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ORIGINAL ARTICLE

Phenotypic and Molecular Characterization of Aminoglycoside Resistance in Clinical *Escherichia coli* Isolates from Patients at Menoufia University Hospitals

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ABSTRACT

Background: *E. coli may develop different complex mechanisms of resistance to various classes of antibiotics.* ***Objectives:*** *To assess the prevalence of E. coli in Menoufia University Hospitals, to evaluate its antibiotic resistance profile and to detect the presence of aminoglycoside resistance genes [aac(3)IIa and aac(6)Ib] by real-time polymerase chain reaction (PCR).* ***Methodology:*** *A total of 200 clinical samples were collected from patients in Menoufia University Hospitals. E. coli was identified by standard microbiological methods. E. coli isolates were tested by the modified Kirby-Bauer disk diffusion method, and for extended-spectrum β-lactamases production. Aac(3)IIa and aac(6)Ib genes were detected by real-time PCR.* ***Results:*** *E. coli forms 25% of the isolated bacterial strains. E. coli isolates showed the highest resistance to pipracillin and amoxicillin-clavulanate (96%). On the other hand, carbapenemes had the least resistance rate (30%) followed by amikacin (44%), gentamicin (60%), tobramycin (60%) and streptomycin (64%). ESβL production was found among 70% of the isolates by the screening and 50% by the confirmatory methods. Real-time PCR showed that 84% of the isolates contain aac(3)IIa, 52% contain aac(6)Ib and 46% contain both genes.* ***Conclusion:*** *E. coli showed resistance to different classes of antibiotics including aminoglycosides with high prevalence of aac(3)IIa and aac(6)Ib genes. Therefore, infection control measures and rationalized use of antibiotics are mandatory.*

INTRODUCTION

E. coli is associated with wide-range of both community-acquired and nosocomial infections including the urinary tract, biliary tract, intravenous catheters, skin and soft tissue. In addition, *E. coli* may cause infections in pregnant women, such as intra-amniotic and puerperal infection, and neonatal infections. These infections may lead to life-threatening septicemia[2].

Misuse of antibiotics is the main cause of antimicrobial resistance limiting the choices of antibiotics available for treating infections[3]. Horizontal gene transfer represents a major tool for spread of resistance against different groups of antimicrobial agents. Plasmids commonly bear several resistance determinants including genes for beta-lactamases, and quinolone and aminoglycoside resistance[4]; single plasmid conjugation is enough to produce a multi-drug resistant strain [5].

In the last two decades, extended-spectrum-beta-lactamase (ESBL)-producing *E. coli* infections have increased worldwide; thus the use of carbapenems has increased. The emergence of carbapenemases, has also been reported. The new-delhi metallo beta-lactamase (NDM)-producing gene was identified in Enterobacteriaceae, mainly in *E. coli and K. pneumoniae*. NDM-1-producing bacteria exhibited a high resistance rate to other classes of antimicrobial agents, such as fluoroquinolones and aminoglycosides[6]. Due to its particular ecology, *E. coli* can be considered as a sensor of the current situation of antimicrobial resistance. Therefore, it is valuable to assess the level of resistance among *E. coli* isolates[7].

Aminoglycosides are valuable in treatment of bacterial infections[1]. Unfortunately, their efficacy has been reduced by the emergence and dissemination of resistance. Resistance to aminoglycosides in *E. coli* occurs by three main mechanisms: production of aminoglycoside modifying enzymes, alteration of outer membrane permeability (OMP) and alteration of receptor protein on the 30S ribosomal subunit *via* mutation[8].

Production of aminoglycoside modifying enzymes (AMEs) is the most important mechanism. There are three classes of AMEs; aminoglycoside nucleotidyl transferases (ANTs), aminoglycoside phospho-transferases (APHs), and aminoglycoside acetyl-transferases (AACs). AAC is the most predominant group. The main gentamicin resistance genes are

aac(3)I, *aac(3)II*, *aac(3)III*, *aac(4)IV*, and *ant(2)* while, amikacin resistance is often mediated by *aac(6)-Ib* and occasionally by *aph(4)II* and *aph(3)VI* [9].

The mechanism of *E. coli* resistance to aminoglycosides isn't well studied in our locality. Therefore, this study was performed to assess the level of aminoglycoside resistance among clinical *E. coli* isolates by phenotypic methods including disc diffusion and agar dilution methods. Detection of the prevalence of *aac(3)IIa* and *aac(6)Ib* genes was performed using real-time PCR.

