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Molecular Evaluation of Aminoglycoside Resistance and Biofilm Formation Potential in *Escherichia coli* Isolates Collected from Hospitalized Patients

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Abstract

Background: Resistance to antibiotics and the ability to develop biofilms, two main virulence determinants of *Escherichia coli*, play a crucial role in the persistence of infections.
Objectives: The aim of this study was to evaluate aminoglycoside resistance and biofilm formation potential in *E. coli* isolates collected from hospitalized patients in the Southwest of Iran.
Methods: A total of 70 *E. coli* clinical isolates from different specimens were collected from Ahvaz teaching hospitals affiliated with Ahvaz Jundishapur University of Medical Sciences. All the isolates were identified as *E. coli* using conventional microbiological tests. Susceptibility to antibiotics was determined using the Kirby–Bauer disk diffusion method. Biofilm formation was assessed using the microtiter plate method. Finally, PCR was conducted to detect virulence gene determinants, including fimbrial genes, aminoglycoside modifying enzymes (AMEs), and 16S rRNA methylase (RMTase) genes.
Results: Among aminoglycoside antibiotics, *E. coli* isolates showed the highest and lowest resistance rates to tobramycin (TOB; 51.4%) and gentamicin (GEN; 24.2%), respectively. Simultaneous resistance to GEN, amikacin, and TOB was observed in 28.5% of the isolates, representing the most common antibiotic resistance pattern. The prevalence of strong biofilm producers was higher in the extensively drug-resistant (XDR) phenotype group compared to the multiple drug-resistant (MDR) group (76.1% vs. 23.8%). Among the 36 isolates resistant to at least one of the aminoglycoside antibiotics, 36.1% had AME-related genes, either alone or in various combinations. Most isolates harboring AME genes were also positive for the presence of biofilm-related genes, including *ecpA* and *fimA*.
Conclusions: The most frequent AME-related genes were *ant(2")*-Ia and *aph(3')-Ia*, followed by *aac(3')-IIa*. The findings of the present study provide probable evidence that GEN is an effective aminoglycoside against biofilm-producing and antibiotic-resistant *E. coli* isolates.

Keywords: *Escherichia coli*,Biofilm, Aminoglycoside-Modifying Enzymes, Antibiotic Resistance, Iran

1. Background

Globally, antimicrobial resistance (AMR) remains a serious challenge to public health in the current century (1). Resistance to antimicrobials has escalated into a worldwide pandemic, presenting a menace to human health and food production (2). Aminoglycosides, fluoroquinolones, and β-lactams, particularly cephalosporins, carbapenems, β-lactam antibiotics, and β-lactamase inhibitor combinations, are broad-spectrum antimicrobials used to treat infections caused by Gram-negative bacteria (GNB) (3).

Aminoglycosides play an essential role in clinical settings and serve as a treatment option for severe and life-threatening infections caused by GNB, especially in hospitals (4). *Escherichia coli* is a prevalent facultative anaerobic and often harmless microbe in the digestive tract of humans and animals. This medically important bacterium causes various significant disorders (5). Numerous monitoring programs utilize *E. coli* because it is ubiquitous in food-producing animals, a marker of fecal contamination, and simple to cultivate. It can also easily develop resistance mechanisms to combat agents active against GNB (6).

Aminoglycosides, powerful bactericidal agents, can hinder bacterial protein synthesis by binding to the 30S ribosomal subunit. These agents act synergistically and are mostly employed in combination with either a glycopeptide or a β-lactam to treat *E. coli* urinary tract infections (7). There are reports of resistance to aminoglycosides, most worryingly in relation to resistance to other antibiotic classes (8). In GNB, resistance to aminoglycosides mainly arises from the production of aminoglycoside modifying enzymes (AMEs) or alteration in the ribosome by acquired 16S rRNA methyltransferases (RMTases). In *E. coli*, the most common resistance mechanism is AME production (9, 10). Aminoglycoside modifying enzymes, based on their catalytic reaction, are classified into three categories: Aminoglycoside N-acetyltransferases (AAC), aminoglycoside O-phosphotransferases (APH), and aminoglycoside O-nucleotidyltransferases (ANT) (11).

