

Research paper

Aminoglycoside Resistance Patterns of Certain Gram Negative Uropathogens Recovered from Hospitalized Egyptian Patients

ABSTRACT

Aims: To determine resistance rates and patterns of certain uropathogens, including *E. coli*, *Klebsiella spp.* and *Pseudomonas spp.*, isolated from hospitalized urinary tract infections patients, to aminoglycoside antibiotics and detection of the most prevalent plasmid-mediated aminoglycoside modifying enzymes (AMEs).

Methods: Uropathogenic isolates (150) were recovered from urine specimens of hospitalized UTI patients in Cairo, Egypt and identified by conventional methods. The recovered uropathogens (*E.coli*, *Klebsiella spp.* and *Pseudomonas spp.*) were tested for their susceptibilities to gentamicin, tobramycin, amikacin, neomycin, netilmicin, and kanamycin by disc diffusion method. Plasmid-mediated aminoglycoside resistance was determined by transformation experiments as well as by using plasmids as templates for PCR screening of the AMEs-coding genes *aph(3')-I*, *aac(6')-I*, *aac(3)-I*, *aac(3)-II* and *ant2"-I* in all resistant isolates.

Results: Of a total of 150 uropathogenic clinical isolates, 110 isolates were of the above mentioned genera and were selected for the current study. Sixty three isolates (57.2%) were resistant to at least one aminoglycoside antibiotic. Highest and lowest resistance rates were observed to kanamycin (53.6%) and amikacin (7.2%), respectively. The resistance rates to gentamicin, neomycin, tobramycin and netilmicin were 33.6%, 24.5%, 23.6% and 14.5%, respectively. AMEs-coding genes were detected on plasmids of 93.6% of resistant isolates with prevalence rates of 53.9% for *ant2"-I*, 38% for both *aac(6')-I* and *aac(3)-II* and 33.3% for *aph(3')-I*, while *aac(3)-I* gene was not detected in any of the tested resistant isolates. Double and triple combinations of AMEs genes were detected in 49.2% of resistant isolates.

Conclusion: A high prevalence of plasmid-mediated resistance to aminoglycoside antibiotics in Gram negative uropathogens from hospitalized patients was observed. Uropathogens may represent a potential reservoir of panaminoglycoside resistance in hospitals, having on their plasmids combinations of AMEs-coding genes. Good infection control measures in Egyptian hospitals together with periodic screening of prevalence rates of different resistance genes are required.

Keywords: Aminoglycoside antibiotics; Interpretative reading; Uropathogens; *aph(3')-I*; *aac(6')-I*; *aac(3)-II*; *ant2"-I*

1. INTRODUCTION

In an era of rapid spread of bacterial resistance to many of antibiotics and decelerated discovery of new antimicrobial agents, the focus has returned to the re-evaluation of the use of old antibiotic compounds, which due probably to limited use for years being displaced by more active and less toxic agents, may retain activity against a large number of currently prevalent resistant bacterial isolates. Aminoglycoside antibiotics are re-emerging as valuable

alternative for the treatment of difficult-to-treat infections, particularly those caused by Gram negative pathogens [1, 2].

Aminoglycosides are a group of clinically important, broad-spectrum antibiotics that inhibit protein biosynthesis in bacteria by selectively binding to the A-site decoding region of the bacterial 16S rRNA within the 30S ribosomal subunit causing mistranslation of mRNA or premature termination of protein synthesis [3]. Advantages for clinical use include: having the properties of bactericidal activity, predictable pharmacokinetics, synergy with other antibiotics and low cost. A particular advantage for urinary tract infections (UTIs) treatment is their ability to attain urine concentrations of 25-100 times that in serum [4, 5]. Concentration-dependant bactericidal activity and postantibiotic effect allowed the once-daily dosing regimen which markedly decreased toxicities that limited their wide use for years [6]. Aminoglycoside monotherapy was found to produce equal efficacy with β -lactam drugs and fluoroquinolones in treatment of UTIs and pyelonephritis [7].

Since introduction into clinical use, three mechanisms are known to be responsible for bacterial resistance to aminoglycoside antibiotics [8]: first; decreased intracellular accumulation by outer membrane permeability alteration [9], diminished inner membrane transport [10], or active efflux [11-13]; second; target modification by mutation of 16SrRNA [14, 15] or ribosomal proteins coding genes [16] or by 16SrRNA methylation, newly identified in clinical isolates [17, 18], and third; enzyme-mediated drug modification resulting in compromised binding to target site, the most prevalent in clinical setting [19]. There are three classes of aminoglycoside modifying enzymes: aminoglycoside phosphotransferases (APHs), aminoglycoside nucleotidyltransferases (ANTs), and aminoglycoside acetyltransferases (AACs). Within each class, there are enzymes with different regiospecificities for aminoglycoside modifications [5].

