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Detection of N-Aminoglycoside Acetyltransferases (3)-IIa and (6`)-Ib Enzymes among Gram Negative Bacilli Causing Nosocomial Infections at Surgical ICU Units of Tanta University Hospital

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A B S T R A C T

Aminoglycosides resistance is a major problem because of their critical role in treating life threatening healthcare associated infections (HCAIs). Therefore, this study aimed to detect aminoglycoside resistant gram-negative bacilli in different clinical isolates in Surgical ICUs of Tanta University Hospital. This study was carried out on 600 patients who had evidence of HCAIs. All specimens were cultured on nutrient, Blood, Mac-Conkey and CLED agars. Out of tested specimens 277 gram-negative bacilli (GNB) isolates were identified by conventional methods of them 100 isolates confirmed by API 20E system and their aminoglycosides resistance was assessed by modified Kirby- Bauer method and E-test. AAC(3)-IIa, AAC(6`)-Ib, APH(3′)-VI (APH A6), APH (3′)-I(APH A1), ANT (3")-Ia (AAD A1) and Arm A genes were detected by conventional (PCR). (39%) of isolates were *Acinetobacter baumanii*, (28%) *Klebsiella pneumoniae*, (25%) *Pseudomonas aeruginosa*, (7%) *Proteus mirabilis* and (1%) *Enterobacter cloacae*. All isolates were MDR with 98% of isolates producing ESBL. Over all aminoglycosides resistance were (96%) to tobramycin, (90%) to gentamycin and (89%) to amikacin. AAC(3)-IIa, AAC(6`)-Ib, APH A6, APH A1, AAD A1 and Arm A were detected in 22%,70%, 76%,,43%,18%, 43% of the isolates respectively.

Introduction

Healthcare associated infections are the major risks associated with critically ill patients of ICU, due to the reduced host immunity, frequent use of invasive medical devices, administration of multiple drugs, cross transmission of pathogens among patients and staffs, and inadequate infection control procedures (Parajuli *et al.*, 2017).

Prevalence of HCAIs is roughly about 10–30% in developing countries and 5–10% in developed

countries (Divatia *et al.,* 2020). Antimicrobial resistance (AMR) is a major cause for global concern due to its potential impact on global population health, costs to healthcare systems reduction of treatment options and treatment failure (Naylor *et al.,* 2018) and is particularly dangerous in healthcare settings, where antimicrobials are heavily used, exerting an important selective pressure on microbes (Comar *et al.,* 2019).

In developing countries, many factors including overuse of antibiotics, limited clinical diagnostic and laboratory capacity, and poor infection control, hygiene, and sanitation have contributed to the emergence and spread of AMR.

Healthcare facilities are high risk environments for the development and spread of drug resistance and frequently have the highest burden of multidrug resistant, extensive drug resistant (XDR) and even pandrug resistant pathogens (Raza & Chaudhary, 2019).

HCAIs causative agents and resistance varies throughout world, however 16–20% of bacterial causative agents are multidrug resistance and most of them are gram negative organisms (Noor *et al.,* 2021).

The API 20E system is very useful for detection of non-fastidious gram-negative bacteria up to species level. Most studies have assessed the ability of this system to recover non fastidious gram-negative bacilli from clinical samples, with higher rates of sensitivity and specificity compared with Mac-Conkey agar and biochemical reaction media (Vithanage *et al.,* 2014).

Strains of gram-negative bacilli producing Aminoglycoside modifying enzymes (AMEs) raise several challenges. First, the gene is carried on plasmids that have spread into multiple sequence types of gram-negative bacilli, which appear to be highly successful and capable of causing nosocomial outbreaks. Second, detection of aminoglycoside resistance genes can be difficult by conventional methods. No aminoglycoside resistance gene confer resistance to all aminoglycoside antibiotics, and so their detection subverts standard laboratory detection protocols (Bi *et al.,* 2017).

The most commonly used phenotypic aminoglycoside resistance screening test is disc diffusion method. E-test is a preferential method over standardized disk diffusion due to its simplicity, accuracy, and reliability. Both have several disadvantages, as those tests require an overnight incubation step, and cannot differentiate the resistance genes producing isolates from each other (Khan *et al.,* 2019).Finally, phenotypic detection of aminoglycoside resistance may be not accurate. Therefore, genotypic detection of aminoglycoside resistance genes is the gold standard, although it only detects a pre-specified set of known genes (Fuhrmeister *et al.,* 2021).

Materials and Methods

This cross-sectional study was carried out in the department of Medical Microbiology & Immunology and Central Research Laboratory, Faculty of Medicine, Tanta University on a total of (100) nonduplicate clinical isolates, that were selected from (277) gram negative bacilli isolates collected from (600) patients admitted at Surgical Intensive Care Units in Tanta University Hospitals during the period of research from January 2019 to December 2019. The study included in patients admitted at these departments for more 48-72 hours, who had infections that fulfilled the criteria for defining the healthcare associated infections (HCAIs).

An informed written consents were obtained from all the participants in this research. Ethical approval for this study was provided by Ethics and Research Committee, Faculty of Medicine, Tanta University.

Collection of samples

Specimens were collected under complete aseptic precautions. The samples included wound swabs,

sputum, endotracheal aspirates, bronchoalveolar lavage, blood as well as urine samples. Specimens were labeled and delivered as soon as possible to the laboratory.

By conventional manual blood culture, blood cultures were carried out in two bottles containing 80 ml each of tryptone soya broth bile broth and an anticoagulant.

When immediate delivery to the laboratory was not possible, the specimen was refrigerated at 4–6 °C (Cheesbrough, 2006).

Culture Identification

All specimens were cultured on blood, nutrient, MacConkey agar and CLED (for urine samples) agars. The plates were incubated at 37°C for 24 hours and blood culture bottles incubated at 37º C and examined daily for 7 days for evidence of growth.