Biofilms, collections of microbial cells, are irreversibly connected to a surface and surrounded by a matrix of materials, primarily polysaccharides. These structures offer a survival strategy to bacteria by facilitating efficient use of available nutrients and preventing access to antimicrobials, antibodies, and white blood cells (12). Biofilms also harbor multiple antibiotic-inactivating enzymes, such as β-lactamases, creating islands of AMR (13). A number of infections caused by *E. coli* are associated with biofilm formation, often leading to an inability to eradicate the infection due to its inherent resistance to high doses of antibiotics. *E. coli* 's ability to form biofilms is a significant virulence feature (14).

2. Objectives

The aim of this study was to evaluate antimicrobial susceptibility, aminoglycoside resistance genes, and biofilm formation in *E. coli* isolates collected from hospitalized patients in educational hospitals in Ahvaz, Khuzestan, Iran.

3. Methods

3.1. Bacterial Isolates

This descriptive cross-sectional study was conducted on 70 clinical isolates of *E. coli* obtained using phenotypic methods. The bacterial isolates were collected from various clinical specimens in Ahvaz teaching hospitals affiliated with Ahvaz Jundishapur University of Medical Sciences. Patients signed informed consent forms before the initiation of this research project. The study was carried out in accordance with the Helsinki declaration.

3.2. Antibiotic Susceptibility Test

The AMR testing was performed using the disk diffusion technique on Mueller-Hinton agar plates (Merck, Germany), as recommended by CLSI in 2021 (15). The antibiotics tested included amikacin (AMK: 30 µg), gentamicin (GEN: 10 µg), tobramycin (TOB: 10 µg), piperacillin-tazobactam (PTZ: 110 µg), imipenem (IMI: 10 µg), meropenem (MER: 10 µg), ertapenem (ERT: 10 µg), cefazolin (CZ: 30 µg), cefoxitin (FOX: 30 µg), cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), ceftriaxone (CRO: 30 µg), cefoperazone (CFP: 75 µg), ciprofloxacin (CIP: 5 µg), azithromycin (AZM: 15 µg), ampicillin-sulbactam (AMP/SL: 20 µg), and tigecycline (TGC: 15 µg) (MAST Diagnostics). *E. coli* ATCC 25922 was used as a quality control strain.

3.3. Measurement of Biofilm Density

The ability of isolates to form biofilm was assessed using the microtiter plate (MTP) technique. The isolates were classified into four categories based on previously described criteria (16): No biofilm ($A \leq Ac$), weak biofilm ($Ac < A \leq 2Ac$), moderate biofilm ($2Ac < A \leq 4Ac$), and strong biofilm ($A > 4Ac$) producers. The optical density value was measured at 570 nm. The PAO1 strain of Pseudomonas aeruginosa was used as a positive control for the biofilm assay. All samples were tested three times.

3.4. Biofilm Elimination Tests

The MTP technique was utilized to test the ability of AMK and GEN to remove biofilms, as previously described (17). After biofilm formation for 48 hours, the medium was discarded, and the wells were washed with PBS to remove non-attached bacteria. Subsequently, the plates were filled with 100 µL of MHB containing 2% glucose and 100 µL of antibiotics at concentrations

Table 1. Sequence of Primers Used in This Study

Genes	Primer Sequence (5'-3')	Product Size (bp)	References
ecpA	F-GCAACAGCCAAAAAAGACACCR-CCAGGTCGCGTCGAACT	477	(19)
mrkA	F-CGGTAAAGTTACCGACGTATCTTGTACTG R-GCTGTTAACCACACCGGTGGTAAC	498	(19)
fimA	F-CGGACGGTACGCTGTATTTT R-GCTTCGGCGTTGTCTTTATC	500	(19)
fimH	F-TGCAGAACGGATAAGCCGTGG R-GCAGTCACCTGCCCTCCGGTA	508	(20)
aac (3′)- IIa	F- ATGCATACGCGGAAGGC R-TGCTGGCACGATCGGAG	822	(21)
ant (2′)- Ia	F-ATCTGCCGCTCTGGAT R-CGAGCCTGTAGGACT	404	(21)
aph (3′)- Ia	F-CGAGCATCAAATGAAACTGCR-GCGTTGCCAATGATGTTACAG	623	(21)
aac (6′)- Ib	F-TATGAGTGGCTAAATCGATR-CCCGCTTTCTCGTAGCA	395	(21)

ranging from 0.25 to 512 µg/mL. After incubation at 37°C for 24 hours, the plates were treated according to the biofilm formation assay described above. The minimal biofilm eradication concentration (MBEC) was defined as the lowest concentration of antibiotics leading to a 100% decrease in the metabolic activity of preformed biofilms. The experiment was carried out in triplicate and repeated three times.

