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Hidden Danger: Superbug *Escherichia coli* Isolated from Urine Isolates of Outpatient Women with Uncomplicated Urinary Tract Infection

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BACKGROUND/AIMS
Escherichia coli (E. coli) is responsible for the vast majority of uncomplicated bacterial urinary tract infection (UTI) cases in women. The high ability of the isolates to develop antimicrobial resistance makes the treatment difficult. In this study, we investigated the presence of plasmid-mediated quinolone resistance (PMQR) genes in *E. coli* isolates and their relationship with extended-spectrum beta-lactamases (ESBL).

MATERIAL and METHODS
A total of 300 *E. coli* isolates from urine specimens of women, including 108 ESBL producers and 192 non-ESBL producers, were analyzed. The ESBL production was examined using the E-test ESBL strips, and the carbapenemase activity was examined using the CarbaNP test. The presence of PMQR genes *(qnrA, qnrB, qnrS, and aac (6´)-Ib)* among urine isolates was investigated using polymerase chain reaction. Conjugation experiments were performed to detect the horizontal transferability of the PMQR-positive plasmid.

RESULTS
Among the ESBL-EC isolates, ciprofloxacin resistance was determined at 69%. Eight isolates were resistant to carbapenems. The *aac(6')-Ib-cr* variant was found in 40% of ciprofloxacin-resistant *E. coli* isolates. None of the isolates harbored the *qnrA, qnrB, or qnrS* gene. The transferability was 14% for *aac(6')-Ib-cr*. The MICs of transconjugants showed increased resistance to fluoroquinolones compared with the recipient *E. coli* J53AzR.

CONCLUSION: This study showed that the frequency of PMQR genes in ESBL-producing superbug *E. coli* isolates reduced therapeutic options for treating community-acquired UTIs in affected women and that a careful use of antibiotics is very important.

Keywords: ESBL-producing *Escherichia coli,* superbug, PMQR genes, *aac(6')-Ib-cr,* female patients with UTI, fosfomycin

INTRODUCTION

All over the world, in outpatient practice, uncomplicated bacterial urinary tract infections (UTIs) are one of the most common community-acquired diseases. *Escherichia coli (E. coli)* is responsible for the vast majority of UTIs, and especially women suffer from UTIs because of the proximity of the urethra to the vagina and the rectum, changes in genital microflora, hormonal influences, and other anatomical and physiological characteristics (1). *E. coli* is a part of the normal flora in the intestinal tract of a healthy human. Uropathogenic *E. coli* is generally acquired from sexual partners, household members, pets, food, toilet, and during travel. However, the high ability of the isolates to develop antimicrobial resistance makes the treatment difficult. These bacteria can transfer the resistance genes to other *E. coli* isolates and different Gram-negative bacteria. Therefore, multidrug-resistant *E. coli* deserves a superbug label. Furthermore, antimicrobial options in treatment are limited due to multidrug resistance (2). Quinolones are the first choice for the treatment of UTIs caused by extended-spectrum-beta-lactamase (ESBL)-producing *E. coli*. However, the widespread use of quinolones for therapeutic and non-therapeutic purposes has led to the rapid spread of quinolone-resistant *E. coli* isolates worldwide (3). The resistance to quinolones usually occurs as a result of "DNA target mutations, overexpression of efflux pumps, loss of porins and mobile genetic elements encoded on plasmids, known as plasmid-mediated quinolone resistance

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(PMQR) genes, namely *qnr, aac (6')-Ib-cr, and qepA"* (4, 5). A series of PMQR determinants within the last 10 years further reveal a new issue about the resistance to quinolones. PMQR genes play an essential role in the development of low-level quinolone resistance and facilitate the emergence of high-level resistance in the presence of quinolones at treatment levels (6). PMQR genes, *qnr (qnrA, qnrB, and qnrS),* which protects the DNA gyrase and Type IV topoisomerase enzymes from quinolone inhibition, and *aac(6')-Ib-cr,* which acetylates quinolones, and efflux by QepA and OqxAB have been reported in clinical isolates of *Enterobacteriaceae,* including *E. coli* (3, 6-8). In many studies, PMQR genes have frequently been shown to be associated with genes encoding ESBL and aminoglycosides on the same plasmid (8, 9). Today, plasmids carrying *qnr* and ESBL determinants represent a concern worldwide. Carbapenems are often the last-choice agents used for the treatment of patients with severe infections. However, carbapenemase-producing *E. coli* has been increasingly reported, especially in clinical settings (2, 10). Fosfomycin, known for over 40 years, has recently become attractive as an alternative agent for the treatment of UTIs (11). "The Infectious Diseases Society of America (IDSA) recommends that physicians obtain information on local resistance rates, the appropriateness of empirical therapy proposals and that ongoing surveillance has been conducted to monitor changes in the susceptibility of uropathogens" (12, 13). This study aimed to investigate the presence of PMQR *(qnrA, qnrB, qnrS, and aac(6')-Ib-cr)* genes, and also their relationship to ESBL among *E. coli* strains isolated from urine samples of outpatient Turkish women, with community-acquired UTI.

