Phenotypic and Genotype patterns of aminoglycoside Resistance in Gram negative bacilli

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Background The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients.Testing is required not only for therapy but also to monitor the spread of resistant organisms or resistant genes throughout the hospital and community. **Objectives**: The purpose of our study is to correlate between the phenotypic and genotypic patterns of amino glycosides resistance in the gram negative bacilli that isolated from Cairo University. The phenotypic pattern is determined by using disc diffusion test to (kanamycin, Tobramycin, Amikacin and Netilmycin) and for genotypic pattern is determined by using molecular techniques**Study design:** prospective study.**Methods**: From total 1559 isolate, 396 isolate were gram negative bacilli collected over a 4 -month period from a Kasr El-Aini hospital as detected by routine conventional biochemical method of identification and the most common isolate was E. coli (157) followed by Klebsiella(153) Two 16S rRNA methylase genes, armA and rmtB, were detected by PCR-based assays. b-Lactamase characteristics were determined by phenotypic methods.**Results**: Of the 45 amikacin resistance isolates arm A gene was detected in seven isolates including one E.coli and six K. pneumoniae and rmt B was detected in five isolates including two E.coli and three K. pneumonia. Almost all the aminoglycoside resistant isolates showed resistance to fluoroquinolones (44) isolates and also the ESBLs production was in (42) isolates .
Conclusions: The spread of the multidrug-resistant isolates producing both ESBLs and 16S rRNA methylases may become a clinical problem.
Keywords: resistance genes, multidrug resistance, armA, rmtB, extended-spectrum b-lactamases.
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1. Introduction:

Infections caused by resistant pathogens result in significant morbidity and mortality, and contribute to escalating healthcare costs worldwide. Despite the availability of newer antibiotics, emerging antimicrobial resistance has become an increasing problem in many pathogens throughout the world (1).

Aminoglycosides are among the most commonly used broad spectrum antibiotics for the treatment of infectious diseases caused by Gram-negative bacilli. They inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal subunit, thereby leading to cell death (2).

The major mechanism of resistance to aminoglycosides of the Enterobacteriaceae is the production of enzymes inactivating these compounds. These enzymes are assigned to three groups: (a) acetyltransferases (acetylation of an amino group/AAC), (b) phosphotransferases (phosphorylation of a hydroxyl group /APH) and (c) adenylyltransferases (adenylylation of a hydroxyl group /AAD or ANT) (2).

These enzymes are often plasmid-encoded but may also be associated with transposable elements. Plasmid exchange and dissemination of transposons facilitate the rapid acquisition of resistance phenotypes not only within a certain species but also among a large variety of bacterial types (3).

Aminoglycoside resistance mechanisms can be ascertained by examining the susceptibility of the strains to a panel of aminoglycosides (phenotypic characterization). It has been shown that phenotypic patterns of aminoglycoside resistance as determined by the disk diffusion test correlate well with the genotypes of the organisms defined using molecular techniques (4, 5).

2. Materials and Methods:

Our studied group included (30/45; 66.7%) male and (15/45; 33.3%) female, with a mean age of 50 years ranging from 39 to 68 years and among the 45 cases the most common underlying problem was the urological problems (34/45; 75.6%) and the most common risk factor was diabetes mellitus (DM)

followed by catheterization which were found in (33; 73.3%).

Clinical isolates

From total 1559 urine samples were cultured from inpatients and outpatients suffering from urinary tract infection (UTI) collected between March to June 2008 in Kasr El-Aini hospital; a 5000 bed Cairo university hospital in Egypt 396 gram negative bacilli (GNB) were isolated. The significant pathogens were identified by standard biochemical procedures (6),157 clinical isolates were E.coli ,153 clinical isolates were klebsilla ,53 clinical isolates were pseudomonas ,12 clinical isolates were acinetobacter , 3 clinical isolates were stenotrophomans maltophillia , 9 clinical isolates were proteus spp.,5 clinical isolates were enterobacter and 4 clinical isolates were citrobacter spp.