3.5. DNA Extraction and Polymerase Chain Reaction

Total DNA was extracted from bacterial isolates using the boiling technique (18). Table 1 lists the specific oligonucleotide primers used to identify AME genes (*aac(3′)-IIa*, *ant(2″)-Ia*, *aph(3′)-Ia*, and *aac(6′)-Ib*) and biofilm formation genes (*fimA*, *fimH*, *mrkA*, and *ecpA*). The quality and quantity of the DNA were assessed using a Nanodrop spectrophotometer (Nanodrop One; Thermo Scientific, Wilmington, USA). DNA samples with a minimum concentration of 50 µg/mL were kept at -20°C until use. The reaction mixture was prepared as follows: 12.5 µL of 2 × Master Mix (Ampliqon, Denmark), 1 µL of each forward and reverse primer, 2 µL of template DNA, and sterile distilled water up to a final volume of 25 µL. The reactions were carried out in the C1000 Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc., USA). The following thermal program was used to amplify the fragments: Initial denaturation at 94°C for 5 min, followed by 32 cycles (denaturation at 95°C for 45 s, annealing at 52 - 58°C for 45 s, extension at 72°C for 60 s), and a final extension at 72°C for 7 min. The PCR products were separated on a 1.5% agarose gel stained with Safe Stain (Sinaclon, Tehran, Iran).

3.6. Statistical Analysis

Descriptive data were analyzed using Microsoft Excel and SPSS version 22 statistical software (IBM Corporation, Armonk, NY, USA). Fisher's exact test was employed to determine significance, with a P-value < 0.05 considered statistically significant. The results are presented as descriptive statistics in terms of relative frequency.

4. Results

4.1. Bacterial Characterization

Between October 2020 and March 2021, a total of 70 *E. coli* clinical isolates were obtained from various clinical specimens, the distribution of which is shown in Table 2. These isolates were recovered from 46 (65.7%) male and 24 (34.2%) female hospitalized patients, with a mean age of 39.5 years (ranging from 11 to 68 years). *Escherichia coli* was mostly isolated from urine [n = 39 (15 females and 24 males); 55.7%], tracheal tube (n = 10; 14.2%), and sputum [n = 8 (5 males and 3 females)]. The remaining specimens were collected from peritoneal aspirate (n = 2; 2.8%), wound (n = 4; 5.7%), and tonsil (n = 7; 10%).

4.2. Antimicrobial Susceptibility Testing

Antibiotic resistance analysis showed that among 70 *E. coli* isolates from clinical specimens, simultaneous resistance to GEN, AMK, and TOB was observed in 28.5% of isolates, making it the most common antibiotic resistance pattern among the studied isolates (Table 3). Among aminoglycoside antibiotics, *E. coli* isolates showed the highest and lowest resistance rates to TOB (n = 36; 51.4%) and GEN (n = 17; 24.2%), respectively. The *E. coli* isolates also displayed different levels of resistance to the following antibiotics: AMK (n = 27; 38.5%), TGC (n = 20; 28.5%), PTZ (n = 31; 44.2%), IMI (n = 55; 78.5%), MER (n = 70; 100%), ERT (n = 42; 60%), CZ (n = 42; 60%), FOX (n = 40; 57.1%), CTX (n = 70; 100%), CAZ (n = 65; 92.8%), CRO (n = 70; 100%), CFP (n = 70; 100%), CIP (n = 20; 28.5%), AZM (n = 5; 7.1%), ampicillin-sulbactam (n = 35; 50%), and clindamycin (n = 15; 21.4%).