MATERIAL and METHODS

Methodology

A total of 300 *E. coli* isolates were obtained from urine samples of outpatient Turkish women with symptoms suggestive of community-acquired UTIs in İstanbul University-Cerrahpaşa, Cerrahpaşa School of Medicine Hospital. In the study, the patients were aged 16-85 years. Patients who were pregnant, with functional, or structural anomalies of the urinary tract, and suffering from an immunocompromized illness, and using immunosuppressants, and who were discharged from the hospital 10–15 days before were excluded from the study. The identification and antimicrobial susceptibility were determined using the BD Phoenix automated identification and susceptibility testing system (BD Diagnostic Systems, Sparks, MD). The isolates resistant or moderately susceptible to tested antibiotics were confirmed using the E-test (bioMerieux, France) method. The susceptibility of ciprofloxacin, carbapenems, tigecycline, colistin, and fosfomycin was determined by E-test (bioMerieux, La Balme-les-Grottes, France) method according to manufacturer's instructions. The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (14). The ESBL production was examined using the double-ended E-test ESBL strips (AB Biodisk, Solna, Sweden) containing gradients of cefotaxime (CT) or ceftazidime (TZ) or cefepime (FEP) at one end and cefotaxime or ceftazidime or cefepime plus clavulanic acid (CTL, TZL, and FEL) at the other, according to the manufacturer's instructions. The carbapenemase activity was investigated using the Carba NP test (RAPIDEC CARBA NP (bioMerieux, La Balmeles-grottes, France) (15). Quinolone-resistant isolates were screened for the presence of PMQR *(qnrA, qnrB, qnrS, and aac(6')-Ib)* genes by the polymerase chain reaction (PCR). DNA was extracted from the fresh culture of *E. coli* colonies according to the protocol performed using the GeneJET Genomic DNA Purification kit (Thermo Scientific, USA). The determination of PMQR *(qnrA, qnrB, qnrS, and aac(6')-Ib)* genes was performed using PCR. All *aac (6')-Ib* positive amplicons were investigated by digestion with BseGI (Fermantas, USA) restriction enzyme to determine the *aac (6')-Ib-cr* variant. The amplification of *qnrA, qnrB, qnrS,* and *aac(6')-Ib* genes was performed using the primers presented in Table I (4, 9, 16-18).

Conjugation Assays Used to Detect PMQR Transferability

Conjugation experiments were performed to detect whether the quinolone resistance could be transferred horizontally to a plasmid-free *E. coli* strain from *E. coli* urine isolates carrying PMQR-positive plasmids. A plasmid-free, sodium azide-resistant *E. coli* J53 (AzR) was used as the recipient, as described previously (19). The recipient (J53) and donor urine isolates were inoculated into the Luria-Bertani (LB) broth (Difco) and grown overnight at 37°C. The equal volumes of the donor and recipient cultures were mixed and incubated overnight at 37°C. The serial dilutions were homogeneously spread onto trypticase soy agar (Oxoid) plates supplemented with sodium azide (150 µg/mL, Sigma-Aldrich) and ciprofloxacin (0.25 µg/mL, Sigma-Aldrich). The transconjugants were selected and collected on the plates. PCR was performed to determine the presence of PMQR determinants (20). Plasmid DNAs of transconjugants and donor isolates were extracted using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Vienna, Austria) according to the manufacturer's instructions. The size of plasmid was estimated by electrophoresis using a 0.7 % (w/v) agarose gel, comparing

