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Vol. 8(27), pp. 2583-2591, 2 July, 2014
DOI: 10.5897/AJMR2014.6878
Article Number: 2880F7345841
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbiological studies on resistance patterns of antimicrobial agents among Gram negative respiratory tract pathogens

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Received 5 May, 2014; Accepted 23 June, 2014

Respiratory tract infections (RTIs) are the most frequently-occurring infections of all human diseases and have been frequently documented. This study investigated the antimicrobial resistance patterns among Gram negative respiratory tract isolates. A total of 309 non replicate Gram negative respiratory tract isolates were collected and identified. Molecular mechanisms of antimicrobial resistance pattern were characterized by phonotypic and genotypic methods including polymerase chain reaction (PCR) amplification and DNA sequencing of isolated genes. Gram negative isolates were *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae*, *Stenotrophomonas maltophilia, Escherichia coli, Enterobacter cloacae, Serratia rubidaea, Haemophilus influenza, Citrobacter koseri, Moraxella catarrhalis, Proteus mirabilis* and *Salmonella typhimurium* with the following frequencies respectively (34.6, 26.6, 13.9, 7.7, 6.4, 5.5, 1.4, 1.3, 1, 1, 0.3 and 0.3%). *S. maltophilia* isolates were the highest that produced extended spectrum beta lactamase (ESβL) with percentage of 75% and metallo beta lactamase (MβL) with percentage of 71%, while *P. aeruginosa* isolates were the highest that produced class C beta lactamase (AmpC) with percentage of 86% and efflux pump with percentage of 73%. This study revealed two common mechanisms of antimicrobial resistance patterns, β-lactamases production and efflux pump, among Gram negative respiratory tract pathogens up to molecular level.

Key words: Resistance pattern, antimicrobial agents, respiratory, pathogens.

INTRODUCTION

The Centers for Disease Control and Prevention (CDC) estimates that more than 100 million antibiotic prescriptions are written each year in the ambulatory care setting. With so many prescriptions written each year, inappropriate antibiotic use will promote resistance. In addition to antibiotics prescribed for upper respiratory tract infections with viral etiologies, broad-spectrum antibiotics are used too often when a narrow-spectrum

antibiotic would have been just effective (Steinman et al., 2003).

Resistance to β-lactam antibiotics occurs primarily through the production of β-lactamases, enzymes that inactivate these antibiotics by splitting the amide bond of the β-lactam ring. β-Lactamases most likely co-evolved with bacteria as mechanisms of resistance against natural antibiotics over time, and the selective pressure exerted by the widespread use of antimicrobial therapy in modern medicine may have accelerated their development and spread. β-Lactamases are encoded either by chromosomal genes or by transferable genes located on plasmids and transposons. In addition, β-lactamase genes (*bla*) frequently reside on integrons, which often carry multiple-resistance determinants. If mobilized by transposable elements, integrons can facile-tate further dissemination of multidrug resistance among different bacterial species (Weldhagen, 2004).

Four major groups of enzymes are defined by their substrate and inhibitor profiles: group 1 cephalo-sporinases that are not well inhibited by clavulanic acid; group 2 penicillinases, cephalosporinases and broad-spectrum β-lactamases that are generally inhibited by active site-directed β-lactamase inhibitors; group 3 metallo β-lactamases that hydrolyze penicillins, cephalo-sporins and carbapenems and that are poorly inhibited by almost all β-lactam-containing molecules; and group 4 oxacillin-hydrolyzing enzymes that are not inhibited by clavulanic acid (Webb, 1984).

Another important mechanism of antibiotic resistance is efflux pumps. In general, multiple antibiotic resistance in Gram-negative bacteria often starts with the relatively limited outer membrane permeability to many antibiotic agents, coupled with the over expression of multi-drug resistance (MDR) efflux pumps, which can export multiple unrelated antibiotics. In addition, by reducing the intracellular concentration of the antimicrobial agent to less than the MIC required for bacterial killing, efflux mechanisms may allow bacterial survival for longer periods, facilitating the accumulation of new antibiotic-resistance mutations (e.g., those encoding topoiso-merase IV or DNA gyrase targets, rendering fluoro-quinolones ineffective) (Piddock, 2006).