Testing the resistance profile to a large panel of antibiotics has been long known to help in prediction of the underlying resistance mechanism in bacterial isolates using "The interpretative reading" described before by Livermore and coworkers [20], ascertained afterwards by molecular techniques, known to be strongly correlated, thereby, minimizing the number of genes tested [21, 22]. The current study has aimed at determining the resistance rates and patterns of certain uropathogens to different aminoglycoside antibiotics as well as detection of the most prevalent plasmid-mediated aminoglycoside modifying enzymes by phenotypic and genotypic methods.

2. MATERIALS AND METHODS

2. 1. COLLECTION, IDENTIFICATION AND STORAGE OF BACTERIAL STRAINS

A total of 150 bacterial isolates were recovered from urine specimens of UTI patients from four different hospitals in Cairo: Al-Demerdash, Al-Qasr Al-Ainy, Al-Hussien and Dar Al-Fouad Hospitals. Bacterial isolates were identified according to the method described by ?. Isolates of the genera *Escherichia*, *Klebsiella* and *Pseudomonas* were selected for this study whose total number was 110 isolates, and they have been stored as glycerol stock at -20°C throughout this study.

2. 2. ANTIMICROBIAL SUSCEPTIBILITY TESTING

Susceptibility of the selected isolates to six aminoglycoside antibiotics was tested using commercial discs (Oxoid, UK) of the following antibiotics (μ g/disc): gentamicin (10), tobramycin (10), amikacin (30), neomycin (30), netilmicin (30) and kanamycin (30) by disc diffusion method on Mueller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Isolates were categorized as susceptible (S), intermediate (I) and resistant (R) depending on the measured inhibition zone diameters [23]. Isolates showing

resistant and intermediate resistance phenotypes were selected for polymerase chain reaction (PCR) assay of AMEs-coding genes. Interpretative reading of resistance profile was used for prediction of the underlying resistance mechanism [20].

2. 3. PLASMID EXTRACTION

Plasmids were extracted from isolates having reduced susceptibility to at least one of the tested aminoglycoside antibiotics using GeneJet Plasmid Miniprep Kit (thermoscientific) according to manufacturer's directions. Presence, number and molecular sizes of plasmids in each isolate were detected by using gel electrophoresis and GelPilot 1 kb (Qiagen, Germany) was used as ladder.

2. 4. LOCALIZATION AND TRANSFERENCE OF RESISTANCE DETERMINANTS

Plasmid localization of resistance genes and their ability to transfer was tested by transformation experiments using *E.coli DH_{5α}* as a recipient strain. Extracted plasmids from resistant isolates were used to transform chemically competent *E.coli DH_{5α}*, prepared according the method described previously [24]. Briefly, 10 µl of the plasmid DNA were added to 200 µl of competent *E. coli DH_{5α}* (10⁸ cfu/ml) and kept on ice for 30 min. Following a heat shock (90 seconds, 42°C), cells were regenerated in 800 µl Luria-Bertani (LB) medium for 1 hour at 37°C at 300 rpm. Transformants were selected by culturing on LB agar plates containing antibiotics to which each donor isolate is resistant (Kanamycin 50µg/ml and gentamicin 50µg/ml). Successful transformation was confirmed by antibiogram analysis and PCR assay.

2. 5. PCR AMPLIFICATION OF GENES ENCODING AMINOGLYCOSIDE MODIFYING ENZYMES

Five AMEs-coding genes, *aac(6')-Ib*, *aph(3')-Ia*, *aac(3)-I*, *aac(3)-II* and *ant2"-I*, were selected for screening in 63 resistant isolates showing reduced susceptibility to at least one of the tested aminoglycoside antibiotics depending on interpretative reading of the most commonly encountered resistance phenotypes [20] and dissemination in Gram negative isolates as reported in literature [19].

Five sets of primers were designed using the open access programs: Primer3web version 3.0.0 (<http://primer3.wi.mit.edu/>) and Primer-BLAST primer designing tool of the National Center of Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Alignments of nucleotide and aminoacid sequences were performed using ClustalW2 of the European Bioinformatics Institute website (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). According to the above mentioned programs, Primer sequences and expected amplicon sizes (bp) were determined as shown in table (1).

PCR assay was carried out in a final volume of 25 µl, consisting of 12.5 µl of 2X DreamTaq™ Green PCR Master Mix (Fermentas, USA), 1 µl (25 picomole) of each primer (Fermentas, USA) and 1µl of plasmid DNA and the reaction mixture was completed to 25 µl with DNA grade water, using various amplification conditions for each primer set. PCR products were detected by electrophoresis on 1% w/v agarose gel containing 0.5 µg/ml ethidium bromide and visualized on UV transilluminator. PCR products sizes were determined using ready-to-use GeneRuler™ 100bp Plus DNA Ladder (Fermentas, USA).