TABLE I. Primers used for the detection of *qnr* and *aac (6')-Ib-cr* genes

Target gene		Primer sequence (5'→ 3')	Gene size	Genebank accession No.	Predicting the size of amplicon (bp)
qnrA	F	TCAGCAAGAGGATTTCTCA	657	KC493127.1	627
	R	GGCAGCACTATTACTCCCA			
qnrB	F	ATGACGCCATTACTGTATAA	681	EF634464.1	562
	R	GATCGCAATGTGTGAAGTTC			
qnrS	F	ACGACATTCGTCAACTGCAA	656	EU391634.1	417
	R	TAAATTGGCACCCTGTAGGC			
aac(6')-Ib-cr	F	TTGCGATGCTCTATGAGTGGCTA	519	Q2143161	482
	R	CTCGAATGCCTGGCGTGTTT			

the known plasmid molecular size markers of *E. coli* V517 harboring plasmids of 54.4, 7.1, 5.6, 5.2, 3.0, 2.7, and 2.1 kb, as previously described (19). MICs of ciprofloxacin were determined for the PMQR gene-positive donors, recipients, and transconjugants using the E-test.

Quality control was performed using standard strains of *E. coli* ATCC 25922, ATCC 35218, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853.

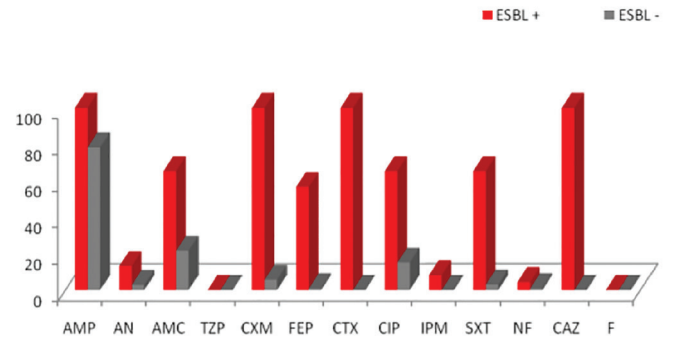

FIGURE 1. Antimicrobial resistance rates of ESBL-EC and non-ESBL-EC isolates
AMP: Ampicillin; AN: Amikacin; AMC: Amoxicillin/Clavulanic Acid; TZP: Piperacillin-Tazobactam; CXM: Cefuroxime; FEP: Cefepime; CTX: Cefotaxime; IMP: Imipenem; SXT: Trimethoprim-Sulfamethoxazole; F: Fosfomycin; NF: Nitrofurantoin; CAZ: Ceftazidime.

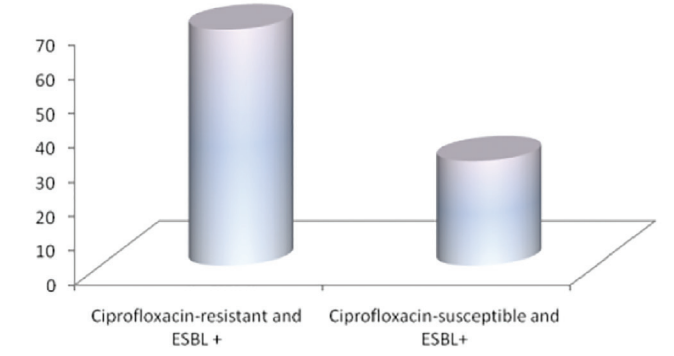

FIGURE 2. Percentage of ESBL production among CREC isolates
ESBL production was significantly more frequent among ciprofloxacin-resistant E. coli isolates than among ciprofloxacin-susceptible isolates (33/108) (p<0.0001).

Statistical Analysis

The statistical analysis of the data was carried out using Fisher's exact test. A p-value of $p<0.05$ was accepted as statistically significant.

RESULTS

A total of 300 *E. coli* isolates were obtained from urine samples of outpatient women with symptoms suggestive of a UTI. The ESBL production was detected in 36% (108/300) of isolates.