Table 2. Distribution of Samples in Terms of Gender [a]

Samples	Gender	
	Male	Female
Urine	24 (34.2)	15 (21.4)
Peritoneal aspirate	1 (1.4)	1 (1.4)
Sputum	5 (7.1)	3(4.2)
Tracheal tube	8 (11.4)	2 (2.8)
Wound	2 (2.8)	2(2.8)
Tonsil	6 (8.5)	1 (1.4)
Total	46	24

[a]Values are expressed as No (%).

Table 3. Distribution of Aminoglycoside Susceptibility Based on Biofilm Formation Ability

Variables	Tobramycin (n)		Amikacin (n)		Gentamicin (n)		P-Value
	N [a]	S [b]	N	S	N	S	
Biofilm							0.008
Producer	31	19	25	25	15	35	0.002
Non- producer	5	15	2	18	2	18	0.1

[a] N, non-susceptible.

[b] S, susceptible.

4.3. Biofilm Formation Assay

According to our results, 50 (71.4%) of the isolates were biofilm producers. Twenty-one (21/70; 30%) of the isolates were strong biofilm producers, while the remaining isolates were considered moderate (29/70; 41.4%) and weak (0/70; 0%) biofilm formers. Although the prevalence of strong biofilm producers was higher among the extensively drug-resistant (XDR) phenotype compared to the multiple drug-resistant (MDR) group (76.1% in XDR vs. 23.8% in MDR), there was no significant relationship between the resistance phenotype and the strength of biofilm formation ($p = 0.59$). Additionally, 50% of non-biofilm producer isolates exhibited the MDR phenotype and were recovered from urine (50%) samples. *E. coli* strains isolated from urine and tracheal tubes from young adults (under 60 years old) were stronger biofilm formers. Biofilm producer strains were significantly more prevalent among aminoglycoside non-susceptible isolates (Table 3). Notably, only 7.1% (5/70) of non-biofilm producer isolates were resistant to aminoglycosides.

4.4. Ability of AMK and GEN in Biofilm Eradication

To investigate the ability of GEN and AMK in eradicating biofilms, we determined the MBEC. After treating biofilms with different concentrations of AMK and GEN, we counted living bacteria in all tested isolates. Table 4 illustrates the ability of these antibiotics to disperse preformed biofilms in *E. coli* isolates. GEN and AMK eradicated the preformed biofilms with MBEC values ranging from 64 µg/mL to 1024 µg/mL ($MBEC_{50}$ = 512 µg/mL and $MBEC_{90}$ = 1024 µg/mL) and from 512 µg/mL to 2048 µg/mL ($MBEC_{50}$ and $MBEC_{90}$ = 2048 µg/mL), respectively.

The data indicated that antibiotic-resistant isolates in the planktonic form did not show increased resistance to antibiotics when grown in the biofilm state. However, stronger biofilm formers exhibited a dramatic increase in resistance to antibiotics when grown in biofilms, compared to moderate and weak biofilm formers. The MBECs of GEN and AMK against strong to moderate biofilm formers were ≥ 512 µg/mL and ≥ 1024 µg/mL, respectively, while against weak to non-biofilm formers, the GEN MBEC was ≤ 512 µg/mL and the AMK MBEC was ≤ 1024 µg/mL.

4.5. Distribution of Aminoglycoside Modifying Enzymes and Biofilm-Related Genes in Escherichia coli

Table 4. Demographic Data, Prevalence of Fimbrial and Aminoglycoside Modifying Enzymes Genes, and Biofilm Production in *Escherichia coli* Isolates