Table 1. Primer sequences and expected amplicon sizes for PCR products

Primer ^a	Primer sequence (5'→3')	Target genes (alternative names) (accession number) ^b	Amplicon Size (bp)
APH(3')I-f	TTATGCCTCTTCCGACCATC	<i>aph(3')-Ia (aphA-1)</i> (V00359) <i>aph(3')-Ic (aphA1-1AB, aphA7)</i> (M37910)	223
APH(3')I-r	GCCTGAGCGAGACGAAATAC		
AAC(6')I-f	TTGCGATGCTCTATGAGTGG	<i>aac(6')-Ib (aacA4)</i> (M21682)	356
AAC(6')I-r	CGTTTGGATCTTGGTGACCT		
AAC(3)I-f	CCACCTACTCCCAACATCA	<i>aac(3)-I (aacC1)</i> (X15852)	329
AAC(3)I-r	TTCCCGTATGCCCAACTTT		
AAC(3)II-f	GCAGAAGGCAATAACGGAG	<i>aac(3)-IIa (aacC3)</i> (X13543) <i>aac(3)-IIc (aacC2)</i> (X54723)	567
AAC(3)II-r	CCAGGCATCGGCATCTCATA		
ANT(2'')II-f	AAGCACGATGATATTGATCTG	<i>ant(2'')-Ia (aadB)</i> (X04555)	288
ANT(2'')II-r	GGCATAGTAAAAGTAATCCCA		

^af, forward primer; r, reverse primer

^bAlternative names and accession numbers of target genes as described by Ramirez and coworkers [19]

2. 6. DNA sequencing

Four amplicons representative of the positive four sets of primers were verified by sequencing on both strands (Macrogen, Korea). The nucleotide sequences of the amplicons were analyzed using the open access software of National Center of Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov>).

3. RESULTS

3. 1. Isolates identification & aminoglycoside resistance profile

Among 110 isolates, 74(67.2%), 24(21.8%) and 12(10.9%) isolates were identified to be *Escherichia coli*, *Klebsiella spp.* and *Pseudomonas spp.*, respectively and these isolates were selected for further studies. Regarding the resistance profile of the isolates, 63(57.2%) isolates had shown resistance to at least one of the six tested aminoglycoside antibiotics. The resistance distribution among the genera was as follows: *Escherichia coli* (64.0%), *Klebsiella spp.* (15.6%) and *Pseudomonas spp.* (18.7%). Regardless of the genera, the highest resistance rate was to kanamycin (53.6%), lowest resistance rate was to amikacin (7.2%). Resistance rates to gentamicin, neomycin, tobramycin and netilmicin were 33.6%, 24.5%, 23.6% and 14.5%, respectively. Resistance rates to aminoglycoside antibiotics differed markedly in isolates of different genera as shown in table (2).

Table 2. Resistance rates among different isolates, including *Escherichia coli*, *Klebsiella spp.* and *Pseudomonas spp.* against the tested aminoglycoside antibiotics

Genus	No. of isolates resistant to each aminoglycoside antibiotic ^{a,b}					
	G	T	A	Nm	Nt	K
<i>Escherichia coli</i> (n=74)	21(28.3%)	12(16.2%)	3(4.0%)	23(31.0%)	5(6.7%)	38(51.3%)
<i>Klebsiella spp.</i>	8(33.3%)	5(20.8%)	1(4.1%)	1(4.1%)	3(12.5%)	9(37.5%)

(n=24)						
<i>Pseudomonas</i>	8(66.6%)	9(75.0%)	4(33.3%)	3(25.0%)	8(66.6%)	12(100.0%)
<i>spp.</i> (n=12)						
Total (n=110)	37(33.6%)	26(23.6%)	8(7.2%)	27(24.5%)	16(14.5%)	59(53.6%)

^aG, gentamicin; T, tobramycin; A, amikacin; Nm, neomycin; Nt, netilmicin; K, kanamycin

^bPercentage of resistance to each antibiotic was calculated as compared to the number of isolates in each genus

3. 2. Transformation

Transformation experiments were successful in only seven isolates (11.1%) out of 63 resistant ones. The transformation was confirmed by antimicrobial susceptibility testing and PCR assay (Table 3). The resistance profile was identical in transformants and donor clinical isolates except in one transformant (116^T) in which resistance to gentamicin was lost, as compared to the donor clinical isolate. Susceptibility to gentamicin was accompanied by absence of *ant(2'')-I* gene despite being detected in the donor isolate (116).

Table 3. Resistance profile and genes detected in donor clinical isolates and transformants

Isolate No.	Donor Clinical Isolate		Transformant	
	Resistance Profile	Genes detected	Resistance Profile	Genes detected
32	Nm ^R -K ^R	<i>aph(3')-I</i>	Nm ^R -K ^R	<i>aph(3')-I</i>
70	Nm ^R -K ^R	<i>aph(3')-I</i>	Nm ^R -K ^R	<i>aph(3')-I</i>
77	Nm ^R -K ^R	<i>aph(3')-I</i>	Nm ^R -K ^R	<i>aph(3')-I</i>
98	T ^R -K ^R	<i>ant(2'')-I</i>	T ^R -K ^R	<i>ant(2'')-I</i>
116	G ^R -Nm ^R -K ^R	<i>aph(3')-I</i> , <i>ant(2'')-I</i>	Nm ^R -K ^R	<i>aph(3')-I</i>
164	G ^R -T ^R -K ^R	<i>ant(2'')-I</i>	G ^R -T ^R -K ^R	<i>ant(2'')-I</i>
152	G ^R -Nm ^R -K ^R	<i>aph(3')-I</i> , <i>ant(2'')-I</i>	G ^R -Nm ^R -K ^R	<i>aph(3')-I</i> , <i>ant(2'')-I</i>