Then the isolates in the primary plates were identified by colony morphology, Gram film and various biochemical reactions according to traditional microbiological methods such as catalase test, oxidase test, urease test, sugar fermentation test, citrate utilization, lysine and ornithine decarboxylation tests, motility tests, and H_2S production test.

These biochemical reactions were done from colonies on nutrient agar (Adesoji *et al.*, 2019). All media, biochemical reactions were quality controlled using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 as reference strains.

Identification of isolates to species level by using API 20E

According to the manufacturer's instructions (bioMérieux® sa, France), a single well isolated colony from a young culture plate carefully emulsified in API NaCl 0.85 % Suspension Medium to achieve a homogeneous bacterial suspension used immediately after preparation and inoculated into tubes of API strip, then incubated at 37°C for 24 hours. After interpretation of results using of API20E system reading table, identification of 7-digit numerical profile was performed using the database (V4.0) with the Analytical Profile Index.

Antibiotic susceptibility testing

Disc diffusion method

All the isolates were tested for antibiotic susceptibility by modified Kirby- Bauer disc diffusion method according to CLSI guidelines (CLSI, 2020). The following antibiotics (Oxoid, UK) were used: Gentamycin 10µg, Amikacin 30µg, Tobramycin 10µg, Aztreonam 30µg, Amoxicillin-Clavulanic acid 30/10 µg, Ampicillin-sulbactam 10/10 µg, Piperacillin-Tazobactam 100/10 µg, Ceftriaxone 30µg, Ceftazidime 30µg, Cefepime30µg, Cefazoline 30µg, Imipenem 10µg, Meropenem 10µg, Cotrimoxazole 25µg, Ciprofloxacin 5µg, Levofloxacin 5µg, Doxycycline 30 µg, Nitrofurantoin 300µg, Ceftriaxone - clavulanic 30µg / 10 µg, Ceftazidime - clavulanic 30µg/10 µg.

Combined disc method

Four discs were placed on the inoculated plates: Ceftazidime (30 µg), ceftazidime / clavulanic acid (30/10 µg), cefotaxime (30 µg), cefotaxime / clavulanic acid (30/10 µg). After overnight incubation, an increase in zone size > 5 mm than zone size once tested alone was accepted as confirmation of ESβL production.

Quality control was performed using *Klebsiella pneumonia* ATCC 700603 (ESBL-producing) and E. coli ATCC 25922 (non-ESBL producing).

Aminoglycosides E-test

MIC breakpoints for Gentamycin, Tobramycin, and Amikacin were detected by E-test strips according to

Manufacturer's instructions (Himedia labs, India). The used MIC range was 0.016-256 µg/ml.

Using sterile forceps, the E- test strips were applied to the inoculated agar surface with the MIC scale facing upwards, followed by incubation at 35ºC for 24 hours. The MIC was read directly from the scale in terms of µg/ml at the point where the edge of the inhibition ellipse intersects with the MIC test strip. MIC values were interpreted and compared with the criteria recommended by CLSI 2020 guidelines.

The quality control (QC) strain (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter* ATCC 196060 were used to monitor the performance of the Disc diffusion method and E- test as reference strains.

Bacterial storage and revival

One ml of fresh saturated bacterial culture grown on Luria Bertani (LB) broth was added to 1 ml of sterile glycerol solution in screw capped tubes. The tubes were stored at -80°C. For bacterial revival one loopful was streaked over blood agar plate and inoculated at 35-37°C for 18-24 h.

Conventional PCR

DNA extraction

According to the manufacture instructions (Thermo Scientific, USA), 10-20 bacterial colonies were suspended in a 1.5- or 2-mL microcentrifuge tube by centrifugation for 10 min at 5000 x g, the pellet was resuspended in 180 µL of Digestion Solution and 20 µL of Proteinase K Solution were added and mixed thoroughly by vortexing to obtain a uniform suspension and incubated at 56 °C while using a shaking water bath for around 30 min, until the cells were completely lysed. 20 µL of RNase A Solution were added and mixed by vortexing.

The mixture was incubated for 10 min at room temperature. 200 µL of Lysis solution was added to the sample, then mixed thoroughly by vortexing for about 15 seconds until a homogeneous mixture was obtained. 400 µL of 50% ethanol were added and mixed by vortexing. The prepared lysate was transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube. The column was centrifuged for 1 min at 6000 x g. The collection tube was discarded.

The GeneJET Genomic DNA Purification Column was placed into a new 2 mL collection tube. 500 µL of Wash Buffer I (with ethanol added) were added and centrifuged for 1 min at 8000 x g then 500 µL of Wash Buffer II (with ethanol added) were added to the GeneJET Genomic DNA Purification Column and centrifuged for 3 min at maximum speed (≥12000 x g).

With each step, the collection tube containing the flow-through solution was discarded. The GeneJET Genomic DNA Purification Column was transferred to a sterile 1.5 mL microcentrifuge tube. 200 µL of Elution Buffer were added to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA, incubated for 2 min at room temperature and centrifuged for 1 min at 8000 x g. the purification column was discarded and the purified DNA was used for genes detection immediately or stored at -20 °C.

Nucleic acid amplification

According to the manufacture instructions (Thermo Scientific, USA) Eight sets of uniplex PCR for AMEs (AAC (6 `)-Ib, AAC (3)-IIa, APH(3′)-VI, APH(3′)-I) and three sets of multiplex (PCRs) for AMEs (ArmA, AADA1) were performed using primers and conditions listed in table (1).

After thawing of DreamTaq Green PCR Master Mix (2X), It was Gently vortexed and briefly centrifuged. The samples were also gently vortexed.