Antimicrobial agents exert strong selective pressures on bacterial populations, favoring organisms that are capable of resisting them. Genetic variability occurs through a variety of mechanisms. Point mutations may occur in a nucleotide base pair, and this is referred to as microevolutionary change. These mutations may alter enzyme substrate specificity or the target site of an antimicrobial agent, interfering with its activity **(**Medeiros, 1997). This study focused on the genetic variability among Gram negative respiratory tract isolates and its relation to antimicrobial resistance including multi-drug resistant isolates.

MATERIALS AND METHODS

Bacterial isolates

A total of 309 non replicate Gram negative respiratory tract isolates from 249 patients: 115 males, 134 females, between the ages of 3 and 50 from medical intensive care unit, MICU and surgical intensive care unit, SICU, with underlying upper and lower respiratory tract diseases with no history of antibiotic administration prior to sample acquisition for three months were collected from King Abdulaziz University Hospital, Jeddah, KSA. From September 2011 to June 2012 according to the generally accepted guidelines for specimen collection and transportation of common specimen types as illustrated in Table 1 (Murray, 2007), clinical specimens collected were isolated, identified using morphological, microscopy, biochemical tests and API kit method as well.

Characterization and molecular mechanisms of antimicrobial resistance pattern of Gram negative respiratory tract pathogens

Isolates that exhibited reduced susceptibility to one or more of ceftazidime, aztreonam, cefotaxime or ceftriaxone were considered as potential producers of ESβL. Double-disk synergy test (Figure 1) was done using ceftazidime and a ceftazidime + clavulanic acid (30 µg/10 µg) discs as confirmatory test for detection of ESβL production (Coudron et al., 1997). Isolates resistant to imipenem or meropenem were considered as suspicious for production of metallo-beta-lactamases (MβL), ethylene diamine tetraacetic acid (EDTA) disc synergy test (Figure 2) was done for detection of metallo-β-lactamases in the imipenem resistant isolates (Yong et al., 2002). Isolates resistant to one or more of cefoxitin, cefotetan, cefotaxime, ceftazidime and aztreonam were considered as suspicious for production of AmpC-beta-lactamases (AmpC-BL), combined disc test (Figure 3) using cloxacillin as inhibitor of AmpC enzymes was done as confirmatory test for detection of AmpC producing isolates (Mirelis et al., 2006). Minimum inhibitory concentration (MIC) of ciprofloxacin against the clinical isolates was determined using the two-fold serial broth dilution method with an inoculum of 1 x 10^6cells/ml. All experiments were done with and without 100 mg/L carbonyle cyanide-m-chlorophenylhydrazone (CCCP). The MIC was taken as the lowest concentration inhibiting visible growth after 18 h incubations at 37°C. CCCP inhibited multi-drug resistant (MDR) efflux pump was inferred if the MIC with CCCP was four-fold or lower than the MIC without CCCP (Omoregie et al., 2007).

Molecular identification of resistance mechanisms by PCR

Plasmid extraction was done using miniprep plasmid DNA purification kit (Sigma-Aldrich, USA), Polymerase chain reaction was carried out in PCR tubes (total volume 25 µl). The reaction mixture contained 2.5 µl 1× Taq DNA polymerase buffer containing 2.5 mM $MgCl_2$, 0.2 µl 1U Taq DNA polymerase, 200 µl mol deoxynucleoside triphosphates (2.5 µl), 15 pmol of forward and

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Table 1. Guidelines for specimen collection and transportation of common specimen types.