*G^R, resistant to gentamicin; T^R, resistant to tobramycin; Nm^R, resistant to neomycin; K^R, resistant to kanamycin

3. 3. Prevalence of AMEs genes in *E. coli*, *Klebsiella spp.* and *Pseudomonas spp.* isolates

Among 63 tested isolates, 59(93.6%) were found to carry AMEs genes on their plasmids. *ant(2'')-I* was the most frequently encountered gene (53.9%) followed by *aac(6')-I* and *aac(3)-II* each were found in 24 isolates (38%). Twenty-one isolates (33.3%) were found to carry *aph(3')-I*, while *aac(3)-I* gene was not detected in any of the tested resistant isolates. Regarding the distribution of the four AMEs genes in resistant isolates of different genera, *ant(2'')-I* was detected in 46.3% of *E.coli* resistant isolates, *aph(3')-I* in 44.1% , while 34.1% and 31.5% of them carried *aac(3)-II* and *aac(6')-I*, respectively. In resistant *Klebsiella spp.* isolates, *ant(2'')-I* was the most commonly detected (70.0%), followed by *aac(3)-II* (60.0%), *aac(6')-I* (40.0%), then *aph(3')-I* was the least commonly detected in 20.0% of *Klebsiella spp.* resistant isolates. The prevalence rates of the four AMEs genes in resistant *Pseudomonas spp.* was 66.6%, 58.3, 33.3% and 16.6% for *ant(2'')-I*, *aac(6')-I*, *aac(3)-II* and *aph(3')-I*, respectively

Twenty-eight isolates (44.4%) had only one AME gene, with *aph(3')-I* being the most common single AME coding gene existing in twelve isolates (19.0%), while 31 isolates (49.2%) had more than one AMEs genes. Coexistence of two and three AMEs genes were detected in 18 isolates (28.5%) and 13 isolates (20.6%), respectively, having a total of nine different AMEs genes combinations. The aforementioned genes could not be detected on the plasmids of only four resistant isolates (6.3%) despite showing resistance to at least one of the tested aminoglycoside antibiotics.

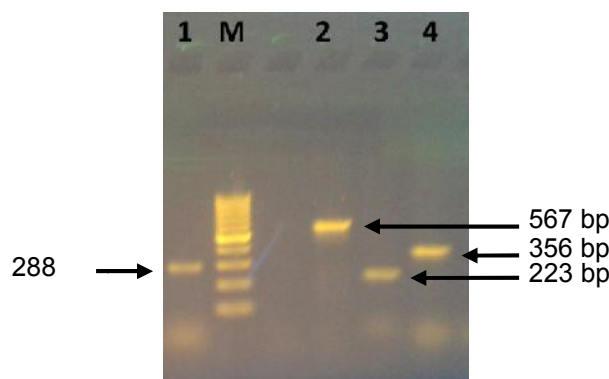


Fig 1. Agarose gel electrophoresis of the amplification products of four positive AMEs-coding genes: lane 1, *ant(2'')*-I gene (288bp); lane M, 100bp ladder; lane 2, *aac(3)-II* gene (567bp); lane 3, *aph(3')-I* (223bp); lane 4, *aac(6')-I* (356bp)

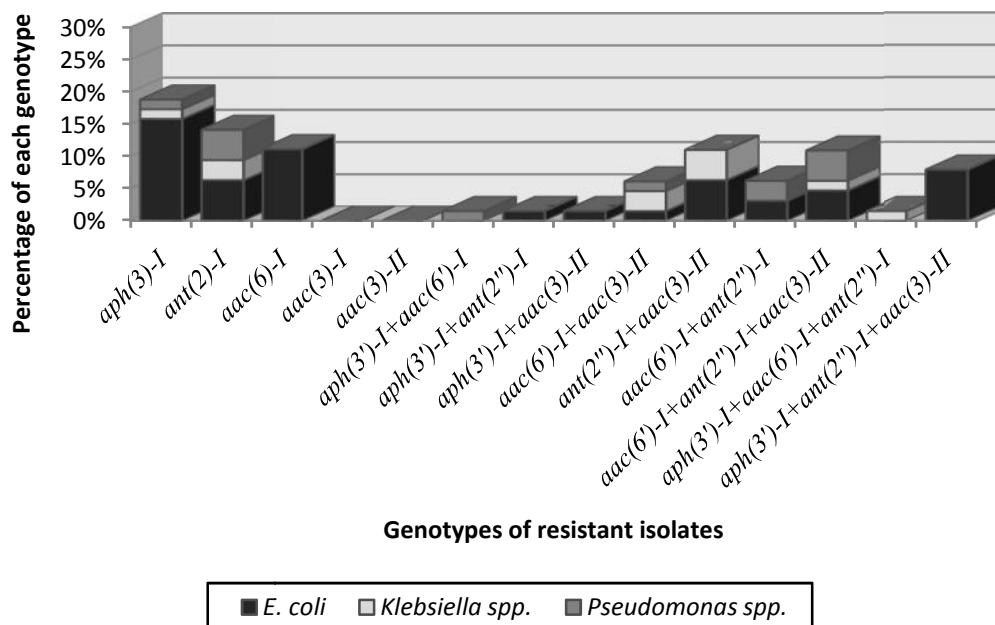


Fig 2. Prevalence rates of different genotypes in resistant isolates of different genera
Percentage of each genotype is calculated as compared to the number of resistant isolates