METHODOLOGY

Patients:

This study was performed in Medical Microbiology and Immunology Department, Faculty of Medicine, Menoufia University. The involved patients (200) were selected from different Departments, Units [especially intensive care units (ICUs)] and Outpatient Clinics. All patients (120 males and 80 females) were subjected to full history taking and thorough clinical examination, their ages ranged from one month to 70 years. The study was approved by the Local Ethics Committee, Faculty of medicine, Menoufia University.

Clinical samples

A total of 200 clinical samples (90 urine, 50 sputum, 10 bronchial aspirate, 20 pus, 12 burn and wound swabs, 10 blood, 6 discharge from drains and 2 from central venous lines) were obtained from infected patients.

Mid-stream urine sample: **(**10–20) ml was collected from un-catheterized patients. For catheterized patients, 5–10 ml was collected after discarding the first few drops of urine [10].

Sputum samples**:** were collected in sterile disposable cups in the morning.

Bronchial aspirates: **(**1-10 ml) were collected form intubated patients.

Venous blood samples: **(**10 ml) were obtained from each adult patient while 2–5 ml were obtained from infants and children and inoculated into blood culture bottles.

Pus**:** was collected by a sterile cotton swab from infected wounds.

Identification of bacterial isolates:

All the specimens were cultured on different media (Oxoid, UK) which were examined by the standard microbiological methods[11] to identify the growing bacteria. The confirmed *E. coli* isolates were preserved in nutrient broth supplemented with 16% glycerol and stored frozen at -80°C [9].

Antimicrobial susceptibility testing and detection of ESBL production:

Disk diffusion method and both dilution:

All the 50 *E. coli* strains were tested for susceptibility to 16 different antibiotics of. Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method using Muller-Hinton agar (MHA) (Oxoid, UK). The tested antimicrobials included Pipracillin (PRL, 100µg), Amoxicillin/Clavulanate (AMC, 10µ/10 Mg), Cefuroxime (CXM, 30µg) Cefotaxime (CTX, 30µg), Ceftazidime (CAZ, 30µg), Ceftazidime/Clavulanate (CTZ, 30/10 Mg), Imipenem (IPM, 10µg), Meropenem (MEM, 10µg), Aztreonam (ATM, 30µg), Gentamicin (GN, L0µg), Amikacin (AK, 30µg), Tobramycin (TOB, 10µg), Streptomycin (S, 30µg), Ciprofloxacin (CIP, 5µg), Levofloxacin (LEV, 5µg), Trimethoprim-Sulfamethoxazole (TMP/SMX, l,25µg- 23.75µg). Both dilution method was used to measure the minimum inhibitory concentrations (MICs) of amikacin and gentamicin because it allows testing of more than one isolate on the same plate[11] The MICs of gentamicin ranged from 0.5 to 32 ug/ml while, that of amikacin ranged from 4 to 256ug/ml. Results were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2018)[12].

Screening of ESβL production by E. coli isolates:

According to CLSI, 2018[12], Ceftazidime (30µg), cefotaxime (30µg), and ceftriaxone (30µg) were used. A zone diameter of ≤ 22 mm for ceftazidime, ≤ 27 mm for cefotaxime, and ≤ 25 mm for ceftriaxone were considered potential ESβL-producers.

Phenotypic confirmation of ESBLs production by E. coli isolates:

Cephalosporin/clavulanate combination disks:

Confirmation of ESβL-production was performed using ceftazidime (30 µg) and cefotaxime (30 µg) disks alone and in combination with clavulanic acid. An increase of at least 5 mm in zone diameter for antimicrobial combination with clavulanic acid *versus* its zone when tested alone confirms ESβL production [12].

The double–disc synergy test [13]

Disc containing ceftazidime as oxyimino β-lactam was placed 30 mm from amoxicillin-clavulanate disc and incubated at 35°C for 24 hours. A clear extension of the edge of the oxyimino β-lactam inhibition zone towards the disc containing clavulanate indicates a positive synergy result [12].