Specimen	Collection methods
Respiratory, Upper	
Nose	Premoistened swab was inserted 1-2 cm into nares and rotated against nasal mucosa.
Nasopharynx	Nasopharyngeal washings and swabs.
Throat or pharynx	The posterior pharynx was swabbed, avoiding saliva.
Respiratory, Lower	
Bronchial alveolar lavage	A large volume of fluid was collected; transported in sterile container.
Sputum (expectorated)	Patient was instructed to rinse or gargle with water to remove excess oral flora; then to cough deeply and expectorate secretions from lower airways; which were then collected and transported in a sterile container.
Sputum (Induced)	Induced with sterile saline using a nebulizer.
Tracheal aspirate	Same tests as expectorated sputum.

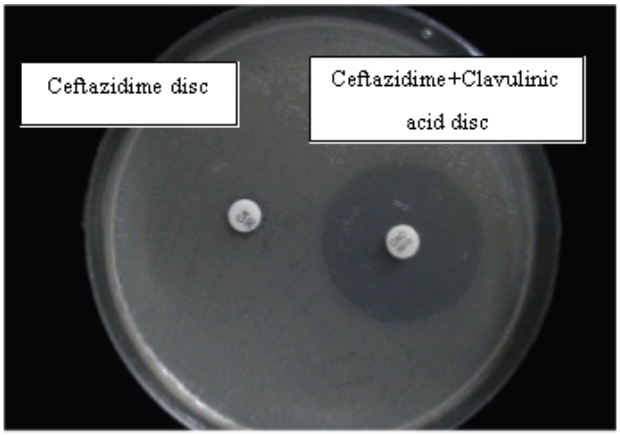

Figure 1. Double-Disc Synergy "DDS" test showing an enhancement in the zone of inhibition between a beta lactam disc and one containing the beta lactamase inhibitor.

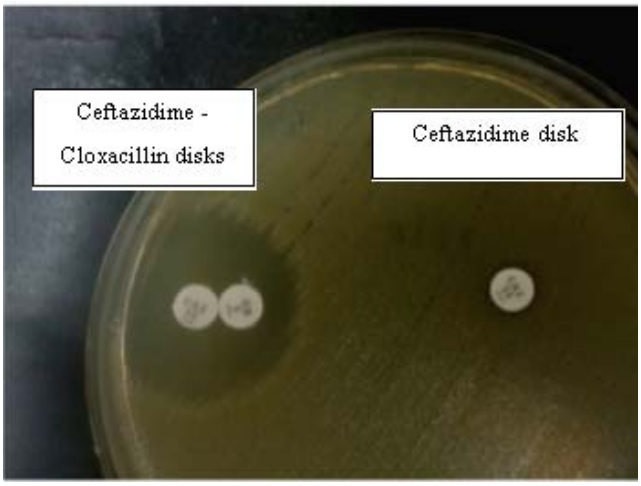

Figure 3. Combined disc test showing an enhancement in the inhibition zone around the combined antibiotic discs.

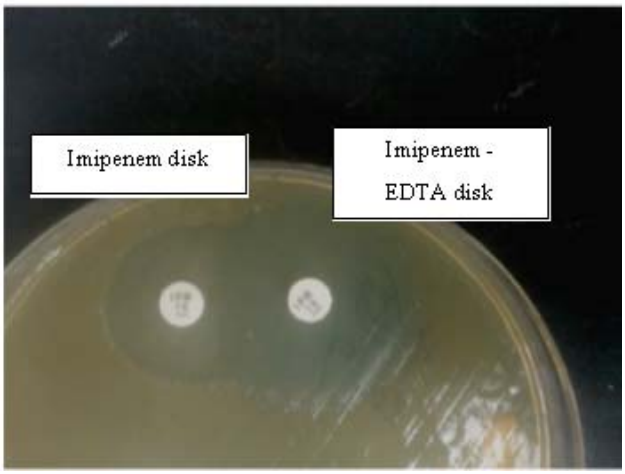

Figure 2. Ethylene diamine tetraacetic acid (EDTA) disc synergy test showing an expanded growth inhibition zone around the Imipenem - EDTA disc.