3. 4. Correlation between AMEs genes and resistance to aminoglycoside antibiotics

Resistance to aminoglycoside antibiotics matched the predicted phenotypes in 53 isolates (84.1%) for gentamicin, 43 isolates (68.2%) for tobramycin, 40 isolates (63.4%) for amikacin, 53 isolates (84.1%) for neomycin, 38 isolates (60.3%) for netilmicin and 59 isolates (93.6%) for kanamycin. Susceptibility to some aminoglycoside antibiotics was retained despite harboring resistance genes in five isolates (7.9%) for gentamicin, twenty isolates (31.7%) for tobramycin, eighteen isolates (28.5%) for amikacin, two isolates (3.1%) for neomycin, 23 isolates (36.5%) for netilmicin and two isolates (3.1%) for kanamycin. In only 11 isolates (17.4% of all resistant isolates), there was 100% concordance between genotype and resistance to all aminoglycoside antibiotics. Frequency of AMEs genotypes and observed

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phenotypes in comparison to expected resistance phenotypes as described before by Ramirez and coworkers [19] were listed in table (4).

Table 4. Frequency of AMEs genotypes, expected resistance phenotypes versus observed phenotypes in tested isolates

AMEs coding genes	Number of isolates n=63(%)	Expected resistance phenotypes ^{a,b}	Observed resistance phenotypes (no. of isolates)
<i>aph(3')-I</i>	12(19.0%)	Nm ^R -K ^R	As expected(8) G ^R -Nm ^R -K ^R (2) K ^R (2)
<i>aac(6')-Ib</i>	7(11.1%)	A ^R -T ^R -Nt ^R -K ^R	As expected(0) T ^R -K ^R (3) K ^R (3) A ^R -K ^R (1)
<i>ant(2'')-I</i>	9(14.2%)	G ^R -T ^R -K ^R	As expected(1) G ^R -A ^R -T ^R -Nt ^R -K ^R (1) G ^R -T ^R -Nm ^R -K ^R (1) T ^R -K ^R (2) K ^R (2) G ^R (1) T ^R -Nt ^R -K ^R (1)
<i>aac(3)-I</i>	0(0.0%)	G ^R	As expected(0)
<i>aac(3)-II</i>	0(0.0%)	G ^R -T ^R -Nt ^R	As expected(0)
<i>aph(3')-I+aac(6')-I</i>	1(1.5%)	A ^R -T ^R -Nt ^R -Nm ^R -K ^R	As expected(0) G ^R -A ^R -T ^R -Nt ^R -Nm ^R -K ^R (1)
<i>aph(3')-I+ant(2'')-I</i>	1(1.5%)	G ^R -T ^R -Nm ^R -K ^R	As expected(0) G ^R -N ^R -K ^R (1)
<i>aph(3')-I+aac(3)-II</i>	1(1.5%)	G ^R -Nm ^R -K ^R	As expected(1)
<i>aac(6')-I+aac(3)-II</i>	4(6.3%)	G ^R -A ^R -T ^R -Nt ^R -K ^R	As expected(0) G ^R -T ^R -Nt ^R -K ^R (1) G ^R -T ^R -K ^R (2) G ^R -Nt ^R -K ^R (1)
<i>ant(2'')-I+aac(3)-II</i>	7(11.1%)	G ^R -T ^R -Nt ^R -K ^R	As expected(1) G ^R -A ^R (1) G ^R -K ^R (4) G ^R -Nt ^R -Nm ^R -K ^R (1)
<i>aac(6')-I+ant(2'')-I</i>	4(6.3%)	G ^R -A ^R -T ^R -Nt ^R -K ^R	As expected(0) G ^R -A ^R -T ^R -Nt ^R -Nm ^R -K ^R (1) G ^R -T ^R -K ^R (1) G ^R -T ^R -Nt ^R -K ^R (2)
<i>aac(6')-I+ant(2'')-I+aac(3)-II</i>	7(11.1%)	G ^R -A ^R -T ^R -Nt ^R -K ^R	As expected(0) G ^R -T ^R -K ^R (2) G ^R -T ^R -Nt ^R -K ^R (3) G ^R -A ^R -T ^R -Nt ^R -Nm ^R -K ^R (2)
<i>aph(3')-I+aac(6')-I+ant(2'')-I</i>	1(1.5%)	G ^R -A ^R -T ^R -Nt ^R -Nm ^R -K ^R	As expected(1)
<i>aph(3')-I+ant(2'')-I+aac(3)-II</i>	5(7.9%)	G ^R -T ^R -Nt ^R -Nm ^R -K ^R	As expected(0) G ^R -Nm ^R -K ^R (5)