Molecular detection of aminoglycoside resistance genes by real-time PCR:

DNA extraction:

Bacterial DNA was extracted and purified using the gene JET™ genomic DNA purification kit (Thermo Fisher Scientific, UK)[14]. Primers were shipped and received in a lyophilized state (Invitrogen, Thermo Fisher, UK). The volume of nuclease-free H_2O added to the lyophilized primer was determined by reading the number of nmol of primers in the tube and multiplied by 10 to make a 100 µmol/L primer stock.

Primer sequence[14]:

	Forward	Reverse
Aac(3)IIa	5`TGAAACGCTGACGGAGCCT 3`	5` GTCGAACAGGTAGCACTGAG 3`
Aac(6)Ib	5`CCGACACTTGCTGACGTACAG3`	5`TGACGGACTCTTGCGCTAAA3`

Real-time PCR program:

It consisted of a 95°C for 10 min to activate the enzyme AmpliTaq Gold DNA polymerase, then cycling parameters; 94°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec for 34 cycles as described by the Manufacturer. Later, standard cycling parameters was tried; holding phase a; 50°C for 2 min, holding phase b; 95°C for 10 min to activate the enzymes, then, cycling parameters; 95°C for 15 sec and 60°C for 1 min for 40 cycles. Double-stranded DNA was detected by SYBER green [14].

Statistical analysis:

Computer SPSS program version 20 was used. The results were expressed as percent. Chi-square test was performed and was considered significant at p value <0.05 [15].

RESULTS

A total of 195/200 specimens (97.5%) showed positive cultures; 190 showed single growth and 5 showed mixed growth (2 isolates for each specimen). *E. coli* represented the most frequent isolated organism (25%) followed by *Klebsiella* (19%), *Enterobacter* (15%), *Staph. aureus* (13%), *Candida* and *Pseudomonas* (9%) and *Acinetobacter* (6%). *Proteus* was the least frequent (4%) as shown in figure (1).

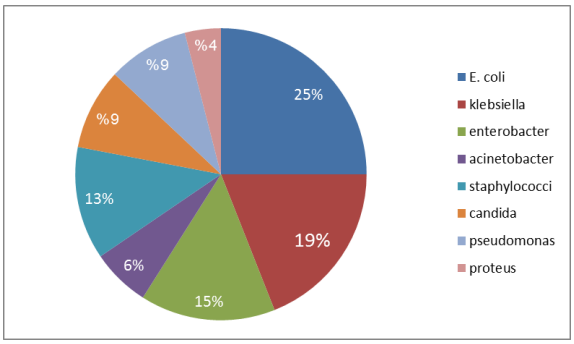

Fig. (1): The isolated organisms from the studied clinical specimens. The most isolated organisms were *E. coli*, *Klebsiella* and *Enterobacter* while, *Proteus* was the least frequent.

The most common source of *E. coli* isolation was from urine (30 isolates) and respiratory secretions (10 isolates), blood culture and swabs (3 for each), central venous line and drains (2 for each). Regarding the hospital departments, the highest isolation rate was from ICUs (34%), while burn unit and chest department showed the least rate (2%) for each.

The disc diffusion method revealed that *E. coli* isolates were highly resistant to pipracillin and amoxicillin-clavulanic acid (96%). On the other hand, the lowest resistance rate was against both imepenem and meropenem (30%). Regarding aminoglycosides resistance, 44% of isolates were resistant to amikacin followed by gentamicin and tobramycin (60%) and finally streptomycin (64%) as shown in table (1).

Table 1: Antibiotic susceptibility pattern of the *E. coli* isolates:

Antimicrobial groups	Antimicrobial Agent	Disk Content (µg)	Clinical isolates (n = 50)						X^2	P value
			S		I		R			
			No	%	No	%	No	%		
Penicillins	Piperacillin	100	1	2	1	2	48	96		
β-Lactam/ β-lactamase inhibitor	Amoxicillin-clavulanic acid	10/10	0	0	2	4	48	96		
Cephalosporins	Ceftazidime	30	12	24	3	6	35	70	14.56	0.005
	Cefotaxime	30	4	8	5	10	41	82		
	Cefuroxime	30	0	0	3	6	47	94		
Monobactams	Azetronam	30	15	30	1	2	34	68		
Carbapenems	Imipenem	10	31	62	4	8	15	30		
	Meropenem	10	32	64	3	6	15	30		
Aminoglycosides	Gentamycin	10	16	32	4	8	30	60	11.56	0.072
	Amikacin	30	25	50	3	6	22	44		
	Tobramycin	10	13	26	7	14	30	60		
	Streptomycin	10	11	22	7	14	32	64		
Fluoroquinolone	Ciprofloxacin	5	13	26	0	0	37	74		
	Levofloxacin	5	14	28	0	0	36	72		
Folate pathway Inhibitors	Trimethoprim Sulfamethoxole	1.25/23.75	10	20	2	4	38	76		

-S= Sensitive I= Intermediate R=Resistant

Statistically significant difference ($p<0.05$). The *E. coli* isolates were most resistant to pipracillin, amoxacillin-clavulanate and cephalosporins and least resistant to carbapenems.