reversed primers (2 µl), 13 µl of deionized water and 5 µl of DNA template. Amplification was carried out using thermocycler (TC-5000, Techine, USA). Reaction products were separated by horizontal electrophoresis for 25 min at 100 V and varying agarose gel density within 1.2–1.7% in dependence of amplicon size. Visualization of bands was carried out after staining with ethidium bromide (0.5 µ g/ml) using an ultraviolet transilluminator and documentation system (G:Box, Syngene, UK) (NCCLS, 1997). Primers used were SHVF: 5'-GATGAACGCTTTCCCATGATG-3', SHVR: 5'-CGCTGTTATCGCTCATGGTAA-3', cycling conditions were 95°C for 5 min; 35 cycles of 95°C for 60 s, 61°C for 60 s, 72°C for 60 s; 72°C for 5 min (Kim et al., 2009), CTX-MF: 5`-TTTGCGATGCATACCAGTAA-3`, CTX-MR: 5`-CGATATCGTTGGTGCCATA-3`, cycling conditions were 95°C for 5 min; 35 cycles of 95°C for 60 s, 60°C for 30 s, 72°C for 60 s; 72°C for 5 min (Amaral et al., 2009), TEMF: 5`-ATGAGTATTCAACATTTCCG-3`, TEMR: 5`-GTCACAGTTACCAATGCTTA-3`, cycling conditions were 95°C for 5 min; 35 cycles of 95°C for 60 s, 58°C for 60 s, 72°C for 60 s; 72°C for 5 min (Kim et al., 2009), VIMF: 5`-CAGATTGCCGATGGTGTTTGG-3`, VIMR: 5`-AGGTGGGCCATTCAGCCAGA-3`, cycling conditions were 95°C

for 5 min; 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. IMPF: 5`-ATGAGCAAGTTATCAGTATTC-3`, IMPR: 5`-GCTGCAACGACTTGTTAG-3`, cycling conditions were 95°C for 5 min; 30 cycles of denaturation at 95°C for 1min, annealing at 57°C for 45 s, extension at 72°C for 45 s and final extension at 72°C for 7 min (Luzzaro et al., 2004), ACCF: 5`-AACAGCCTCAGCAGCCGGTTA-3`, ACCR: 5`-TTCGCCGCAATCATCCCTAGC-3`, LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1F: 5`-TGGCCAGAACTGACAGGCAAA-3`, LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1R: 5`-TTTCTCCTGAACGTGGCTGGC-3`, DHA-1, DHA-2F: 5`-AACTTTCACAGGTGTGCTGGGT-3`, DHA-1, DHA-2R: 5`-CCGTACGCATACTGGCTTTGC-3`, ACT-1F: 5`-TCGGTAAAGCCGATGTTGCGG-3`, ACT-1R: 5`-CTTCCACTGCGGCTGCCAGTT-3`, FOX-1 to FOX-5bF: 5`-AACATGGGGTATCAGGGAGATG-3`, FOX-1 to FOX-5bR: 5`-CAAAGCGCGTAACCGGATTGG-3`, MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11F: 5`-GCTGCTCAAGGAGCACAGGAT-3`, MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11R: 5`-CACATTGACATAGGTGTGGTGC-3`, cycling conditions were initial denaturation at 95°C for 2 min, 30 cycles of DNA denaturation at 94°C for 45 s, annealing at 62°C for 45 s, extension at 72°C for 1 min. Final extension at 72°C for 5 min (Perez-Perez and Hanson, 2002), AdeBF: 5`-GTATGAATTGATGCTGC-3`, AdeBR: 5`-CACTCGTAGCCAATACC-3`, AdeJF: 5`-TTCTTTGGTGGTACAACAGG-3`, AdeJR: 5`-GCTGCAATCAGTTTCTCATG-3`, AbeMF: 5`-TGCAACGCAGTTTCATTTTT-3`, AbeMR: 5`-CGATGTTTCATCGGCTTTTT-3`, MexAF: 5`-ACCTACGAGGCCGACTACCAGA-3`, MexAR: 5`-GTTGGTCACCAGGGCGCCTTC-3`, MexXF: 5`-CATCAGCGAACGCGAGTACAC-3`, MexXR: 5`-CAATTCGCGATGCGGATTG-3`, MexCF: 5`-AGCCAGCAGGACTTCGATACC-3`, MexCR: 5`-ACGTCGGCGAACTGCAACCGCTG-3`, MexEF: 5`-GTCATCGAACAACCGCTG-3`, MexER: 5`-GTCGAAGTAGGCGTAGACC-3`, cycling conditions were 10 min denaturation at 95°C, 30 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C. Final extension at 72°C for 10 min (Perez-Perez and Hanson, 2002).