In a thin-walled PCR tube the reaction was performed at a defined volume of 25 µl and included 6 µl of sample DNA, 12 µL of DreamTaq Green PCR Master Mix (2X), Forward primer 1µM,

Reverse primer 1µM and 5µl of nuclease-free water for a single gene detection.

For 2 genes detection, the multiplex reaction was performed at a defined volume of 25 µl and included 6 µl of sample DNA, 12 µL of DreamTaq Green PCR Master Mix (2X), Forward primer 1 µM, Reverse primer 1 µM of one gene and Forward primer 1 µM, Reverse primer 1 µM of the second gene and 3µl of nuclease-free water, where the differences in melting temperatures (Tm) between the two primers did not exceed 5 °C.

A negative control was prepared by the addition of the same contents to the tube with water instead of the extract.

Amplification was performed on Biometra thermal cycler (Analytik Jena, Germany) by 1 cycle of Initial denaturation and Enzyme activation at 95°C for 2 minutes, followed by 30 cycles of Denaturation at 95°C for 30s, Annealing for each gene according to table NO. 1 for 15s and extension at 72°C for 10s. lastly 1 cycle of final extension at 72 °C for 10 minutes (Bamigboye et al 2018).

Agarose gel electrophoresis of the amplified DNA

The PCR products were visualized and photographed on a 302 nm UV transilluminator. After electrophoresis for 45 minutes at 100 V through 1% agarose gel containing ethidium bromide (1µg/ml).

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Qualitative data were described using number and percent. Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean ± standard deviation, median and interquartile range (IQR). P value less than 0.05 was considered significant (Washington *et al.,* 2020).

Results and Discussion

Out of samples of 600 patients with (HCAIs), 277 isolates of gram-negative bacilli identified by conventional biochemical methods.

API 20E system identification

API 20E system identification was used to confirm species identification and to avoid the variability in findings of manual biochemical tests.

For an organism identified by API 20E, it was excluded from this study if the percentage of identification was less than 90%, and so out of 277-gram negative isolates, 100-gram negative isolates included in the study.

Acinetobacter baumannii isolates required complementation with other biochemical analysis such as growth at 42 ºC to be identified as *A. baumannii*.

A. baumanii was the most frequently isolated (39%), followed by *Klebsiella pneumoniae* (28%), *Pseudomonas aeruginosa* (25%), and *Proteus mirabilis* (7%). *Enterobacter cloacae* (1%).

Antimicrobial susceptibility testing

All the isolates in our study were MDR, showing (100%) resistance to Amoxicillin-Clavulanic acid, Ampicillin-sulbactam, Piperacillin-Tazobactam, Cefazoline, Ciprofloxacin, Levofloxacin, Nitrofurantoin, Cotrimoxazole, and Aztreonam (not tested for *Acinetobacter baumanii*).

They showed (96%) resistance to 3rd& 4th generation Cephalosporins (Ceftriaxone, Ceftazidime, Cefepime), and (92.9%) resistance to meropenem, (74.6%) to imipenem and (75.9%) resistance to doxycycline (not tested for *Pseudomonas aeruginosa*). (96%) and (90%) of isolates were resistant to tobramycin, and gentamycin respectively and (89%) were resistant to amikacin. All results listed in table (2).

Combined disc method

(98%) of the isolates phenotypically produced ESBL. 100% of *Acinetobacter baumanii, Proteus mirabilis,* and *Pseudomonas aeruginosa* isolates and (96.4%) of *Klebsiella pneumoniae* isolates produced ESBL.

Susceptibility pattern of isolates to (tobramycin, gentamycin, and amikacin) by E-test

By using E-test, isolated organisms showed high rates of aminoglycoside resistance (96%) and (90%) resistance to tobramycin, and gentamycin (MICs ≥ 16 µg/ml) respectively and (89%) resistant to amikacin (MICs ≥ 64 µg/ml)).

(80%) of isolates showed resistance to all tested aminoglycosides (tobramycin, gentamycin& amikacin). The results listed in table (3).

Conventional PCR results

Among 100 isolates, APH A6 enzyme was the most common detected gene (76%), followed by AAC(6′)-Ib enzyme (70%), APH A1(43%), AAC(3)-IIa (22%) and AAD A1(18%) either singly (8 isolates) or in combinations (27 different combination patterns, maximum of five genes/isolate).

Singly, AAC(3)-IIa, Arm A, and APH A1 was detected in one case, AAC(6′)-Ib in two cases and APH A6 in three cases. AAD A1 was not detected singly.

The distribution of different resistant genes among different isolates were listed in table (4).

HCAIs are considered the most common complications of hospital care (Haque *et al.,* 2018).

Antimicrobial resistance (AMR) represents one of the major threats for human health, especially in healthcare settings, as a high-risk environment for the development and spread of drug resistance (Comar *et al.,* 2019). Not surprisingly, AMR is strongly associated with HCAIs severity, as many HAIs are caused by multidrug resistant (MDR) or even pan-drug resistant (PDR) microbes particularly Gram-negative microorganisms (Irek *et al.,* 2018).

The aminoglycoside antibiotics are commonly used in the treatment of HCAIs from gram-negative bacilli. (Kishk *et al.,* 2021). However, different resistance mechanisms had been developed against aminoglycosides such as, enzymatic modification (the most common), impermeability, the activity of efflux pumps, biofilm formation and the activity of 16S rRNA methylase (Zarei *et al.,* 2019).