The clinical isolates isolated from Inpatients samples were 74% (293/396) and were 26% (103/396) from outpatients samples .

Our study focused on 45 (45/396) GNB isolates that show resistance to all aminoglycosides used in our study Amikacin (AK), Gentamicin (GM), Tobramycin (TOB) and Netilmicin (NET) based on CLSI criteria for the disc diffusion(7) .The susceptibilities of each gram negative bacterial isolate were determined by the disk diffusion method (Kirby – Bauer method).

All media, biochemical reactions and susceptibility testing were quality controlled using **ATCC** 25922 (American Type Culture Collection) *Escherichia coli* and *Pseudomonas aeruginosa* **ATCC** 27853 as reference strains.

b-Lactamase characterization

ESBL production was detected by the confirmatory disc diffusion tests recommended by the CLSI (7). ESBL detection occur on two steps, the first step done by measuring disk diameter of third generation cephalosporins antibiotics and it is called screening test then followed by second step called confirmatory test as follow.

A-Screening test:

Each gram negative bacilli were isolated considered a potential ESBL-producer according to the CLSI recommendation, if the zone diameter of the following antibiotics result were as in table (1).

Table (1): The disk diffusion diameters for screening of ESBLs.

Antibiotic name	***Zone dimeter***
Cefpodoxime (30 u)	≤ 22 mm
Ceftazidime(30 u)	≤ 22 mm
Aztreonam(30 u)	≤ 27 mm
Cefotaxime(30 u)	≤ 27 mm
Ceftriaxone(30 u)	≤ 25 mm

B-Confirmatory test (the Double disk synergy test):

CLSI recommends performing phenotypic confirmation of potential ESBL-producing isolates of *GNB by* testing both cefotaxime and ceftazidime in combination with 4 µg/ml clavulanic acid and kept 30 mm apart from center on inoculated Mueller- Hinton Agar (MHA).

Testing can be performed by disk diffusion. For disk diffusion testing, a ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organism (7)

Detection of methylase genes

The armA and rmtB genes were detected by conventional PCR. A fresh bacterial colony was suspended in 100mL of sterile distilled water and boiled at 100 ^{o}C for 10 min. After centrifugation, the supernatant was removed for PCR assays. Primers for armA gene amplification were (5-CCGAAATGACAGTTCCTATC- 3 and 5-GAAAATGAGTGCCTTGGAGG-3), which amplify a 846- bp fragment within armA (8). Primers for rmtB gene amplification were 5-ATGAACATCAACGATGCCCT-3, and 5-CCTTCTGATTGGCTTATCCA-3 which amplify a 769- bp fragment within rmtB (8). Reactions for both genes were run on a GeneAmp PCR system 480 (PE Applied Biosystems, Foster City, CA, USA) with the GeneAmp DNA amplification kit containing AmpliTaq polymerase (PE Applied Biosystems) under the following conditions: 12 min at 95 ^{o}C; 35 cycles of 1 min at 94 ^{o}C, 1 min at 55 ^{o}C, and 2 min at 72 ^{o}C; and finally, 7 min at 72 ^{o}C. PCR products were electrophoresed in 1.5% agarose gels and visualized under UV light (8).

3. Results:

Prevalence of methylase genes

Of the 45 amikacin resistance isolates from (Kasr El-Aini) hospital using conventional PCR technique for detection of 16S rRNA methylases (arm A and rmt B), arm A gene was detected in (21.2%) isolates including (3%) E.coli and (18.2%) K. pneumoniae and rmt B was detected in (15.1%) isolates including (6.1%) E.coli and (9.1%) K. pneumoniae.