DNA sequencing

After initial screening for the amplification of β-lactamases and efflux pump genes on both chromosomal and plasmid, the plasmid and chromosomal borne genes were subjected to nucleic acid sequencing. The initial PCR amplified products were purified and treated with QIAquick PCR Purification Kit (QIAgen Inc.,Valencia CA, USA).

Direct sequencing of each amplicon was carried out using the Sanger dideoxynucleotide chain termination method with the ABI Prism Big Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Inc., Foster City, CA, USA) on an ABI Prism 3500 Automated Sequencer. Using data collection software version 2.0, and sequencing analysis software 5.1.1, for each sequencing reaction, 2 µl purified PCR product were added to a final reaction volume of 10 µl containing 1× of sequencing buffer; 4 µl BigDye reaction mix; and 3.2 pM of each of the Forward and Reverse primer. The sequencing cycle was composed of two stages; stage one is denaturing at 96°C for 1 min, while stage two is composed of 25 cycles of denaturing at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min (Sabate et al., 2000).

Each cycle sequence product was purified by BigDye XTerminator Purification Kit. The purified PCR product was then placed in the DNA analyzer. The DNA sequences obtained were compared with those in the GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/).

RESULTS

Distribution of Gram negative microorganisms among respiratory tract isolates

Gram negative isolates were were *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae*, *Stenotrophomonas maltophilia, Escherichia coli, Enterobacter cloacae, Serratia rubidaea, Haemophilus influenza, Citrobacter koseri, Moraxella catarrhalis, Proteus mirabilis* and *Salmonella typhimurium* with the following frequencies respectively (34.6, 26.6, 13.9, 7.7, 6.4, 5.5, 1.4, 1.3, 1, 1, 0.3 and 0.3%. The distribution of organisms harboring β-lactamases and efflux pump among Gram negative respiratory tract isolates are illustrated in Table 2.

Detection and prevalence of beta-lactamases and efflux pump genes in Gram negative respiratory tract isolates

PCR and sequence analysis indicated the presence of bla_{SHV}, bla_{CTX-M}, bla_{TEM}, bla_{IMP}, bla_{VIM}, *ACC*, *DHA*, *AdeJ*, *MexX* and *MexE* genes in the isolated respiratory tract isolates with distribution illustrated in Table 3.

DNA sequencing results

Nucleotide composition analysis of some *A. baumannii* isolates showed that, the RND family drug transporter (*AdeJ*) gene detected was of GCwith value of 41.5 and the detailed composition was: T (30.7), C (20.8), A (27.8) and G (20.8). Among the studied 659 nucleotide bases compromising for *AdeJ* gene, 655 bases were conserved while only 4 sites were variable. Surprisingly, 3 out of the four base substitutions were transitional changes, from T→C (356 and 389) and C→T (566). Only one base substitution was transversional change from G→T (449) (Figure 4). Nucleotide composition analysis of some *P. aeruigenosa* isolates showed that, the multidrug efflux membrane fusion protein encoding gene (*MexE*) detected was of high GC with value of 71.1 and the detailed composition was: T (10.7), C (38.7), A (18.2) and G (32.4). Among the studied 458 nucleotide bases compromising for *MexE* gene, 455 bases were conserved while only 3 sites were variable. Two out of the three base substitutions were transitional changes, from A→G (15 and 39). Only one base substitution was transversional change from C→A (77) (Figure 5).