^aG^R, resistant to gentamicin; T^R, resistant to tobramycin; A^R, resistant to amikacin; Nm^R, resistant to neomycin; Nt^R, resistant to netilmicin; K^R, resistant to kanamycin

^bExpected phenotypes as described by Ramirez and coworkers [19]

4. DISCUSSION

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting all age groups either in community or healthcare setting [25]. A limited geographical variability of uropathogen occurrence has been demonstrated by different studies being dominated by Gram negative pathogens, most commonly *Escherichia coli*, *Klebsiella spp.* and *Pseudomonas spp.* in hospitalized patients [26]. Other than representing a therapeutic threat on critically ill UTI patients and those in long-term care facilities, resistant uropathogens may become potential reservoirs of certain resistance phenotypes among hospitalized patients [26].

In the current study, resistance profiles and rates of the three Gram negative uropathogens, *Escherichia*, *Klebsiella* and *Pseudomonas*, to aminoglycoside antibiotics were tested in 110 isolates selected from a total of 150 urine specimens from UTI patients in Egyptian hospitals. In 110 isolates and in agreement with other studies [26], the distribution of the three genera was as follows: *Escherichia coli* was the most common (67.2%), followed by *Klebsiella spp.* (21.8%), while *Pseudomonas spp.* was the least common (10.9%). The highest resistance to aminoglycoside antibiotics was exhibited by *Pseudomonas spp.* isolates which were all resistant to kanamycin.

Although variable degrees of resistance to aminoglycoside antibiotics have been shown by isolates of different genera, a high overall resistance of 57.2% was detected. Out of six tested aminoglycoside antibiotics, amikacin was the most active against all isolates while kanamycin has encountered the highest resistance, which is consistent with the results of different previous studies [27-29].

Resistance rates to different aminoglycoside antibiotics among *E. coli* and *Klebsiella spp.* isolates recorded in the current study differed from previous studies in Egypt. In a study carried out in Upper Egypt in 2011 by Gad and coworkers [29], higher resistance rates were detected to gentamicin (36.0%), tobramycin (30.0%), amikacin (16.0%) and neomycin (48.0%) compared to our study (28.3%, 16.2%, 4.0% and 31.0%, respectively) in *E.coli* isolates, but lower resistance rate was detected to kanamycin (44.0%). According to the same study, all previously mentioned aminoglycoside antibiotics encountered higher resistance rates (62.5% to gentamicin, 50% to tobramycin, 25% to amikacin, 62.5% neomycin and 75% to kanamycin) in *Klebsiella spp.* isolates compared to our study (33.3%, 20.8%, 4.1%, 4.1%, and 37.5% respectively). Another Egyptian study on Gram negative uropathogens carried out by Wassef and coworkers [30] has reported resistance rates to aminoglycoside antibiotics that are significantly higher than the two studies, where the highest resistance rate among *Klebsiella spp.* was to netilmicin (80.4%), followed by tobramycin to which resistance rate was 71.9% then gentamicin to which 69.3% resistance was reported. Amikacin was the most active against *Klebsiella spp.* isolates (17% resistance). Although resistance rates to gentamicin (54.2%), tobramycin (47.1%) and netilmicin (55.4%), according to the same study, were higher in *E. coli* isolates than those recorded in the current study, lower amikacin resistance (2.6%) was encountered. In comparison to the results of the SENTRY antimicrobial surveillance program, monitored antimicrobial resistance among uropathogens worldwide over a 4-year period (1997-2000), *E. coli* uropathogens in the current study has recorded higher resistance rates to amikacin, gentamicin and tobramycin than detected in each of North America, Latin America and Asia-West Pacific area. While resistance rates of *Klebsiella spp.* isolates to the same antibiotics in our study were lower than those in Latin America, they were higher than those encountered in both North America and Asia-West Pacific area as reported by the SENTRY program [26, 31, 32].

Several previous studies have examined the resistance of *Pseudomonas spp.* to different aminoglycoside antibiotics in Egypt and other countries worldwide. Resistance rates