100 isolates included in our study, were based on API 20E system results, we excluded any isolate that the percentage of identification was less than 90%. All isolates were had excellent, very good and good identification to the species level. *K pneumoniae* isolates revealed 5 different biotypes. The most prevalent were 5215773 and 5214733, which occurred at a prevalence of 50 % and 35.7 %, respectively. These results were compatible with El-Badawy *et al.,* (2017) who detected; by API 20E system, 13 different biotypes of their *K. pneumoniae* clinical isolates, the most prevalent were 5215773 and 5205773, which occurred at a prevalence of (56%) and (29%), respectively. For an organism identified by API 20E. This is in agreement with Robinson *et al.,* (1995), and Peleg *et al.,* (2008) who reported that API 20E, was found to be reliable system for the identification, and biotyping of Enterobacterales and nonenteric gram-negative bacilli. On contrary, Bergogne-Bérézin. (2007) noticed that the (API 20E and 20NE) are not considered a sufficiently reliable identification method.

In the present study, *Acinetobacter baumanii* was the most frequently isolated organism from the studied patients (39%), followed by *Klebsiella pneumoniae* (28%), *Pseudomonas aeruginosa* (25%), and *Proteus mirabilis* (7%). The least isolated organism *Enterobacter cloacae* (1%). These results are nearly similar to that obtained by Zhou et

al. (2019), and Parajuli *et al.,* (2017) who found that, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, were the most prevalent pathogens resulted in HCAIs. On the other hand, El-Badawy *et al.,* (2017), and Gashaw *et al.,* (2018) found that, the most recovered isolates from HCAIs patients were *Klebsiella* species and *Escherichia coli*.

In our study, the antibiotic resistance patterns of 100 clinical isolates were determined by kirby bauer disc diffusion which, by virtue of its convenience, flexibility, efficiency and low-cost is probably the most widely used AST method worldwide. All the isolates in our study were MDR, showing (100%) resistance to Amoxicillin-Clavulanic acid, Ampicillin-sulbactam, Piperacillin-Tazobactam, Cefazoline, Ciprofloxacin, Levofloxacin, Nitrofurantoin, Cotrimoxazole, and Aztreonam (not tested for *Acinetobacter baumanii*). They showed (96%) resistance to 3rd & 4th generation Cephalosporins (Ceftriaxone, Ceftazidime, Cefepime), and (92.9%) resistance to meropenem, (74.6%) to imipenem and (75.9%) resistance to doxycycline (not tested for *Pseudomonas aeruginosa*). Daef & Elsherbiny. (2012) reported that, Gram negative bacteria showed very high resistance (50-100%) to many groups of antimicrobials, as penicillins, cephalosporins, quinolones, aminoglycosides. Kumari *et al.,* (2007), Parajuli *et al.,* (2017), Siwakoti *et al.,* (2018), and Wani *et al.,* (2021) observed that, nearly (96%), (87.07%) (89.5%), and (85.1%) of the Gram-negative bacterial isolates causing nosocomial infections were found multidrug resistant. On the other hand, Christoff *et al.,* (2010), and Eldegla *et al.,* (2016) detected lower resistance to imipenem (11.2%), and (14.3%) respectively among Gram negative bacilli. El-Badawy *et al.,* (2017) reported much lower rates of resistance to imipenem and meropenem (25%), and quinolones among *K. pneumoniae* isolates. Asghar & Ahmad. (2018) reported that, (46.1%) of *Pseudomonas aeruginosa* isolates were resistant to one or more antibiotics.

In our study, phenotypically 100% of *Acinetobacter baumanii*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* isolates and (96.4%) of *Klebsiella pneumoniae* isolates produced ESBL. These results were compatible with, Dalhoff. (2012) reported that 94.9% of the Enterobacterales strains of HCAIs were ESBL-positive. Eldegla *et al.,* (2016) reported that (85%) *K. pneumoniae* isolates were ESBL producers. Fallah *et al.,* (2014), and Ranjbar & Farahani. (2019) reported that, (84.2%) and (85%) of isolated *A. baumannii* strains respectively were ESBL producers Tchakal-Mesbahi *et al.,* (2021), Adjei *et al.,* (2018).and Chen *et al.,* (2015) found that ESBLs production among *Pseudomonas aeruginosa* isolates was (60%), (76%), and (85%) respectively. Whereas, lower rates detected by Onduru *et al.,* (2021) reported that of (35%) of *Klebsiella* spp. isolates were ESBL producing. While Uddin *et al.,* (2021) reported that, only (8.8%) isolates of *P. aeruginosa* were ESBL producers.

In our study, by using E-test, isolated organisms showed high rates of aminoglycoside resistance (96%) and (90%) resistance to tobramycin, and gentamycin (MICs $\geq$ 16 µg/ml) respectively and (89%) resistant to amikacin (MICs $\geq$ 64 µg/ml)), the same aminoglycoside susceptibility patterns obtained by the disc diffusion method. (80%) of isolates showed resistance to all tested aminoglycosides (tobramycin, gentamycin& amikacin), All isolates showed resistance at least to one of the tested aminoglycosides.

(96.4%) of *Klebsiella pneumoniae* isolates in our study were resistant to tobramycin, (92.9%) to amikacin, and (75%) of them were resistant to gentamycin. The isolates of *Proteus mirabilis* showed (100%) resistance to gentamycin and tobramycin, and (85.7%) of them were resistant to amikacin. The one isolate of *Enterobacter cloacae* showed (100%) resistance to tobramycin, and (0%) resistance to gentamycin, and amikacin. Similar findings reported by Estabraghi *et al.,* (2016) and Galani *et al.,* (2019) that, (93%) and (92%) of *K. pneumoniae* were resistance against amikacin respectively. Lower rates of resistance detected by Nasiri *et al.,* (2018), El-Badawy *et al.,* (2017), and

Eftekhar and Seyedpour (2015), found that, (49.2%), and (60%), and (53.2%) respectively of *K. pneumoniae* isolates were resistant to gentamicin. Gür *et al.,* (2020) in Turkey found that, among Enterobacterales isolates, resistance rates against all of the tested aminoglycosides remained below (10.0%).