Table 2. Screening for ESβL, metallo β lactamase, Amp C and efflux pump production among Gram negative respiratory tract isolates.

Isolate	No. of isolates	ESβL		Metallo β lactamase		Amp C		Efflux pump	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
P. aeruginosa	107	39	68	55	52	92	15	78	29
A. baumannii	82	61	21	44	38	65	17	41	41
K. pneumoniaea	43	12	31	13	30	5	38	5	38
S. maltophilia	24	18	6	17	7	18	6	4	20
E. coli	20	10	10	9	11	5	15	8	12
E. cloacae	17	10	7	0	17	13	4	1	16
S. rubidaea	4	0	4	0	4	0	4	0	4
H. influenza	4	1	3	0	4	2	2	1	3
C. koseri.	3	0	3	0	3	1	2	1	2
M. catarhalis	3	1	2	2	1	2	1	1	2
P. mirabilis	1	1	0	1	0	1	0	1	0
S. typhimurium	1	0	1	0	1	0	1	1	0

Table 3. Prevalence of beta-lactamases and efflux pump genes in Gram negative respiratory tract isolates.

Name of organism	*bla*$_{SHV}$	*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV}$ and *bla*$_{CTX-M}$	*bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{IMP}$ and *bla*$_{VIM}$	*ACC*	*DHA-1, DHA-2*	*AdeJ*	*MexX*	*MexE*
A. baumannii	12	24	2	-	1	12	5	-	34	4	12	-	-
C. koseri	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli	1	2	5	-	-	1	1	3	-	-	-	-	-
E. cloacae	6	1	1	-	-	-	-	-	-	-	-	-	-
H. influenza	-	-	-	-	-	-	-	-	-	-	-	-	-
K. pneumoniaea	3	1	4	-	-	-	-	-	-	1	-	-	-
M. catarhalis	-	-	-	-	-	-	-	-	-	-	-	-	-
P. mirabilis	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	12	4	2	-	10	24	-	33	12	-	36	3
S. typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-
S. maltophilia	-	-	6	-	-	-	-	-	-	-	-	-	-

Nucleotide composition analysis of some *P. aeruigenosa* isolates showed that, the periplasmic multidrug efflux lipoprotein encoding gene (*MexX*) detected was of high GC with value of 71 and the detailed composition was: T (13.3), C (37.4), A (15.3) and G (34). Among the studied 413