recorded in the current study were higher than those reported by Gad and coworkers [29] who detected lower resistance rates to kanamycin (80.0%), gentamicin (55.6%), tobramycin (22.2%), and amikacin (20.0%) but higher resistance rate to neomycin (77.8%). Testing the susceptibility of the same genus to gentamicin, tobramycin, netilmicin and amikacin by Wassef and coworkers [30] revealed lower resistance rates to tobramycin (68.0%) and amikacin (24.5%), and higher resistance rates to gentamicin (77.4%) and netilmicin (83.0%). In comparison to other studies on aminoglycoside resistance in *Pseudomonas spp.* urinary isolates conducted worldwide, resistance to amikacin, gentamicin, and tobramycin in this study were higher than those in Europe (19.4%, 46, and 35.3%, respectively) [33], Asia-West Pacific area (8.5%, 24.5%, and 17.9%, respectively) [26], and North America (6.7%, 13.3%, 8.8% and , respectively) [31] but *Pseudomonas spp.* from urinary isolates in Latin America had shown lower resistance rates to both gentamicin (57.6%) and tobramycin (54.5%) and higher resistance rate to amikacin (51.5%) compared to our findings [32, 34]. Interpretative reading of resistance profile has been widely used before for detection of aminoglycoside resistance mechanisms in different studies [29, 35, 36], as recommended by Livermore and coworkers [20], but co-production of more than one aminoglycoside-modifying enzyme in the same isolate and concurrent development of complex resistance phenotypes together with the poor correlation between genotype and resistance profile, according to the current study and previous studies [37], genotypic detection of underlying resistance mechanism is a must. For proper interpretation of resistance patterns and rates to different aminoglycoside antibiotics, isolates were screened for the presence of five AMEs-coding genes, known to be widely distributed among Gram negative bacilli by PCR assay. So far, enzyme-mediated modification has been known to be the most important mechanism of resistance to aminoglycoside antibiotics [19]. More than 85 AMEs are known to be currently existent, capable of inactivating aminoglycosides by one of three mechanisms: acetylation, adenylation or phosphorylation. Of all known AMEs only a limited number has undergone selection and became responsible for the majority of aminoglycoside antibiotics resistance [6, 38]. Miller and coworkers [38], brought together results of different studies of resistance to aminoglycoside antibiotics in different countries worldwide, have reported higher complexity of aminoglycoside resistance mechanisms detected in studies carried out in 1980s than detected in earlier studies. The author has explained this finding by existence of combinations of gentamicin-modifying enzymes and AAC(6)-I that inactivates amikacin. In the current study, 44.4% of tested Gram negative isolates had a single AME gene while the majority (55.6%) had a combination of two or three. Out of five tested AMEs genes, *ant(2'')-I* was the most prevalent (53.9%) either individual, in 14.5%, or combined with other AMEs genes in 39.6% of resistant isolates of different genera, followed by both *aac(6')-I* and *aac(3)-II* each detected in 38.0% of resistant isolates, *aac(6')-I* was detected as an individual resistant mechanism in 11.1% of resistant isolates and combined to other AMEs genes in 26.9% of them, while *aac(3)-II* existed in combination to other AMEs in its all positive isolates. Regarding the order of prevalence of the four detected genes in the three genera, *ant(2'')-I* was the most prevalent in all genera followed by *aph(3')-I*, *aac(3)-II*, then *aac(6')-I* in *E.coli* isolates, *aac(3)-II*, *aac(6')-I* , then *aph(3')-I* in *Klebsiella spp* isolates and *aac(6')-I*, *aac(3)-II* then *aph(3')-I* in *Pseudomonas spp.*. A recent study in France has reported the predominance of *aac(6')-I* in 36.5% of *Pseudomonas spp.* isolates and *ant(2'')-I* in 21.2% of isolates. *aac(3)-II* was the least common among AMEs genes detected in only 7.7% [39]. In another study in Tahrán, *aac(6')-II* was the most common AME gene detected in *Pseudomonas spp.* isolates followed by *ant(2'')-I*, *aph(3')-VI* then *aac(6')-I* [27]. Despite being the least prevalent, *aph(3')-I* was the most frequent individual AME gene in *E.coli* isolates, while *ant(2'')-I* was the most common individual AME gene detected in isolates of the genera *Klebsiella* and *Pseudomonas*. APH(3')-I enzyme is responsible for resistance to kanamycin and neomycin, the phenotype most frequently detected in Gram negative isolates in Gad and co-workers study [29]. In contrast to our finding concerning *aph(3')-I* gene, it was reported as a rare mechanism among Gram negative isolates tested

for aminoglycoside susceptibility in Turkey by Over and coworkers [28]. The finding of high frequency of *aph(3')-I* in urinary isolates in the current study and the previous Egyptian study [29] is not of great importance, due to lack of kanamycin use in clinical setting [6]. Older studies from different countries around the world in the period from 1988 to 1993 has reported *ant(2'')-I* as the most common individual resistance mechanism to aminoglycoside antibiotics among *Enterobacteriaceae* isolates in Turkey [28, 40], USA, and Japan and *aac(3)-II* in Europe, Latin America and South Africa [38, 41], while *ant(2'')-I* was reported to be the most prevalent individual resistance mechanism in *Pseudomonas spp.* isolates in USA and *aac(6')-II* was the most common in Europe, Latin America, South Africa and Japan in the same period.