Isolate Number	Ward	Sample	Patient's Gender	Age	Biofilm	MBEC		Resistotype	Presence of AME Genes
						Gentamicin	Amikacin		
E1	ICU	Sputum	Female	40	Non	512	512	MDR	aac (3')-IIa
E2	ICU	Sputum	Female	31	Moderate	512	1024	MDR	*FimA*
E3	ICU	Sputum	Male	45	Strong	1024	2048	XDR	*FimA*
E4	ICU	Tracheal tube	Female	26	Moderate	1024	1024	MDR	*ecpA*, *FimA*, ant (2")-Ia
E5	Surgery	Peritoneal aspirate	Female	52	Moderate	1024	1024	MDR	*ecpA*, *FimA*, *FimH*, ant (2")-Ia, *aph(3')-Ia*,
E6	Men	Urine	Male	41	Non	64	512	XDR	*ecpA*,
E7	Men	Urine	Male	37	Moderate	128	1024	MDR	*ecpA*
E8	Burn	Wound	Female	67	Strong	512	2048	MDR	*FimA*
E9	Burn	Wound	Male	38	Non	128	512	MDR	ant (2")-Ia
E10	ENT	Tonsil	Male	35	Strong	1024	2048	MDR	*ecpA*, ant (2")-Ia
E11	ENT	Tonsil	Male	41	Moderate	512	1024	MDR	*ecpA*
E12	ENT	Tonsil	Male	29	Non	512	1024	MDR	*ecpA*, *FimA*, aac (6')-Ib
E13	Men	Urine	Male	38	Strong	1024	2048	XDR	*ecpA*
E14	Surgery	Sputum	Female	42	Strong	1024	2048	XDR	*FimA*
E15	ICU	Sputum	Male	50	Non	512	1024	MDR	*ecpA*, aac (3')-IIa, ant (2")-Ia, aph (3')-Ia
E16	internal medicine	Urine	Female	48	Moderate	512	2048	MDR	*ecpA*, *FimA*, ant (2")-Ia
E17	Men	Urine	Male	43	Moderate	512	2048	XDR	*ecpA*, *FimA*, *FimH*, aac (3')-IIa
E18	Women	Urine	Female	20	Moderate	512	2048	MDR	*ecpA*, ant (2")-Ia, aph (3')-Ia
E19	ICU	tracheal tube	Male	68	Non	128	1024	MDR	*ecpA*
E20	Men	Urine	Male	35	Moderate	1024	2048	MDR	*ecpA*, aac (3')-IIa, ant (2")-Ia
E21	internal medicine	Urine	Female	32	Strong	1024	2048	MDR	*ecpA*, *FimA*, aph (3')-Ia
E22	Women	Urine	Female	41	Moderate	512	2048	XDR	*ecpA*, ant (2")-Ia
E23	Women	Urine	Female	27	Moderate	512	2048	XDR	aac (3')-IIa, ant (2")-Ia
E24	Men	Urine	Male	42	Non	512	512	XDR	*ecpA*, ant (2")-Ia
E25	Surgery	Tonsil	Male	51	Non	512	1024	XDR	*ecpA*
E26	Urology	Urine	Female	34	Non	512	1024	XDR	*FimA*
E27	Urology	Urine	Female	22	Strong	512	2048	XDR	*ecpA*, *FimA*, aac (3')-IIa, ant (2")-Ia ,
E28	Urology	Urine	Female	40	Strong	512	2048	XDR	*ecpA*, ant (2")-Ia, aph (3')-Ia
E29	ICU	Sputum	Male	31	Strong	512	2048	XDR	*FimA*, *FimH*, aph (3')-Ia, aac (6')-Ib
E30	Men	Urine	Male	45	Non	1024	512	MDR	*ecpA*
E31	Urology	Urine	Female	26	Moderate	64	512	XDR	aac (3')-IIa
E32	internal medicine	Urine	Male	45	Moderate	512	2048	MDR	*ecpA*, ant (2")-Ia
E33	internal medicine	Urine	Male	26	Moderate	512	2048	MDR	*ecpA*, ant (2")-Ia
E34	ICU	Tracheal tube	Male	52	Non	128	512	MDR	*ecpA*, ant (2")-Ia