Isolate	Nucleotide Sequence
	10 20 30 40 50 60 70 80 90
	....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
A. baumannii isolate	CTGCAATGGT TCTGTCGTTA ATTGTAGCGT TGACGTTCAC ACCGGCACTT TGTGCAACTA TCTTGAAACA GCATGATCCT AATAAAGAAC
A. baumannii ATCC 17978	CTGCAATGGT TCTGTCGTTA ATTGTAGCGT TGACGTTCAC ACCGGCACTT TGTGCAACTA TCTTGAAACA GCATGATCCT AATAAAGAAC
	100 110 120 130 140 150 160 170 180
	....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
A. baumannii isolate	CAAGCAATAA TATCTTTGCG CGTTTCTTTA GAAGCTTTAA CAATGGTTTT GACCGCATGT CGCATAGCTA CCAAAATGGT GTTAGCCGCA
A. baumannii ATCC 17978	CAAGCAATAA TATCTTTGCG CGTTTCTTTA GAAGCTTTAA CAATGGTTTT GACCGCATGT CGCATAGCTA CCAAAATGGT GTTAGCCGCA
	190 200 210 220 230 240 250 260 270
	....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
A. baumannii isolate	TGCTTAAAGG CAAAATCTTC TCTGGCGTGC TCTATGCTGT TGTAGTTGCC CTTTTAGTCT TCTTGTTCCA AAAACTCCCG TCTTCATTCT
A. baumannii ATCC 17978	TGCTTAAAGG CAAAATCTTC TCTGGCGTGC TCTATGCTGT TGTAGTTGCC CTTTTAGTCT TCTTGTTCCA AAAACTCCCG TCTTCATTCT
	280 290 300 310 320 330 340 350 360
	....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
A. baumannii isolate	TACCAGAAGA AGATCAGGGT GTGGTCATGA CACTTGTACA ATTACCACCA AATGCAACGC TTGACCGTAC CGGTAAAGTG ATTGATACCA
A. baumannii ATCC 17978	TACCAGAAGA AGATCAGGGT GTGGTCATGA CACTTGTACA ATTACCACCA AATGCAACGC TTGACCGTAC CGGTAAAGTG ATTGACACCA
	370 380 390 400 410 420 430 440 450
	....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
A. baumannii isolate	TGACTAACTT CTTTATGAAT GAAAAAGATA CCGTGGAATC TATTTTCACT GTTTCTGGTT TCTCATTCAC AGGTGTTGGT CAAAACGCGG
A. baumannii ATCC 17978	TGACTAACTT CTTTATGAAT GAAAAAGACA CCGTGGAATC TATTTTCACT GTTTCTGGTT TCTCATTCAC AGGTGTTGGT CAAAACGCTG
	460 470 480 490 500 510 520 530 540
	....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
A. baumannii isolate	GTATTGGCTT CGTTAAGTTG AAAGACTGGA GCAAACGTAC GACACCAGAA ACTCAAATTG GTTCATTGAT TCAGCGTGGT ATGGCATTAA
A. baumannii ATCC 17978	GTATTGGCTT CGTTAAGTTG AAAGACTGGA GCAAACGTAC GACACCAGAA ACTCAAATTG GTTCATTGAT TCAGCGTGGT ATGGCATTAA
	550 560 570 580 590 600 610 620 630
	....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
A. baumannii isolate	ATATGATCAT TAAAGATGCA TCATACGTTA TGCCGTTACA GCTTCCAGCA ATGCCTGAAC TTGGTGTAAC TGCCGGATTT AACTTGCAGC
A. baumannii ATCC 17978	ATATGATCAT TAAAGATGCA TCATATGTTA TGCCGTTACA GCTTCCAGCA ATGCCTGAAC TTGGTGTAAC TGCCGGATTT AACTTGCAGC
	640 650
	....|....| ....|....| ....|....
A. baumannii isolate	TTAAAGATTC AAGTGGTCAA GGCCATGAG
A. baumannii ATCC 17978	TTAAAGATTC AAGTGGTCAA GGCCATGAG

Figure 4. Multiple DNA sequence alignment of AdeJ gene isolated from *A. baummanii* tested isolate and retrieved sequences from Genbank.