Antimicrobial Susceptibility Test

The antimicrobial resistance rates were significantly higher in ESBL-producing *E. coli* (ESBL-EC) isolates than in non-ESBL-EC isolates ($p<0.05$) (Figure 1). Thirty-five percent (105/300) were resistant to ciprofloxacin. Ciprofloxacin had MIC ranges of 0.008 to ≥32 µg/mL with MIC50 at 0.5 µg/mL and MIC90 at 1 µg/mL. Among the ESBL-EC isolates, ciprofloxacin resistance was determined at 69% (75/108). The ESBL production was significantly more frequent among ciprofloxacin-resistant *E. coli* (CREC) isolates than among ciprofloxacin-susceptible isolates (33/108) ($p<0.0001$) (Figure 2). Sixty-five percent (68/105) of CREC isolates belonged to women aged >40 years. Eight isolates were resistant to carbapenems, and the MICs of the isolates were determined ≥32 µg/mL for imipenem, meropenem, and ertapenem, and their carbapenemase activities were positive. These isolates were both resistant to ciprofloxacin and positive for ESBL production. One of the 8 isolates belonged to a 51-year-old woman, and others belonged to young women (average 25 years old).

ESBL-CREC isolates were highly resistant to ampicillin, cefuroxime, cefotaxime, ceftazidime (100%), amoxicillin/clavulanic acid,

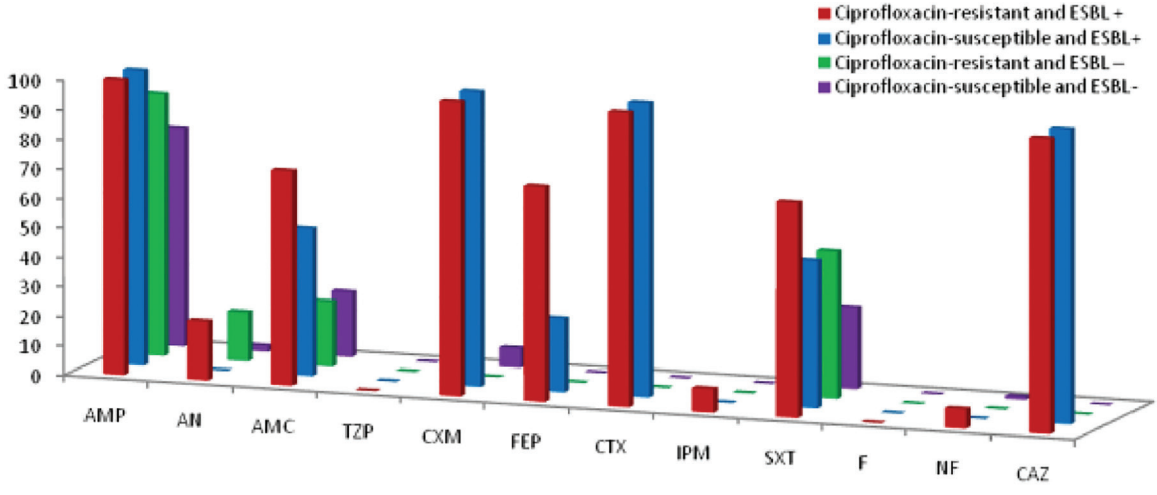

FIGURE 3. Antimicrobial resistance rates of *E. coli* isolates according to the presence or absence of ciprofloxacin resistance and ESBL production
AMP: Ampicillin; AN: Amikacin; AMC: Amoxicillin/Clavulanic Acid; TZP: Piperacillin-Tazobactam; CXM: Cefuroxime; FEP: Cefepime; CTX: Cefotaxime; IMP: Imipenem; SXT: Trimethoprim-Sulfamethoxazole; F: Fosfomycin; NF: Nitrofurantoin; CAZ: Ceftazidime.

FIGURE 4. PCR amplification products of the *aac(6')-Ib-cr* gene in CREC isolates

cefepime, and trimethoprim–sulfamethoxazole (73.3%). The resistance was lower to amikacin (20%) and nitrofurantoin (6.6%) (Figure 3). The combined resistance to third-generation cephalosporins, carbapenems, ciprofloxacin, amikacin, trimethoprim–sulfamethoxazole, and nitrofurantoin was detected in 2.6% (8/300) of urine isolates. Fosfomycin, tigecycline, and colistin resistance was not detected in any of the isolates.

Prevalence of PMQR Genes

Forty percent (42/105) of CREC isolates were positive for *aac(6')-Ib-cr* variant, of which 50% (21/42) were ESBL producers, and 28% (6/21) of these isolates were also resistant to carbapenems. None of the isolates harbored *qnrA, qnrB, and qnrS* genes.