The isolates of *Acinetobacter baumanii* in our study showed (100%) resistance to gentamycin, (94.9%) to tobramycin and (84.6%) of isolates were resistant to amikacin. Nearly similar results obtained by Akers *et al.,* (2010) reported that the aminoglycoside antibiotics susceptibility was 96.6% to gentamicin and 77.5% to tobramycin. In divergence of our results, (Kishk *et al.,* 2021) found that, *Acinetobacter* isolates showed lower rates of resistance (9.6%) to Amikacin.

Pseudomonas aeruginosa isolates in our study showed (96%) resistance to tobramycin and amikacin and (92%) resistance to gentamicin. This is in agreement with Poonsuk *et al.,* (2013) that reported high resistance rates to tobramycin (96%) gentamicin (95%), and. amikacin (92%), On the other hand, Asghar & Ahmad. (2018), reported that, the detected resistance rates against the aminoglycosides; amikacin, gentamicin, and tobramycin among *P. aeruginosa* clinical isolates were (43%), (18.5%), and (17%) respectively. The difference between the present study results and the other researchers can be explained by the antibiotic prescribing habits, empirical hospital antibiotic policies, nature of infections, sample size, and the sticking to infection control measures.

There are several noteworthy findings in our study. We observed that, aminoglycoside resistance in clinical isolates is predominantly caused by the production of AMEs (99% of isolates), while the occurrence of 16S rRNA methyltransferase genes (Arm A) was observed in (43%). Nearly similar results obtained by Galani *et al.,* (2019) in Greek, where aminoglycoside resistance is mainly caused by the production of AMEs (85.3% of isolates).

In our study, a remarkable AME diversity was observed. APH A6 enzyme was the most common detected gene (76%), followed by AAC(6′)-Ib enzyme (70%), APH A1(43%), AAC(3)-IIa (22%) and AAD A1(18%) either singly or in combinations. Singly, AAC(3)-IIa, Arm A, and APH A1 was detected in one case, AAC(6′)-Ib in two cases and APH A6 in three cases. AAD A1 was not detected singly.

In the present study, among *Klebsiella pneumoniae* isolates, AAC(6′)-Ib was the most detected gene (89.3%), followed by APH A6 (67.9%). Nearly similar results noticed by El-Badawy *et al.,* (2017) and Nasiri *et al.,* (2018). detected that among aminoglycoside-resistant *K. pneumoniae* isolates tested positive for ACC(6′)Ib gene were (88%) and (91.5%) respectively On the other hand, Latifi *et al.,* (2021) reported that, AAC(6′)Ib gene detected in (21.2%)of *Klebsiella pneumoniae* isolates.

In the present study results, among 8 isolates of *Proteus mirabilis* and *Enterobacter cloacae* APH A1was the most detected gene (75%), followed by AAC(6′)Ib and APH A6 (62.5%) respectively. Nearly similar results obtained by Gür *et al.,* (2020), and Sacha *et al.,* (2012) found that, The most common determinant of aminoglycoside resistance among *P. mirabilis* was AAC(6')-Ib (75%), and (71.43%) respectively.

On the other hand, Michalska *et al.,* (2014) found that, The most prevalent gene encoding aminoglycoside resistance among *P. mirabilis* was ant(2″)-Ia, present in(80.3%).

Table.1 Gene detection primers, their sequences, annealing temperatures, and amplicon sizes.

Gene	Primer Sequences (5′–3′)	Annealing temperature Tm in (°C)	Amplicon size in bp	Refrence
AAC (6 `)-Ib	F: TTGCGATGCTCTATGAGTGGC TA R: CTCGAATGCCTGGCGTGTTT	54	482	(Nasiri *et al.*,2018)
AAC(3)-IIa	F: TAGCTTGTCCATCGTGACTC R: ATCGAACAG GTAGCACTGAG	50	740	(Nasiri *et al.*,2018)
ArmA	F: ATTCTGCCTATCCTAATTGG R: ACCTATACTTTATCGTCGTC	54	315	(ELsheredy *et al*,2021)
AAD A1	F: ATGAGGGAAGCGGTGATCG R: TTATTTGCCGACTACCTTGGTG	55	792	(ELsheredy *et al*,2021)
APH (3′)-VI	F: ATGGAATTGCCCAATATTATTC R: TCAATTCAATTCATCAAGTTTTA	54	780	(ELsheredy *et al*,2021)
APH(3′)-I	F: CAACGGGAAACGTCTTGCTC R: ATTCGTGATTGCGCCTGAG	55	455	(ELsheredy *et al*,2021)

Table.2 Susceptibility pattern of isolated organisms to different antimicrobial agents by modified Kirby-Bauer disc diffusion method

Antibiotic	*Klebsiella pneumoniae (n=28)*		*Proteus mirabilis and Enterobacter cloacae (n=8)*		*Acinetobacter baumanii (n=39)*		*Pseudomonas aeruginosa (n=25)*	
	No.	%	No.	%	No.	%	No.	%
AMC	28	100.0	8	100.0	-	-	25	100.0
SAM	28	100.0	8	100.0	39	100.0	25	100.0
TZP	28	100.0	8	100.0	39	100.0	25	100.0
CZ	28	100.0	8	100.0	-	-	-	-
CTX	27	96.4	7	87.5	39	100.0	25	100.0
CAZ	27	96.4	7	87.5	39	100.0	25	100.0
FEB	27	96.4	7	87.5	39	100.0	25	100.0
IPM	20	71.4	5	62.5	33	84.6	20	80.0
MEM	25	89.3	7	87.5	37	94.9	25	100.0
AZT	28	100.0	8	100.0	-	-	25	100.0
TOB	27	96.4	8	100.0	37	94.9	25	100.0
GEN	21	75.0	7	87.5	39	100.0	23	92.0
AK	26	92.9	6	75.0	33	84.6	24	96.0
CIP	28	100.0	8	100.0	39	100.0	25	100.0
LEV	28	100.0	8	100.0	39	100.0	25	100.0
DOX	25	89.3	8	100.0	39	100.0	-	-
F (n= 6)	6	100.0	8	100.0	8	100.0	5	100.0
SXT	28	100.0	5	100.0	39	100.0	25	100.0