MICs of amikacin and gentamicin were measured by agar dilution method; (48%) of *E. coli* strains were sensitive to amikacin (MIC≤16_ug/ml) while, 36% of isolates were sensitive to gentamicin (MIC ≤4). There was no significant difference between the disc diffusion and both dilution methods.

The isolated 50 *E. coli* strains were screened for ESβL-production by ceftazedime disc diffusion method where 35/50 (70%) were potential ESβL producers. 25/50 (50%) of *E. coli* isolates were confirmed to be ESβL-producers by double disc diffusion (ceftazedime + ceftazedime-clavulanate) and synergy test (figure 2). ESβL-producers confer resistance to different classes of antibiotics. Regarding aminoglycosides; 92% and 84% of ESβL-producers were not susceptible to gentamicin and amikacin respectively (table 2).

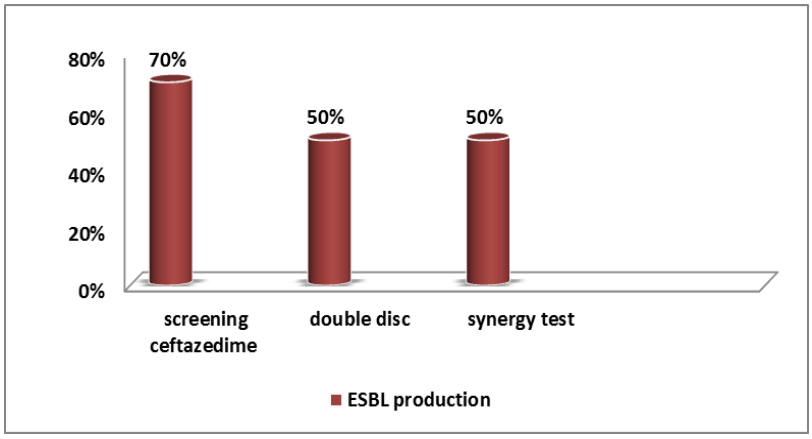

Fig. 2: Comparison between screening and confirmatory methods of ESβL production. ESBL production was detected among 70% and 50% of the isolates by the screening and confirmatory methods respectively.

Table 2: Relation between ESβL production and aminoglycoside resistance among the isolated *E. coli* strains

Aminoglycoside susceptibility	ESβL production				X^2	P value
	ESβL (25)		Non ESβL(25)			
	NO	%	NO	%		
Gentamicin not susceptible	23	92%	9	36%	20.05	0 .00001
Gentamicin-sensitive	2	8%	16	64%		
Amikacin not susceptible	21	84%	4	16%	23.12	0.00001
Amikacin-sensitive	4	16%	21	84%		

-Not susceptible= resistant + intermediate resistant. Statistically significant difference ($p < 0.05$). About 92% and 84% of ESBL-producers were not susceptible to gentamicin and amikacin respectively.

Real-time PCR showed the presence of *aac(3)IIa* gene in 84% of isolates and *aac(6)Ib* in 52% of isolates. Both genes were detected in 46% of *E. coli* strains (figure 3). Table (3) shows that 76% of strains that were resistant to both gentamicin and amikacin contained both genes. Table (4) shows that ESβL production is commonly associated with presence of aminoglycoside resistance genes, 80% of ESβL-producers contained both *aac(3)IIa* and *aac(6)Ib*.