Conjugation Experiments

Transconjugants (with plasmid sizes 54-100 kb) were successfully obtained from 6 of 42 *aac(6')-Ib-cr* gene-positive *E. coli* isolates used as donors. The *aac(6')-Ib-cr* gene was successfully transferred from 6 *aac(6')*-Ib-cr gene-positive *E. coli* urine isolates to their transconjugants. Transferability was 14% (6/42) for *aac(6')-Ib-cr. E. coli* isolates that harbored *aac(6')-Ib-cr* were resistant to ciprofloxacin (MICs 32-256 µg/mL). The MICs of ciprofloxacin for the 6 transconjugants ranged from 0.25 to 1 µg/mL, or were 31- to 125-fold higher than that for the recipient *E. coli* J53AzR (MIC 0.008µg/mL).

The PCR amplification products of *aac(6')-Ib-cr* gene in CREC isolates are shown in Figure 4.

DISCUSSION

UTIs are the most common infections in women, and over 50% of women experience UTI at least once in their lifetime. UTI can significantly affect the quality of life. *E. coli* is the most common causative agent in the UTIs of women. These bacteria can easily become resistant. Many reports have shown that the prevalence of multidrug-resistant *E. coli* isolates is increasing worldwide because of the dissemination of mobile genetic elements (21-24). A surveillance study conducted in Europe between 2004 and 2010, including Turkey, reported that the ESBL production is positive in the mean 15% of *E. coli* isolates from different samples, and Turkey has the highest percentage with 25% (23). In the present study, the ESBL production was 36% among the urine isolates of *E. coli* from outpatient women patients.

An increase in quinolone resistance among ESBL-EC isolates has been reported all over the world. In an antimicrobial resistance surveillance study report on ECDC in 2012, the average percentage of resistance to quinolone was 22%, and it was predominant in Italy and Cyprus (42%), and Slovakia (41%) (21), and in Turkey (52%) (22). In a study conducted in our hospital in 2009, the rate of ciprofloxacin resistance among ESBL-EC blood isolates was 57.6% (24). In the present study, ciprofloxacin resistance was determined as 69% (75/108) in ESBL-EC urine isolates. The ESBL production was significantly more frequent among our CREC isolates than among ciprofloxacin-susceptible isolates (p<0.0001).

PMQR genes may facilitate the spreading and increase the prevalence of quinolone-resistant isolates. The *aac(6')-Ib-cr* encodes a bifunctional aminoglycoside 6'-N-acetyltransferase capable of acetylating both aminoglycosides and fluoroquinolones (25). In the many studies conducted on *E. coli* in different countries, the frequency rates of the *qnr* gene were reported at rates 11%-75% (26-28). In studies conducted in Turkey, the most prevalent PMQR determinant was *aac(6')-Ib-cr* (at rates 46%–60%) (29-31). In the present study, we observed that the frequency of *aac(6')-Ib-cr* was 40% (42/105) in CREC isolates. Several studies demonstrated the association between *aac(6')-Ib-cr* and the ESBL (31-33). Similarly, we determined that 50% of *E. coli* isolates harboring *aac(6')-Ib-cr* were ESBL producers. The conjugation experiments demonstrated that *aac(6')-Ib-cr* was transferable. The MICs of ciprofloxacin for transconjugants harboring *aac(6')-Ib-cr* were 31- to 125- fold higher than the MIC for the recipient *E. coli* J53AzR. In conjugation experiments, we showed the possibility that the *aac(6')-Ib-cr* gene could be transferred horizontally among isolates of *E. coli,* causing uncomplicated community-acquired UTI in Turkish women.

Several reports of *E. coli* resistant to aminoglycosides are increasing worldwide (21, 22, 24). The resistance to amikacin was reported as 11% for ESBL-EC in the United States (34). In the EARSS Annual Report, the resistance to aminoglycosides in *E. coli* isolated from different samples was 35% in Turkey (22). In the current study, amikacin resistance was detected at 20% in urine isolates.