Table.3 Susceptibility pattern of isolates to (tobramycin, gentamycin, and amikacin) by E-test

Antibiotic MIC µg/ml		Organism									
		Klebsiella pneumoniae (n= 28)		*Acinetobacter baumanii* (n= 39)		*Proteus mirabilis* (n= 7)		*Pseudomonas aeruginosa* (n= 25)		*Enterobacter cloacae* (n= 1)	
		No.	%	No.	%	No.	%	No.	%	No.	%
Tobramycin	R ≥16	27	96.4	37	94.9	7	100.0	24	96.0	1	100.0
	S ≤ 4	1	3.6	2	5.1	0	0.0	1	4.0	0	0.0
Gentamycin	R ≥16	21	75.0	39	100.0	7	100.0	23	92.0	0	0.0
	S ≤ 4	7	25.0	0	0.0	0	0.0	2	8.0	1	100.0
Amikacin	R≥64	26	92.9	33	84.6	6	85.7	24	96.0	0	0.0
	S ≤ 16	2	7.1	6	15.4	1	14.3	1	4.0	1	100.0

Table.4 Distribution of aminoglycosides resistance genes among the isolated organisms

Resistance gene	Organism										χ^2	^{MC}p
	Klebsiella pneumoniae (n = 28)		*Acinetobacter baumanii* (n = 39)		*Proteus mirabilis* (n = 7)		*Pseudomonas aeruginosa* (n= 25)		*Enterobacter cloacae* (n = 1)			
	No.	%	No.	%	No.	%	No.	%	No.	%		
AAC(6′)Ib	25	89.3	26	66.7	4	57.1	14	56.0	1	100.0	9.064*	0.042*
AAC(3)IIa	10	35.7	6	15.4	1	14.3	5	20.0	o	0.0	6.724	0.131
Arm A	4	14.3	31	79.5	1	14.3	7	28.0	0	0.0	35.557*	<0.001*
AAD A	6	21.4	8	20.5	2	28.6	2	8.0	0	0.0	3.440	0.504
APH A6	19	67.9	31	79.5	5	71.4	21	84.0	0	0.0	5.021	0.269
APH A1	8	28.6	13	33.3	6	85.7	16	64.0	0	0.0	13.852*	0.005*