Thus, among the 153 K. pneumoniae isolates and 157 E. coli isolates collected from Cairo university, the overall prevalence rates of 16S rRNA methylases were 3.9% % in K. pneumoniae and 1.2% in E. coli, the prevalence rates of armA were 3.9% in K. pneumonia and 0.6% in E. coli, and the prevalenace rates of rmtB were 1.9% in K.

pneumoniae and 0.6% in E. coli as shown in table (2).

Since armA and rmt B had not been detected in Acinetobacter and P. aeruginosa isolates collected between March and June at Kasr El-Aini , our study indicates that clinical E. coli and K. pneumoniae isolates that produced 16S rRNA methylases remained rare at Kasr El-Aini and suggesting that armA is more prevalent than rmtB amongst Enterobacteriaceae isolates in Cairo .

Table (2):The prevalence rate.,

Organism	No	Prevalence rate
Overall		
K. pneumonia	153	3.9%
E.coli	157	1.2%
arm A positive		
K. pneumonia	6	3.9% (as isolates harboring both genes)
E.coli	2	0.6%
rmtB positive		
K. pneumonia	3	1.9%
E.coli	2	0.6%

b-Lactamase characterization

ESBL producing organisms were 42 (93.3%) of the 45 isolates that showed high-level amikacin resistance,(23;92%) in K. pneumonia, (3;75%) in E.coli , (12;100%) in Pseudomonas and (4;100%) in Acinetobacter spp. Isolates as shown in table (3).

Table(3): Percentage of ESBL production.

Organism	% of ESBL producer
Pseudomonas	100
K. pneumonia	92
E.coli	75
Acinetobacter	100

Antimicrobial susceptibility testing

The resistance patterns of the 12 armA-positive and rmtB-positive isolates are summarized in Table (4). All 12 isolates displayed high-level resistance to gentamicin, kanamycin, and tobramycin in addition to amikacin, were resistant to trimethoprim/ sulfamethoxazole , and were susceptible to imipenem . All 12 isolates were ESBL-producing isolate. Of the 7 armA-positive isolates, all isolates were resistant to chloramphenicol and tetracycline. The 33 ESBL-producing isolates demonstrated reduced susceptibilities to cefotaxime, ceftazidime , aztreonam , and cefepime.

Since 42 (93.3%) of the 45 isolates with high-level aminoglycoside resistance were ESBL producers and ciprofloxacin- resistant, the spread of such multidrug-resistant organisms may pose a formidable challenge in the management of seriously ill patients. Therefore, continuous surveillance of such organisms is needed.

Table (4)

Organism	No	Resistance
arm A positive		
K. pneumonia	6	R ESBL CIP SXT CHL AMX CIP FOX TET
E.coli	1	R ESBL CIP SXT CHL AMX CIP FOX TET
rmtB positive		
K. pneumonia	3	R ESBL CIP SXT CHL AMX CIP FOX TET
E.coli	2	R ESBL CIP SXT CHL AMX CIP FOX TET

Resistance to gentamicin, tobramycin, kanamycin and amikacin.ESBL, ESBL production detected by the CLSI confirmatory test and reduced susceptibilities to ceftazidime, cefotaxime, aztreonam, or cefepime. AMX, amoxicillin; CHL, chloramphenicol; CIP, ciprofloxacin; FOX, cefoxitin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.

The impact of the aminoglycosides used in our study differ according to the antimicrobial drug potency in the following pattern, AK (349/396; 88.1%) followed by TOB (163/396; 41.1%) as shown in table (5)

Table (5): Comparison between the impacts of different sensitive aminoglycosides on different gram negative bacilli.