Carbapenems are also considered among the last-resort antibiotics in the treatment of serious infections caused by multidrug-resistant members of the *Enterobacteriaceae,* including *E. coli.* However, because of the global increase of carbapenem resistance, these bacteria have become a worldwide problem (21). In the present study, 8 isolates were resistant to carbapen-

ems (MICs>32 µg/mL). These isolates were both resistant to ciprofloxacin, and ESBL was positive.

The percentage of combined resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides was 4% in Europe (21). In the current study, the percentage of combined resistance, including carbapenems and nitrofurantoin among the ESBL-EC isolates in urine samples of women with symptoms suggestive of a community-acquired UTIs was 2.6%.

Tigecycline was approved by the Food and Drug Administration (FDA) in 2005, and it, like "old" antibiotics, phosphomycin and colistin, is among the remaining options in clinical use for the treatment of UTIs caused by multidrug-resistant *E. coli* isolates (35). In the last decade, colistin has been increased for the treatment of multidrug-resistant Gram-negative bacilli, especially in combination with other drugs (35).

In the 2013 report by IDSA, ESBL-EC was listed among the 6 drug-resistant microbes urgently needed in new treatments (36). These data led to reconsidering nontraditional antibiotics such as fosfomycin, a phosphonic acid derivative approved by the FDA for the treatment of uncomplicated UTIs in women. Recent reports have shown that it has *in vitro* activity against multidrug-resistant pathogens, including ESBL-CREC (37, 38). As it was also seen in the present study, fosfomycin has shown good *in vitro* activity against ESBL-CREC isolates. It may be a promising treatment option. However, clinical data regarding the use of fosfomycin in the treatment of UTIs caused by multidrug-resistant pathogens are still limited, and concerns about the widespread use of fosfomycin include tolerability, cost, and resistance (39). A recent analysis reports a fosfomycin resistance rate of 0.5% in community-acquired *E. coli* UTI in women in the United Kingdom (11). A systematic review of data, mainly from Europe and Asia, showed that 97% of ESBL-EC was susceptible to fosfomycin. Data from both *in vitro* and clinical studies are suggesting that fosfomycin should be used with caution in infections caused by ESBL-EC. In these studies, it is emphasized that the reason for the emergence of resistance to fosfomycin in ESBL-EC may be related to the increased use of this agent (37).

The ability of *E. coli* to transfer resistance genes to other bacteria causes the spread of antimicrobial resistance. This situation threatens the effectiveness of existing antibiotics. High rates of recurrent UTIs suggest that antibiotics are not an effective therapy for all UTIs, and UTIs are resulting in billions of dollars in health care costs annually.

In conclusion, our findings indicate that the rates of ciprofloxacin resistance among urine isolates of *E. coli* in women are high, that CREC isolates carry a transferable *aac(6')-Ib-cr* gene, and that they have a combined drug resistance (2.6%), including carbapenems of ESBL-EC urine isolates. These data point out that the multidrug resistance has the potential to spread among *E. coli* isolates from urine samples of outpatient women with community-acquired UTIs. We observed it had a low resistance for nitrofurantoin (6.6%). None of our multidrug-resistant *E. coli* urine isolates showed resistance to fosfomycin, tigecycline, or colistin. There is a need for accurate epidemiological data for appropriate empirical treatment in patients with both the community and the hospital hospital-acquired infections in each country. Therefore, it is crucial to apply antimicrobial resistance prevention and control strategies to reduce morbidity, mortality, and health care costs; limit the potential spread of resistance genes; and ensure careful antibiotic use in UTIs caused by *E. coli* with superbug potency.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of the İstanbul University-Cerrahpaşa, Cerrahpaşa School of Medicine.

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer review: Externally peer reviewed

Author Contributions: Concept - S.Ö., O.A., A.Ö., İ.H.Ç., F.K.Ç.; Design - S.Ö., O.A., A.Ö., İ.H.Ç., F.K.Ç.; Supervision - S.Ö., O.A., A.Ö., İ.H.Ç., F.K.Ç.; Data Collection and/or Processing - S.Ö., O.A.; Analysis and/or Interpretation - S.Ö., O.A.; Literature Search - A.Ö., İ.H.Ç., F.K.Ç.; Writing Manuscript - A.Ö., İ.H.Ç., F.K.Ç.; Critical Review - S.Ö., O.A., A.Ö., İ.H.Ç., F.K.Ç.

Conflict of Interest: The authors have no conflicts of interest to declare.

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