Photo.1 API 20E strip of *Klebsiella pneumoniae*

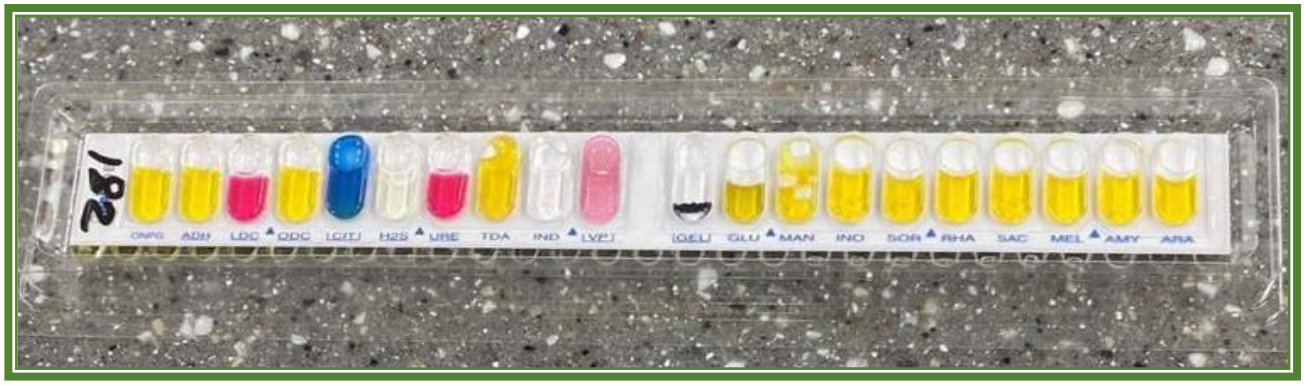


Photo.2 API 20E card of *Klebsiella pneumoniae*

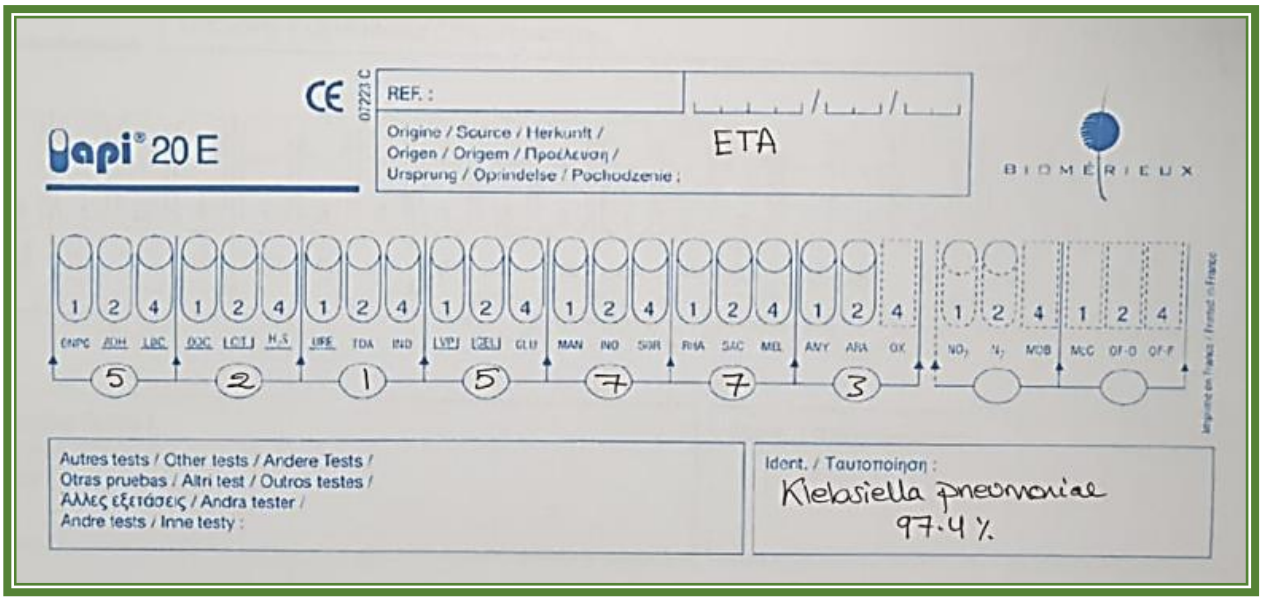


Photo.3 Gentamycin E-test showing sensitive isolate of *Pseudomonas aeruginosa* (MICs ≤ 4 µg/ml).

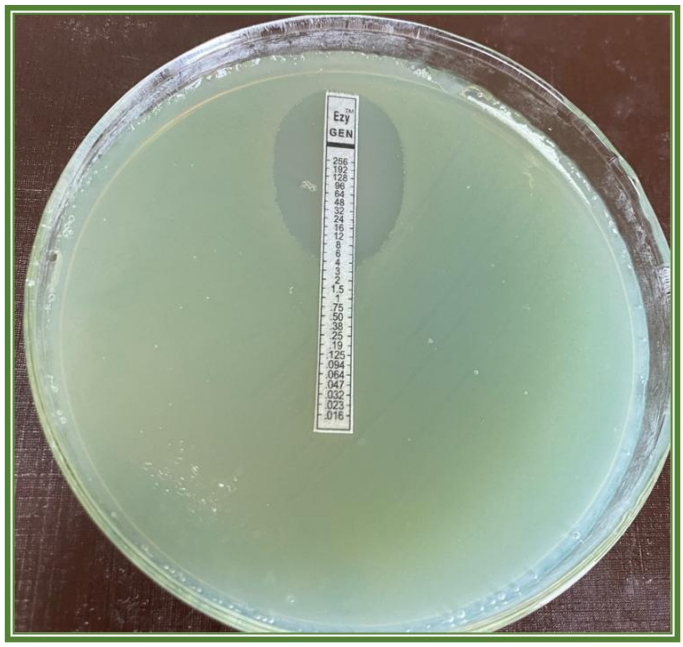
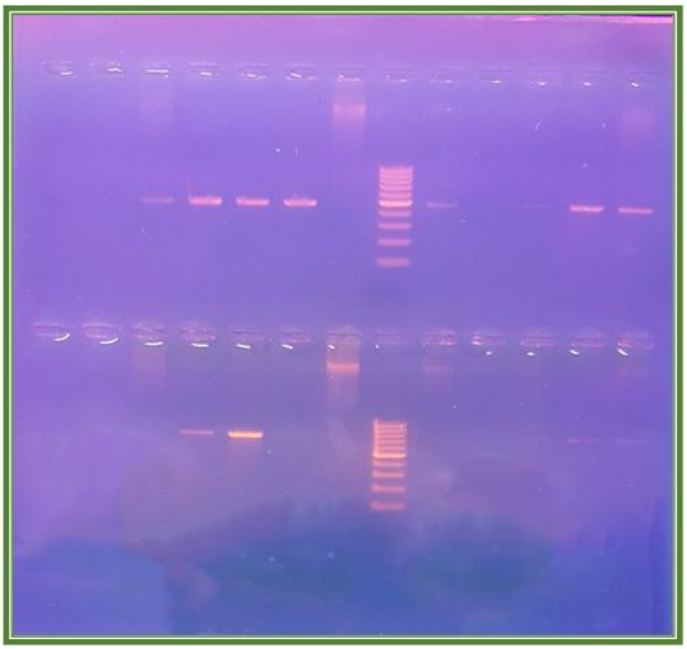
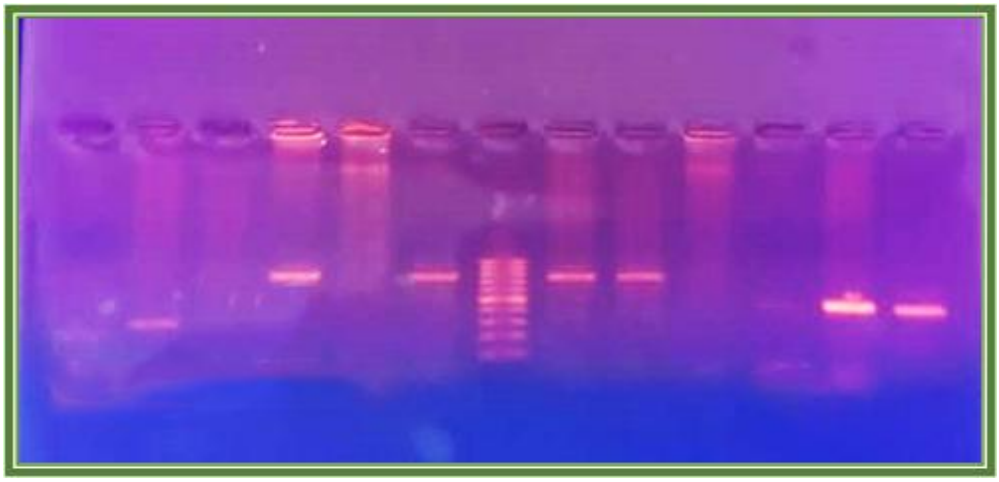