Organisms	No	AK		GM		TOB		NET	
		No	%	No	%	No	%	No	%
E coli	157	153	97.4%	86	54.8%	83	52.9%	70	44.6%
Klebsiella	153	127	83%	47	30.7%	43	28.1%	30	19.6%
Pseudomonas	53	40	75.5%	12	22.6%	17	32%	9	17%
Acinetobacter	12	8	66.7%	3	25%	6	50%	0	0%
Stenotrophomonas	3	3	100%	1	33.3%	2	66.7%	1	33.3%
Enterobacter	5	5	100%	3	60%	3	60%	2	40%
Proteus	9	9	100%	6	66.7%	6	66.7%	4	44.4%

Citrobacter	4	4	100%	4	100%	3	75%	3	75%
Total	396	349	88.1%	162	40.9%	163	41.1%	119	30%

Amikacin showing the highest impact (90%) and effectiveness on gram negative bacilli among aminoglycosides used in our study (Fig 1).

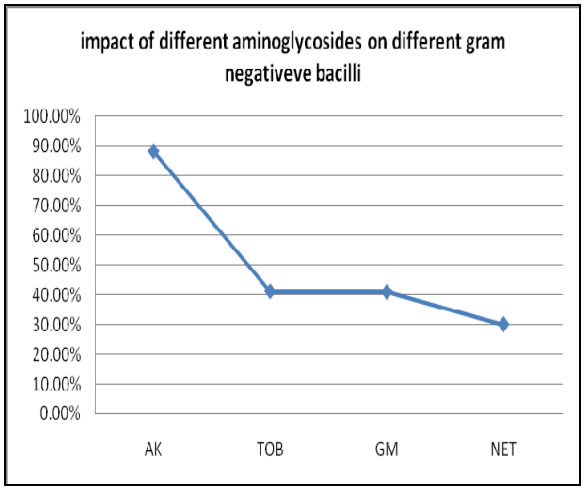

Fig (1): Curve showing the overall aminoglycosides effectiveness on the isolated gram negative organisms.

4. Discussion:

The prevalence of antimicrobial resistance among microorganisms that cause UTI is increasing worldwide and is a major factor in selecting antibiotics for treatment.

The laboratory has a major role in epidemiological evaluations of the nosocomial infections. This includes accurate identification and susceptibility testing of organism causing nosocomial infection, storage of data and isolates and microbiologic typing by phenotypic and genotypic methods (9).

Aminoglycosides continue to play an important role in antimicrobial therapy against both gram-negative and gram-positive pathogens, usually in combination with β-lactam agents. Resistance to the class can be widespread and has primarily been the result of aminoglycoside inactivation through the chemical processes of acetylation, phosphorylation, and/or adenylation, with varying effects depending upon the particular agent (10).

Since 2003, methylation of 16S rRNA has emerged as a serious threat to the class through the action of plasmid mediated methyltransferase enzymes. Alteration of the A site of the 16S rRNA of the bacterial 30S ribosomal subunit by these enzymes (designated ArmA, RmtA, RmtB, RmtC, RmtD, and NpmA) confers resistance to almost all aminoglycosides, including arbekacin, by limiting the binding of these agents to ribosomal target sites following methylation of specific nucleotides. ArmA (Enterobacteriaceae, Acinetobacter spp.) and rmtB (Enterobacteriaceae) have been detected primarily in Asia and Europe (11).

ArmA has also been reported from a clone of Acinetobacter baumannii found in the United States (Pennsylvania) (10).

A sixth plasmid-mediated methyltransferase enzyme, NpmA, is unique in that it produces a broad resistance phenotype to aminoglycosides including apramycin and neomycin, due to methylation of the A1408 position at the A site of 16S rRNA; all other methyltransferases described to date from clinical isolates methylate the G1405 position (12).

Our study aimed to detect the presence of armA and rmtB genes in the isolated aminoglycosides resistant isolates of (Klebsiella, E coli and Acinetobacter), and to correlate the phenotypic pattern of resistance detected by the disk diffusion method and the genotypic pattern of resistance detected by the conventional PCR technique.

Therefore we analyzed 1559 cultured urine samples collected over four months period suffering from UTI in Cairo University Hospital (Kaser El-Eini) .A total of 396 gram negative bacterial isolate were further identified and susceptibility tests were done according to the guidelines of National Committee for Clinical Laboratory Standards (CLSI,2006).

