that revealed only 30% of cases showed bacterial isolates.[9] However, the urine culture positivity rate of 59% in patients with kidney stones in Thailand is quite similar to the results of our study.[10] Our findings indicate a high incidence of persistent urinary tract infections linked to urolithiasis. These results may be due to geographic variations, demographics of patients, and, crucially, distinct pathogenic processes underlying urolithiasis.

In our study, *E. coli* was the most frequently isolated pathogen with 36 samples, whereas *P. mirabilis* was isolated from 17 samples. The other agents isolated were *S. aureus* (n:3), *K. pneumoniae* (n:2) and *P. aeruginosa* (n:2). These results confirm that *E. coli* is the most common cause of urinary tract infections suspected to be kidney stones and *P. mirabilis* is also a frequently isolated causative agent.[11, 12]

When the results of different studies were analyzed, *E. coli* and *P. mirabilis* were found to be the most common causative agents of urinary tract infections in patients with kidney stones and this result is consistent with the data obtained in our study.[13-15] The presence of *E. coli* in the human intestinal flora and its ability to adhere easily to the walls of the urinary tract due to surface proteins on their fimbriae can frequently result in its detection in patients with urolithiasis and urinary tract infections. The frequent detection of *P. mirabilis* in patients with urolithiasis may be attributed to its ability to produce high levels of the urease enzyme. This enzyme plays a crucial role in hydrolyzing urea molecules in urine, leading to the release of ammonia. As a result, the pH of the urine increases, fostering conditions conducive to the formation of urinary system stones.[16]

According to a study conducted in Egypt, *E. coli* (57%) and *S. aureus* (20%) were identified as the dominant bacteria associated with the formation of kidney stones.[9] A different study reported that *Corynebacterium* spp. and *E. coli* were the most commonly detected microorganisms, at rates of 23.5% and 21.5%, respectively.[10] The results obtained differ from our study. The characteristics of the stone found in patients, as well as the size, composition and mechanism of stone formation may contribute to variances in predominant bacterial species. Furthermore, there may be differences in the bacterial species isolated from infections associated with kidney stone formation due to studies conducted in different patient populations in different regions.

It is a fact that the rising antimicrobial resistance of clinically important bacteria, such as *E. coli* in infectious diseases is becoming increasingly worrying. Many studies around the world have shown that *E. coli* isolates develop resistance to various antibiotics. In a study conducted in Algeria, 86% of *E. coli* isolates were resistant to 1st generation cephalosporin group antibiotics, followed by ticarcillin with 83%, ampicillin with 73%, and amoxicillin-clavulonic acid with 58%.[17] Similarly, a study conducted in Iraq has shown that approximately 90% of *E. coli* isolates were resistant to quinolone group antibiotics.[18]

In our study, the antimicrobial resistance rates of *E. coli* isolates obtained from urine samples were higher against piperacillin (78%), ticarcillin (78%) and trimethoprim-sulfamethoxazole (58%). On the other hand, the lowest resistance was observed against imipenem (12%) and amikacin (24%). These results are consistent with those published by the authors who identified *E. coli* as the dominant uropathogen.[9, 10] Inappropriate use of antibiotics may lead to increased antimicrobial resistance and reduced treatment options.

P. mirabilis is one of the most important etiological factors of urinary tract infections and is known to show high level resistance to some antibiotics. Some studies show that it develops resistance to some common antibiotics such as cephalosporins,

fluoroquinolones, and aminoglycosides.[19] In a recent study conducted in China focusing on patients with urinary stones, Gram-negative bacilli isolated from these individuals exhibited notable resistance patterns. Our findings resonate with this, affirming that Gram-negative bacilli in our study displayed resistance to first, second, and third generation cephalosporins, quinolones, tetracycline, cotrimoxazole, and nitrofurantoin. Contrarily, these bacteria showed marked sensitivity to drugs containing β-lactamase inhibitors, and carbapenems.[20] Although there are differences between the results obtained from the studies, the results of our study are generally compatible with the findings of other studies in the literature.[19, 20] Treatment options may be limited and infections can be difficult to control due to *P. mirabilis* resistance to antibiotics. Hence, surveillance and control of antibiotic resistance are critical.