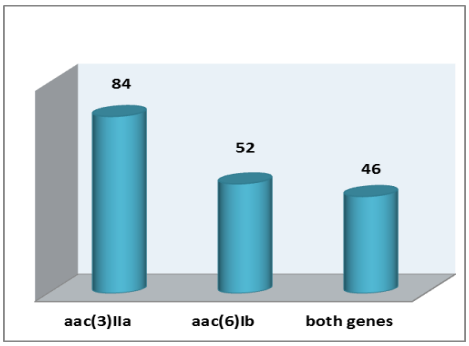

Fig. 3: Prevalence of *aac(3)IIa* and *aac(6)Ib* genes among the isolated 50 *E. coli* strains. Genes were detected by real-time PCR where, 84% of isolates carry *aac(3)IIa* gene, 52% of isolates carry *aac(6)Ib* and 46% of isolates carry both genes.

Table (3): Relation between presence of *aac(3)IIa* and *aac(6)Ib* and aminoglycoside resistance by disc diffusion method:

Gene	Disc diffusion				X^2	P value
	GN R/ AK R (23)	GN R/AK S (11)	GN S/AKR (2)	GN S/AKS (14)		
Aac(3)IIa	2 (8.7%)	8 (72.7%)	0	7 (50%)	26.76	0.0015
Aac(6)Ib	1 (4.3%)	0	1(50%)	2 (14.3%)		
Both	19 (82.6%)	2 (18.1%)	1(50%)	1 (7%)		
No	1 (4.3%)	1 (9%)	0	4 (28.5%)		

GN = Gentamicin AK= Amikacin R=Resistant S=Sensitive. Statistically significant difference ($p<0.05$). There was a significant association between presence of *aac(3)IIa* and *aac(6)Ib* genes and aminoglycoside resistance.

Table (4): Relation between ESβL production and aminoglycoside resistance genes.

Gene	ESβL-positive(25)	ESβL-negative(25)	X^2	P value
Aac(3)IIa	**4 (16%)**	**15 (60%)**	20.80	0.0001
Aac(6)Ib	**1 (4%)**	**4 (16%)**		
Both	**20 (80%)**	**3 (12%)**		
No	**0**	**3 (12%)**		

-Statistically significant difference ($p<0.05$). 80% of ESBL-producers carry both genes and 100% of ESBL-producers carry at least one gene.

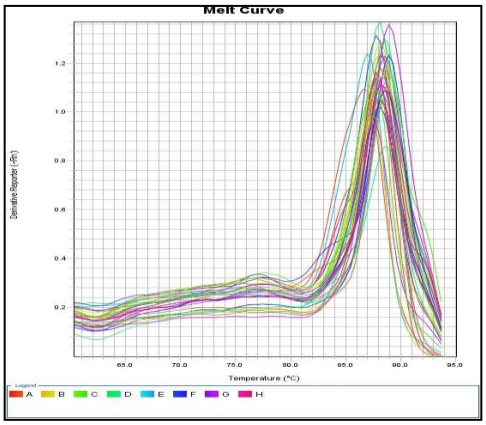

Fig. 4: Melting curve of *aac(3) IIa* gene by real-time PCR.

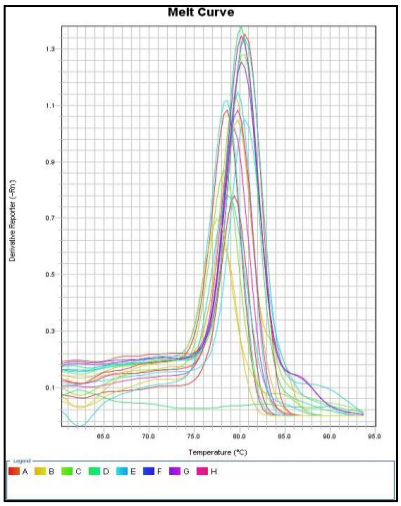

Fig. 5: Melting curve of *aac(6)Ib* gene by real-time PCR.

DISCUSSION

Continuous development of antibiotic-resistant bacterial infections is one of the major worldwide threats nowadays [16]. *E. coli* is one of the most prevalent bacteria in different specimens where they develop complex mechanisms of resistance to various antibiotics [17].

In this study, 50 *E. coli* strains were isolated from 200 clinical samples (25%). Similar rates were reported by several investigators; (28 %[18], 28% [19] and 20%[20]). On the other hand, higher rates were reported (41% [21] and 41.8%[22]). These differences may be due to regional variations in application of infection control measures and resistance to antibiotics. In this study, 34% of *E. coli* isolates were obtained from ICUs where *E. coli* represented 18.8% of all the isolates from ICUs in agreement with the finding reported by others (12%[23] and 15.2%[24]).