Nine different combinations of AMEs were encountered in the current study of which, both [*aac(6')-I+ant(2'')-I+aac(3)-II*] and [*ant(2'')-I+aac(3)-II*] were the most common, being equally detected in 11.1% of all resistant isolates followed by the combination [*aph(3')-I+ant(2'')-I+aac(3)-II*]. The two combinations [*aac(6')-I+aac(3)-II*] and [*aac(6')-I+ant(2'')-I*] were equally existent in 6.3% of isolates. Other rare combinations each detected in 1.5% of resistant isolates include [*aph(3')-I+aac(6')-I+ant(2'')-I*], [*aph(3')-I+ant(2'')-I*], [*aph(3')-I+ant(2'')-I*] and [*aph(3')-I+aac(6')-I*]. Of all combinations detected, those of *aac(6')-I* and gentamicin-modifying enzymes coding genes (*ant(2'')-I* and *aac(3)-II*) are the most important, as described before [38], and were found in 25.3% of resistant isolates. The great attention to the *aac(6')-I* gene arises from several factors among which are: being the most common cause of amikacin resistance in members of the family *Enterobacteriaceae* [36]; having a wide spectrum of activity (Phenotype: ATNtK) [19]; when combined with a gentamicin-modifying enzyme, resistance to almost all aminoglycoside antibiotics may develop [36]; continuous evolution of gene variants whose products have wider substrate spectrum. Among these variants products are: AAC(6')-Ib-cr, an enzyme that includes fluoroquinolones as substrates [42] and AAC(6')-Ib', an enzyme capable to modify GEN instead of AMK (AAC(6')-II phenotype), with difference in amino acid residue in position 119 from leucine to serine. Both AAC(6')-I proteins, harbouring either a leucine or a serine at position 119, may co-exist in the same strain, conferring the broad resistance profile G^R-T^R-A^R-Nt^R-K^R [39]; and finally frequent existence with other antibiotic inactivating enzymes as β -lactamases or carbapenamases [43, 44].

It is noteworthy that two of the isolates that have shown resistance to all tested aminoglycoside antibiotics had the genotype [*aac(6')-I+ant(2'')-I+aac(3)-II*]. Being the most prevalent combination in resistant isolates and carried on plasmids, dissemination of panaminoglycoside resistance among clinical isolates is a potential threat.

A notable finding, revealed by this study, was the poor correlation between the resistance profiles and the genotypes of tested resistant isolates. Resistance profiles of only 17.4% of resistant isolates were 100% concordant with their genotypes. This was accompanied by retained susceptibility to one or more antibiotics despite existence of AMEs gene to which they are known to be substrates. This was evident for amikacin in 75.0% of *aac(6')-I* gene carrying isolates, a similar finding was reported before [45, 46]. Gentamicin is a substrate of enzymes coded by both *ant(2'')-I* and *aac(3)-II*. Susceptibility to gentamicin was detected in 14.7% of ANT(2'')-I producers while none of *aac(3)-II* carriers were sensitive to gentamicin. Similarly, tobramycin remained active against 20.8%, 44.1%, and 54.1% of *aac(6')-I*, *ant(2'')-I*, and *aac(3)-II*-carrier isolates, respectively. 50.0% of *aac(6')-I* carriers and 62.5% *aac(3)-II*-carriers have lost their ability to inactivate netilmicin. On the other hand, there was a conserved ability to modify kanamycin in 100% of isolates carrying either *aph(3')-I* or *aac(6')-Ib*, while 5.8% of *ant(2'')-I* carriers could not inactivate kanamycin. Based on the above mentioned findings, the loss of potential of some AME to modify certain aminoglycoside antibiotics is responsible for difference in resistance rates to aminoglycosides in isolates harboring same resistance AMEs genes and consequently the overall resistance to different aminoglycosides.

Low success rate (11.1%) of plasmid-localization of resistance genes by phenotypic transformation experiments, despite detection by PCR assay on plasmids of resistant isolates may be due to the following reasons: the dramatic reduction in transformation frequencies with larger plasmids (>15 kb) using heat shock-mediated transformation [47], plasmid-mediated resistance genes, as confirmed by PCR, may be carried by a plasmid other than that succeeded to transform *E.coli* DH_{5α} and the last reason, based on the finding that all successful transformations were achieved in *E.coli* clinical isolates, is failure of expression of acquired plasmids of *Klebsiella* and *Pseudomonas*-origin in *E.coli* DH_{5α} host strain.

5. CONCLUSION

This study provided information about resistance to aminoglycoside antibiotics and most common resistance determinants providing useful comparative data for future studies. High prevalence of plasmid-mediated aminoglycoside resistance in uropathogens particularly *Pseudomonas spp.* isolates was observed. Combinations of different AMEs-coding genes was highly disseminated in resistant isolates most importantly amikacin and gentamicin-modifyin g enzymes coding genes. Proper infection control measures and periodic monitoring of resistance to aminoglycoside antibiotics, using interpretative reading guided molecular techniques, in clinical isolates in Egyptian hospitals is required to minimize further dissemination and preserve the usefulness of this important class of antibiotics for complicated infections.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

"This work was carried out in collaboration between all authors. Author SMH performed the experiments, wrote the first draft of the manuscript, and search the literature. Author KMA designed the experiments, wrote the protocol, and managed the analysis of the study. Authors WFE and author MSA managed the literature search. All authors read and approved the final manuscript."

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