One of the mechanisms by which bacteria cause urinary tract infections is biofilm formation. Of the 36 *E. coli* isolates, 16 had high, 11 had moderate and 5 had weak biofilm formation capacity. In total, 89% of the *E. coli* isolates were capable of producing biofilm. When we look at the results of another study conducted in Iraq, it was found that 93% of *E. coli* isolates were capable of biofilm formation.[21] According to the findings of another study on biofilm formation in uropathogenic *E. coli* isolates in Iran, 92% of the biofilms were found to be positive.[22] Another study carried out in Iraq found that 90% of the isolates had the potential for biofilm formation.[23]

The ability of *P. mirabilis* strains to form biofilms is a topic of interest to researchers in recent years. Tabatabaei et al. concluded that half of the *P. mirabilis* strains isolated from patients with urinary tract infections were capable of biofilm formation.[24] Similarly, studies conducted in Iraq and Poland revealed that all *Proteus* spp. isolates from patients with urinary tract infections were capable of biofilm formation.[25, 26] Biofilm formation was detected in 94.1% of 17 *P. mirabilis* strains isolated in our study. The results of our study are in agreement with the findings of the studies in the literature which determined the biofilm formation ability of *P. mirabilis* strains.[24-26] It is thought that *P. mirabilis* can easily sustain infections thanks to its unique ability to form biofilms. This may help us understand how the bacteria can cause such infections despite the host's strong immune response.

Various virulence factors such as adhesion molecules are involved in the attachment of bacterial cells to the urinary system and biofilm development. The findings from our investigation revealed that 24 (66.7%) *E. coli* strains isolated from patients with urolithiasis had *foc* gene and 20 (55.6%) had *sfa* gene. These findings suggest that virulence genes, particularly those associated with biofilm formation, play a crucial role in the development of urinary tract infection. Several studies have reported the role of *sfa* and *foc* genes in encoding adhesion molecules involved in the pathophysiology of urinary tract infections caused by *E. coli*.[7, 27, 28] These results suggest that *sfa* and *foc* genes play a critical role in the early stages of biofilm development of *E. coli* isolates obtained from patients with urinary tract infections by increasing adhesion.

In our study, the *pmfA* gene was detected in 12 (70.6%) and the *mrpA* gene was detected in 10 (58.8%) *P. mirabilis* strains. A study exploring virulence genes in *P. mirabilis* strains isolated from patients with urinary tract infections in Brazil found that all isolates positive for *mrpA* and *pmfA* genes.[29] A study by Amina et al. found that 38% of *P. mirabilis* strains from patients with urinary tract infections tested positive for the *mrpA* gene, while 46% tested positive for the *pmfA* gene.[30] During a study carried out in Bangladesh, the *pmfA* gene was identified in 10 out of 29 biofilm-producing *P. mirabilis* isolates. Additionally, the *mrpA* gene was present in 16 of the isolates.[31] In a comparable study performed in Iraq, the *pmfA* gene was detected in 41% and the *mrpA* gene in 35% of *P. mirabilis* strains that were isolated.[32] The results indicate that the *pmfA* and *mrpA* genes are likely prevalent in *P. mirabilis* strains and potentially linked to virulence traits.

Limitations

While our study provides valuable insights into the association between urinary tract infections and kidney stones, it is important to acknowledge certain limitations. Firstly, the cross-sectional nature of the study design limits our ability to establish causation or infer the temporal sequence of events. Longitudinal studies would be beneficial to better understand the dynamic relationship between urinary tract infections and the presence of kidney stones over time. Another limitation of our study is the absence of a control group, which restricts our ability to make direct comparisons and draw more definitive conclusions regarding the observed association between urinary tract infections and kidney stones.

Conclusion

In summary, identifying high-risk pathogens in terms of antibiotic resistance and biofilm formation is critical, as is developing effective treatments against such pathogens. The results of our study suggest the potential significance of proper antibiotic selection in treating urolithiasis-related urinary tract infections, particularly in the context of preventing recurring infections caused by *E. coli* and *P. mirabilis* pathogens. Further research is required to comprehend the function and mechanisms of virulence genes present in pathogens that participate in the development of kidney stones and the creation of biofilms.

Ethics Committee Approval

Ethical committee approval was received from the Ethics Committee of Kirkuk Health Office (Date: March 1, 2022, Number: 149). The study was conducted under the principles of the Declaration of Helsinki.

Informed Consent

Informed consent was obtained from the individuals participating in the study.

Authors Contrubituons

All of the authors contributed at every stage of the study.

Conflict of Interests

There is no conflict of interest to declare.

Financial Disclosure

No person/organization is supporting this study financially.

Statements

This study was presented as a Master of Science Thesis at Çankırı Karatekin University, Institute of Science and Technology.

Peer-review

Externally peer-reviewed.

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