E35	Men	Urine	Male	41	Moderate	1024	2048	XDR	*ecpA*, *FimA*, *FimH*, ant (2")-Ia, *aac(6')-Ib*
E36	women	Sputum	Male	37	Strong	1024	2048	XDR	*ecpA*
E37	urology	Urine	Male	67	Moderate	1024	2048	MDR	*ecpA*, *aac(3')-IIa*, *ant(2")*-Ia
E38	surgery	Sputum	Male	38	Moderate	1024	2048	MDR	*ecpA*
E39	internal medicine	Urine	Male	35	Non	512	1024	XDR	*ecpA*, *FimA*
E40	infectious diseases	Urine	Female	41	Moderate	1024	2048	XDR	*ecpA*, ant (2")-Ia
E41	infectious diseases	Urine	Female	29	Strong	1024	2048	XDR	-
E42	urology	Urine	Female	38	Non	512	512	XDR	-
E43	Women	Urine	Female	42	Strong	1024	2048	XDR	-
E44	Women	Urine	Female	50	Moderate	1024	2048	XDR	*ecpA*, ant (2")-Ia
E45	men	Urine	Male	48	Non	128	512	XDR	aac (3')-IIa, ant (2")-Ia
E46	internal medicine	Urine	Female	38	Strong	512	2048	XDR	*ecpA*, ant (2")-Ia
E47	Men	Urine	Male	43	Strong	512	2048	XDR	*ecpA*
E48	Men	Urine	Male	42	Non	1024	512	XDR	*ecpA*, ant (2")-Ia
E49	men	Urine	Male	36	Moderate	64	2048	XDR	*aac(3')-IIa*, ant (2")-Ia
E50	pediatrics	Urine	Male	13	Moderate	512	2048	XDR	*ecpA*, ant (2")-Ia
E51	pediatrics	Urine	male	11	Moderate	1024	2048	XDR	*ecpA*
E52	ICU	Urine	male	56	Strong	512	2048	XDR	*ecpA*, ant (2")-Ia
E53	ICU	Urine	Male	74	Moderate	1024	1024	XDR	*aac(3')-IIa*, *ant(2")*-Ia
E54	ICU	Tracheal tube	Female	40	Moderate	1024	2048	XDR	*ecpA*, ant (2")-Ia
E55	surgery	Tracheal tube	male	31	Strong	1024	2048	XDR	*ecpA*, *FimA*, *FimH*, ant (2")-Ia, *aph(3')-Ia*, *aac(6')-Ib*
E56	men	Urine	Male	45	Moderate	128	1024	XDR	*ecpA*, *FimA*
E57	men	Urine	Male	26	Non	128	512	XDR	*ecpA*
E58	burn	Wound	Female	52	Strong	1024	1024	XDR	-
E59	burn	Wound	Female	41	Non	512	1024	-	*ecpA*, ant (2")-Ia
E60	ENT	Tonsil	Male	37	Strong	1024	2048	MDR	*ecpA*, ant (2")-Ia
E61	men	Tonsil	Male	67	Strong	1024	2048	XDR	*ecpA*, ant (2")-Ia
E62	ENT	Tonsil	Female	13	Moderate	1024	2048	MDR	*ecpA*, *FimA*, *FimH*, ant (2")-Ia, aac (6')-Ib
E63	men	Urine	Male	29	Non	1024	512	MDR	*ecpA*
E64	men	Urine	male	42	Moderate	1024	2048	XDR	*ecpA*, aac (3')-IIa, ant (2")-Ia
E65	Surgery	Peritoneal aspirate	male	51	Non	128	512	MDR	*ecpA*, ant 2")-Ia
E66	Male	Tracheal tube	Male	34	Moderate	1024	1024	MDR	aac (3')-IIa, *ant(2")*-Ia
E67	Male	Tracheal tube	Male	40	Strong	64	2048	MDR	*ecpA*, ant (2")-Ia
E68	Male	Tracheal tube	Male	31	Non	512	512	XDR	*ecpA*, *FimA*, ant (2")-Ia, aph (3')-Ia
E69	Male	Tracheal tube	Male	45	Moderate	1024	2048	MDR	*ecpA*, ant (2")-Ia
E70	Male	Tracheal tube	Male	23	Strong	1024	1024	XDR	*ecpA*, ant (2")-Ia