Photo.4 Gel electrophoresis of aminoglycoside resistance genes AAC(6ˋ)-Ib and AAC (3)-IIa



Lane 8,21: DNA ladder, lane (3,4,5,6,9,12,13): showed uniplex conventional PCR positive bands at 482 bp for AAC(6ˋ)-Ib gene, lane (17,18,25,26) showed uniplex conventional PCR positive bands at 740 bp for AAC (3)-IIa.

Photo.5 Gel electrophoresis of aminoglycoside resistance genes Arm A, AAD A_1, APH A_1 and APH A_6



Lane 7: DNA ladder, lane (2): showed multiplex PCR positive bands at 315 bp for Arm A gene, lane (4): showed multiplex PCR positive bands at 792 for $AADA_1$ gene, lane (6,8,9): showed uniplex PCR positive bands at 780 bp for APH A_6, lane (12,13): showed uniplex PCR positive bands at 455 bp for APH A_1.

In our results, *Acinetobacter baumanii* (APH A_6) and (Arm A) were the most found ones (79.5%, each) followed by ACC(6′)-Ib (66.7%). This is in concordance with Costello *et al.,* (2019) found that, APH A_6 is commonly associated with *Acinetobacter* spp., and was detected in (46.9%) of *A. baumannii* isolates. ELsheredy *et al.*, (2021), and Lee et al. (2018) revealed that, ArmA gene was detected in (83%), and (42.4%) of *Acinetobacter* isolates and its presence was associated with high-level of

aminoglycoside resistance. But, Kishk *et al.,* (2021) and Farsiani *et al.,* (2015) found that AAC C1 and APH A1 genes were the predominant AMEs gene.

Among *Pseudomonas aeruginosa* isolates of our study, the most prevalent resistance genes were and APH A_6 (84%) APH A_1 (64%), followed by AAC(6′)-Ib (56%). These findings were compatible with Beigverdi *et al.,* (2020), Kim *et al.,* (2007) reported that the most frequently detected AMEs gene from was APH A_6 in (63%), and (38%) of *Pseudomonas aeruginosa* isolates respectively. On the other hand, Poonsuk *et al.,* (2013) found that, the most detected gene among *Pseudomonas aeruginosa* isolates in Thailand was $AADA_1$ (84%).

Also, we found that, 27 different AMEs & ArmA combination patterns (maximum of five genes/isolate) correlating with resistance to different agents of aminoglycoside were identified. The most frequently detected combinations were (AAC(6′)Ib and APH.A_6), (APH.A_6 and APH.A_1) and (AAC(6′)Ib, Arm.A and APH.A_6) each in 8 isolates. Nearly similar results detected by Galani *et al.,* (2019) where 23 different AMEs patterns were identified. Higher rates of genes combinations observed by Costello *et al.,* (2019) that, over 60 profiles were observed. While, Gür *et al.,* (2020) reported that, the most common combination was AAC(6')-Ib + AAC(3)-IIa found in (44.6%) of Enterobacterales isolates.

Correlation analysis between phenotypic pattern and genotypic pattern showed the presence of significant correlation between tobramycin antibiotic resistance and AAC(6′)Ib gene, gentamicin antibiotic resistance and (ArmA) gene, and amikacin antibiotic resistance and (AAC(6′)Ib) and (APH A_1) genes.

Despite these correlations, the aminoglycoside resistance phenotype was not always a reliable predictor of the aminoglycoside resistance genotype. For instance, in the present study, it was observed that, in 90 gentamicin resistance isolates the AAC(3′)-IIa gene detected in only (22) of the isolates respectively, while ArmA were detected in 10% of the gentamicin susceptible isolates. Also, we detected the presence of AAC(6′)Ib, and ArmA in (27%) and (36%) of Amikacin susceptible isolates, although AAC(3′)-IIa, and AAC(6′)Ib genes confer resistance to gentamicin, and amikacin respectively while ArmA gene confers resistance to all clinically significant aminoglycosides. This is consistent with some studies, Galani *et al.,* (2019) where, in 49.7% of the gentamicin non-susceptible isolates the AAC(3′)-IIa, ANT(2′)-Ia, AAC(3′)-Ia or AAC(3)-IV genes, were not detected, while AAC(3′)-IIa was detected in 4% of the gentamicin susceptible isolates. All amikacin non-susceptible isolates harbored the AAC(6′)-Ib or an RMT gene, while (6.7%) amikacin susceptible isolates susceptible isolates harbored also the AAC(6′)-Ib gene. In Atassi *et al.,* (2021) study, the successful prediction of aminoglycoside resistance phenotypes from the presence of aminoglycoside resistance genes was limited. In disagreement with us, de Oliveria Santos *et al.,* (2018) also reported an isolate that was phenotypically susceptible to gentamicin and resistant to amikacin, and it carried APH(3')-IIb and APH(3')-VI, which confer resistance to amikacin but not gentamicin.

The explanation for this may be due to the contribution of multiple concurrent resistance mechanisms and differentiations in catalytic activity of aminoglycoside resistance genes. Also, these genes were often detected in combination, which makes it difficult to predict the aminoglycoside resistance profile based on the presence of a single gene. Furthermore, there are multiple variants of same gene (Galani *et al.,* 2019).

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Conflict of interest

There is no conflict of interest between the authors.

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