Forty five; 11.4% gram negative isolates were resistant to aminoglycosides and considered our study group, included (30; 66.7%) male and (15; 33.3%) female, with a mean age of 50 years ranging from 39 to 68 years.

Our study group included (6; 13.3%) outpatients and (39; 86.7%) inpatients, the resistance was higher in inpatients and the most resistant organism was Klebsiella (20; 44.4%).

In our study the most common isolated UTI pathogen was E coli (157; 39.6%) followed by Klebsiella (153; 38.6%) while the least common pathogen was Stenotrophomonas (3; 0.7%).

In our study the aminoglycosides resistance rate among the isolated 396 bacterial isolate detected by disk diffusion method was (45; 11.4%). Bogaerts et al., 2007[11] in a Belgium study found that the aminoglycosides resistance rate was (22; 0.14%) detected by disc diffusion method .The higher percentage founded in our study than Europeans results may be due to miss use or over use of antibiotics in our country .

Our study showed that amikacin was the most potent aminoglycoside its overall potency over the isolated gram negative organisms was 88.1%, while Gm was 40.9%, TOB 41.1% and finally NET 30%.

Similar studies in Europe showed that amikacin exhibited activity against gram-negative bacilli superior to those of gentamicin and tobramycin (13).

In our study among the 153 Klebsiella isolates and 157 E coli isolates the overall prevalence rate of the 16S rRNA methylases were 3.9% in Klebsiella and 1.2% in E coli the prevalence rates of armA alone were 3.9% in Klebsiella and 0.6% in E coli while the prevalence rate of rmtB alone were 1.9% in Klebsiella and 0.6% in E coli, thus the armA prevalence rate was higher in Enterobacteriaceae isolates than the rmtB in our study.

Jing et al., 2004 in the Taiwanese study found that the overall prevalence rates of 16S rRNA methylases were 1.2% in K. pneumoniae and 0.4% in E. coli, the prevalence rates of armA were 0.9% in K. pneumoniae and 0.4% in E. coli, and the prevalence rates of rmtB were 0.3% in K. pneumoniae and 0.04% in E. coli.

So the Taiwanese study indicates that E. coli and K. pneumoniae isolates that produced 16S rRNA methylases remained rare and suggests that armA is more prevalent than rmtB amongst Enterobacteriaceae isolates in Taiwan.

The apparently higher prevalence of armA compared with that of rmtB in our study and the Taiwanese study could be due to its association on the same conjugative plasmid with the gene for CTX-M-3 and its location on the functional transposon Tn 1548 (14).

Kunikazu et al., 2007 in Japanese study found that armA and rmtB were found both in Klebsiella spp. and E coli in prevalence rate 0.008% in Klebsiella and 0.02% in E coli .while armA alone was found in Acinetobacter baumannii with prevalence rate 0.13%.

In our study there was no correlation between the phenotypic pattern of aminoglycosides resistance detected by disk diffusion test and the genotypic pattern of resistance that was detected in only (8/33; 24.2%) bacterial isolate.

The most correlation recorded was in the surveillance study in Europe, North America, and Latin America 95.5% because of usage of nine aminoglycosides including arbekacin.

Arbekacin is a semisynthetic aminoglycoside belonging to the kanamycin group, requires 2 modifications at the (6′) aminogroup and the (2″) hydroxyl group for inactivation, so this agent is not inactivated by known plasmid-mediated aminoglycoside modifying enzymes. Therefore, a high-level arbekacin resistance (MIC >512 mg/L) was used as a marker for screening the 16S rRNA methylase-producing strains (15).

We suggest that the absence of correlation in our study was because of presence of other causes of aminoglycosides resistance in the PCR negative isolates such as the enzymatic modification. Also usage of arbekacin in the selective citeria will enhance the chance for detection of the 16S rRNA methyltransferases causative genes.

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