Abbreviations: MBEC, minimal biofilm eradication concentration; AME, aminoglycoside modifying enzymes.

Among the 36 isolates that were resistant to at least one of the aminoglycoside antibiotics, 13/36 (36.1%) isolates had AME-related genes either alone or in various combinations. The presence of these genes was not confirmed in the remaining aminoglycoside non-susceptible isolates (23/36; 63%) by PCR and sequencing. Of the 47 positive isolates for at least one of the AME genes, 23 isolates carried one gene encoding AMEs, 8

Table 5. The Distribution of Aminoglycoside Modifying Enzymes and Biofilm Formation Genes in *Escherichia coli*

AMEs and RMTase Genes	No. (%)
fimA	5 (7.1)
ecpA	13 (18.5)
ant (2″)- Ia + ecpA	19 (27.1)
ant (2″)- Ia	1 (1.4)
aph (3′)- Ia	2 (2.8)
ant (2″)- Ia + ecpA + fimA	1 (1.4)
aac (6′)- Ib + aph (3′)- Ia	1 (1.4)
ant (2″)- Ia + aac (6′)- Ib	3 (4.2)
ant (2″)- Ia + aph (3′)- Ia	4 (5.7)
aph (3′)- Ia + ant (2″)- Ia + aac (6′)- Ib	1 (1.4)

Abbreviation: AMEs, aminoglycoside modifying enzymes; RMTase, rRNA methyltransferases.

isolates carried two genes, and 1 isolate carried three genes. Overall, 15 different combination patterns were determined in the mentioned isolates (Tables 4 and 5).

The prevalence of genes encoding AMEs among the aminoglycoside non-susceptible *E. coli* isolates was as follows: *ant(2″)*-Ia (n = 36; 51.4%), *aac(3′)-IIa* (n = 10; 14.2%), *aac(6′)-Ib* (n = 5; 7.1%), and *aph(3′)-Ia* (n = 8; 11.4%). Our findings revealed that the most frequent AME-related genes were *ant (2")-Ia* and *aph(3′)-Ia*, followed by *aac(3′)-IIa*. As depicted in Table 5, ant *(2")-Ia* was found to coexist with *aac(3′)-IIa* (n = 9; 12.8%), *aph(3′)-Ia* (n = 4; 5.7%), and *aac(6′)-Ib* (n = 3; 4.2%). These coexistences were the most common combinations in TOB and GEN non-susceptible isolates.

In the current study, the prevalence of genes involved in biofilm formation and virulence (*ecpA*, *fimA*, and *fimH*) was detected in all biofilm-producing *E. coli* isolates, whereas *mrkA* was not found in any isolates. *EcpA* and *fimA* were also identified in 75% (15/20) and 20% (4/20) of non-biofilm-producing isolates, respectively. Most isolates harboring AME genes were also positive for biofilm-related genes, including *ecpA* and *fimA*. Table 4 lists all the isolates analyzed, along with their origin, genotype(s), and phenotype(s).

5. Discussion

Escherichia coli remains one of the significant causes of hospital-acquired infections, leading to infections in the urinary tract, surgical wounds, the circulatory system, and pneumonia (22). Aminoglycosides are important drugs in treating *E. coli* infections; however, resistance to these drugs has recently increased in ESBL-producing *E. coli* (22). Various reasons have been attributed to the emergence of resistance against aminoglycosides, the most important of which entail chromosomal mutation and the acquisition of mobile genetic elements with resistance genes (22). Aminoglycoside modifying enzymes -producing genes are found on plasmids carrying ESBL (22). This study aimed to identify aminoglycoside resistance and biofilm formation genes in *E. coli*, and the results confirmed the prevalence of biofilm-producing isolates among XDR isolates. Resistance to aminoglycosides in different regions of Iran exhibits diverse patterns, ranging from 0.00% to 77.27% (23). In our study, the lowest and highest antibiotic resistance among aminoglycosides was related to GEN and TOB, with prevalence rates of 24.2% and 51.4%, respectively. Sometimes, doctors fail to conduct necessary tests to confirm bacterial infections, leading to the unnecessary prescription of antibiotics and contributing to the increased incidence of antibiotic resistance (23).

The prevalence of resistance genes varies between countries. In a study conducted by Ojdana and his colleagues in Poland in 2018, the prevalence of *aac(6′)-Ib* and *ant(2″)-Ia* genes was reported as 59.2% and 4.6%, respectively. Among the isolated isolates, 79.5% were resistant to aminoglycosides. Additionally, the highest resistance was observed in TOB (70.5%), GEN (59%), netilmicin (43.2%), and AMK (11.4%) (19). In the study by Abo-State et al. in Egypt, the most common aminoglycoside resistance genes were reported in the following order: *aac(3′)-IIa* (40%), *aac(6′)-Ib* (30%), *aph(3′)-Ia* (23.3%), *ant(2″)-Ia* (20%), *aph(3′)* (13.3%), and *aac(3′)-Ib* (6.6%). They identified AMK as the most effective antibiotic against *E. coli* (24). In Iran, the prevalence of resistance genes has been reported differently (23). In the present study, ant (2″)-Ia was the most prevalent among the isolated isolates.