The urinary tract was the main site of *E. coli* infections where *E. coli* represented 33.3% of all cases of urinary tract infection, a finding which is similar was reported (38.2%) [25]. However, higher results were found by others (60% [26], 72.45%[8], 52.7% [27] and 50%[28]).

This study showed that the highest resistance rate was against pipracillin and amoxicillin-clavulanic acid (96%) in agreement with that demonstrated by others (94% [29] and 96.6%[30]). However, lower figures were reported (42% [31] and 46.7%[1]). Over use of the β-lactam antibiotics due to their broad-spectrum and wide safety margin lead to extensive resistance. Resistance to β-lactam drugs is attributed to several factors including production of β-lactamases, active expulsion by efflux pump and presence of β-lactam-resistant

transpeptidases. However, production of the β-lactamases remains the most important mechanism of resistance to these drugs[32].

Our study showed that the most efficient group of the tested antibiotics was carbapenemes (resistance rate 30%). These results agree with another study in India (30%) [33]. Alyamani et al [30] found that all *E. coli* isolates were sensitive to meropenem and Rahim et al [34] showed that all *E. coli* isolates were sensitive to impenem. Carbapenemes are now considered the last resort for treatment of infections caused by multidrug-resistant organisms. Therefore, carbapenem resistance should be seriously considered [22]. In this study, MICs of gentamicin and amikacin were determined by agar dilution method. It was found that 44% and 60% of the isolated strains were resistant to amikacin and gentamicin by the disc diffusion method. However, 38% and 56% were resistant to gentamicin and amikacin respectively by the agar dilution method with no significant difference.

Ceftazedime disc diffusion was used as a screening test for ESBL production. This method showed that 70% of our isolates were potential ESβL-producers. ESβL production was confirmed by double disc diffusion and synergy test, where only 50% of the isolated strains were ESβL-producers. There was a significant difference between the two methods (p=0.04). Similar findings were demonstrated by some investigators (61%[24], 40.8% [35] and 55%[28]). However, lower rates were found (18% [36,37], 23.8%[25] and 36%[23]). On the other hand, higher rates were detected (67%[1] and 66.7%[33]). ESβLs are the most important group of β-lactamases that confer resistance to penicillins, cephalosporins and azetreonem [5]. Therefore, ESβL production is often associated with resistance to different antibiotics. Regarding aminoglycosides, 96% and 84% of ESβL-producers were not susceptible to gentamicin and amikacin respectively. These results agree with that of ***Rahim et al*** [34] (82% and 92%) but higher than that of ***Al-Agamy et al***[36] (83% and 54%)**.** ESBL coding genes are often carried on plasmids which may also carry other resistance genes. Therefore, ESβL-producers have limited therapeutic options[30]**.**

In this study, the real-time PCR results showed that *aac(3)IIa* and *aac(6)Ib* genes were found among 84% and 52% respectively of our isolates. On the other hand, 46% of the isolates contained both genes. Our results are similar to that previously reported (91%)[9]. However, lower rates were detected by others (47.8%[38], 39.4%[39]and 40%[21]). The percent of detection of *aac(6)Ib* (52%) in our study agrees with the results reported by ***Soleimani et al***[38](47%) and ***Alyamani et al***[30] (43%) but higher than that of ***Kim et al*** [40](20%).These variations may be attributed to differences in application of infection control measures, misuse of antibiotics and other environmental factors. The high prevalence in our study may be explained by inappropriate antibiotic prescription[3,4].

Presence of both *aac(3)IIa* and *aac(6)Ib* is often associated with gentamicin and amikacin-resistant phenotypes. Our findings showed that 96% of isolates that were resistant to both gentamicin and amikacin contained at least one gene. This finding agrees with that previously reported by Ho et al., (92%) and (96.7%) [9]. These results may be explained by the fact that AMEs are the main mechanism of resistance and that *aac(3)IIa* mainly mediates gentamicin resistance[9,14].

In conclusion, *E. coli* is the most frequently isolated organism in Menoufia University Hospitals. Antibiotic resistance is very common among our isolated *E. coli* strains. Aminoglycoside modifying enzymes are the most important cause of aminoglycoside resistance where *aac(3)IIa* gene is the most common. Therefore, infection control measures should be followed and antibiotic policy must be applied to limit the spread of antibiotic resistance.

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