In a study conducted in the southwestern region of Iran on the prevalence of virulence genes in biofilm-forming *E. coli*, Boroumand and his colleagues found *iutA*, *FimH* (93%), *ompT, PAI*, and *TraT* genes to be strong biofilm-producing strains. In addition, among the isolated strains, 19.4%, 23.8%, and 56.3% were strong, medium, and weak producers, respectively (25). In another study by Tajbakhsh et al., in Iran, 87% of the strains were resistant to GEN, and 70% were resistant to AMK. Moreover, the prevalence of biofilm producers among the isolated isolates was 61.53%, among which 18.75% were strong, 25% were intermediate, and 56.25% were weak producers. Similarly, the prevalence of the *FimH* gene was 93.33%, and biofilm production showed a significant relationship with the presence of the virulence genes *FimH*, *pap*, *sfa*, and *afa* (26). Katongole et al. explored that biofilm-producing *E. coli* is associated with the MDR phenotype in such a way that 78% of biofilm-producing *E. coli* were MDR and 87% were resistant to GEN. The prevalence of the *fim* gene was 53.5%, which was the most common virulence factor among biofilm-producing strains (13).

In Karigoudar et al.'s study, 94.2% of biofilm producers were resistant to GEN, and a significant link was found between biofilm production and antibiotic resistance (27). Apart from the aforementioned investigations confirming the relationship between biofilm production and the presence of antibiotic resistance in *E. coli* isolates, Behzadi and associates did not find any significant connection between biofilm production and resistance to several antibiotics (28). In a systematic review and meta-analysis conducted in 2020, the incidence rate of biofilm in uropathogenic *E. coli* was 84.6%. The rate of strong producers was 24.8%, while those of medium and weak producers were 26.1% and 44.6%, respectively. In addition, there was a significant relationship between biofilm production and the presence of virulence genes and the occurrence of antibiotic resistance (29).

In our study, *FimH*, *FimA*, and *ecp* genes were present in all biofilm-producing strains. Altogether, biofilm-producing strains show higher antibiotic resistance compared to non-producing strains (30). The persistence of urinary infections in patients is related to the presence of biofilm-producing strains, as these strains tend to have a higher prevalence of antibiotic resistance (26). Enhanced knowledge of detecting biofilms in *E. coli* contributes to more effective management of infections caused by this bacterium. Furthermore, by identifying biofilms and antibiotic sensitivity patterns, it is feasible to choose the most effective antibiotic treatment (27).

5.1. Conclusions

In 2014, the World Health Organization (WHO) introduced *E. coli* as a major concern in causing hospital- and community-acquired infections (31). However, a 2015 report from the Eastern Mediterranean Regional Office of WHO demonstrated that none of the participating countries had a national action plan to combat antibiotic resistance, which could serve as a priority and indicator for control measures (23). The present study reported a high prevalence of antibiotic resistance, particularly varying rates among aminoglycosides. Considering that aminoglycosides are effective drugs in the treatment of *E. coli* infections, preventing resistance against these antibiotics is important. Studies found a high prevalence of resistance genes in the isolated strains, along with virulence factors related to binding factors in all strains.

Various factors inhibit the establishment of an effective program to combat antibiotic resistance in Iran and the Eastern Mediterranean countries. In Iran, many antibiotics can easily be purchased from pharmacies without a prescription. People in the Eastern Mediterranean region often use antibiotics to treat febrile illnesses and obtain them without a prescription. Moreover, low-quality and counterfeit antimicrobial drugs are abundantly found in these areas (23).

Footnotes

Authors' Contribution: S. A., S. Kh., Z. F. contributed to the study conception and design. Data collection and analysis were performed by S. A., S. Kh., M. K., E. M. A., H. M., M. S., M. M., S. M., S. D., M. A. and Z. F. The first draft of the manuscript was written by S. A., S.Kh. and Z. F. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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