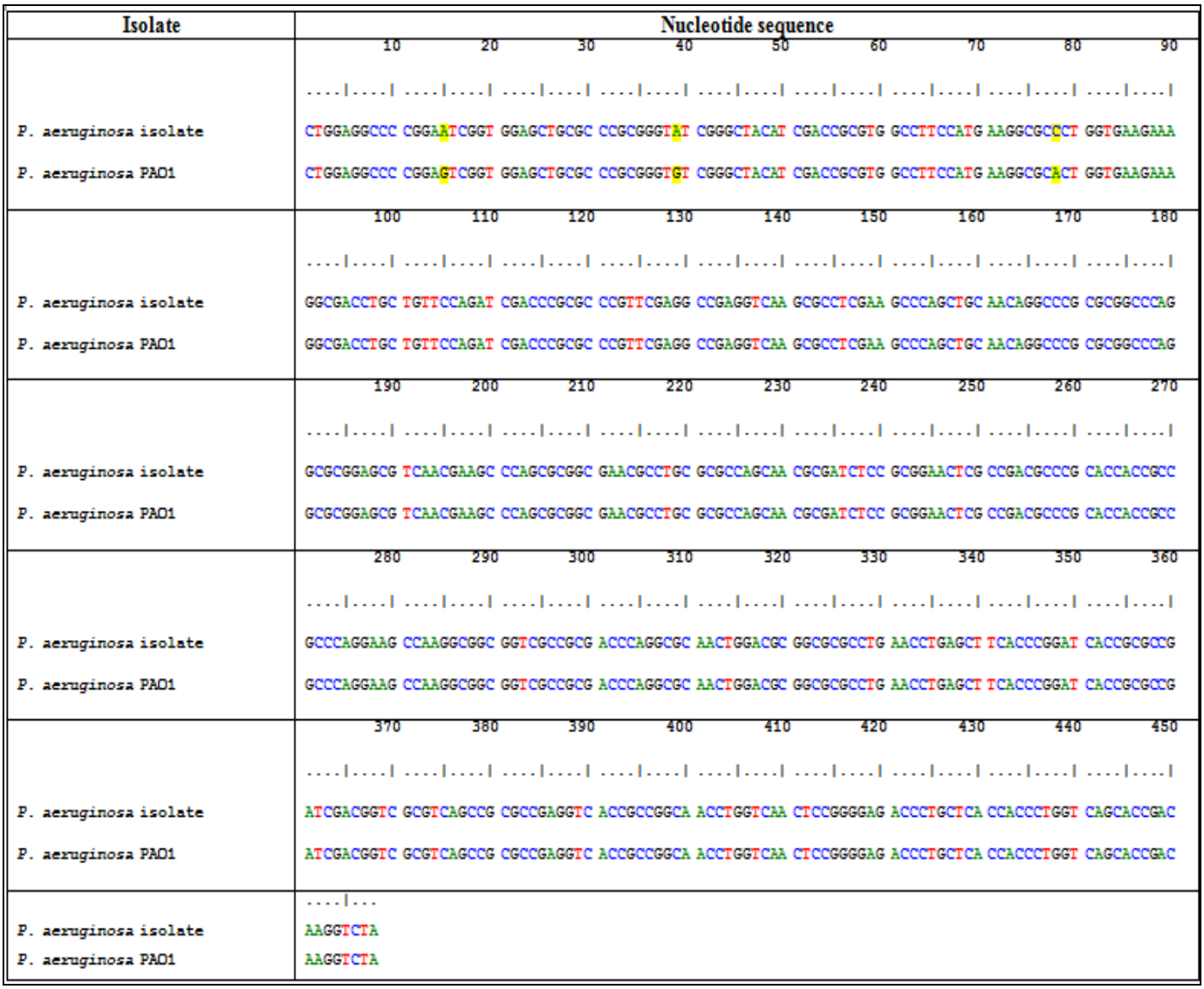

Figure 5. Multiple DNA sequence alignment of MexE gene isolated from *P. aeruginosa* tested isolate and retrieved sequences from Genbank.

nucleotide bases compromising for *MexX* gene, 411bases were conserved while only 2 sites were transitional changes, from T→C (20) and C→T (404) (Figure 6).

DISCUSSION

The present study proposes a combined phenotypic and genotypic approach for the specific diagnosis of antibiotic resistance mediated by β-lactamases and efflux pump system harboring Gram negative respiratory tract isolates.

In the present study, bla_{CTX-M} genes were predominant in *A. baumannii* and *P. aeruginosa* isolates with percentage of 39 and 31% respectively, followed by bla_{SHV} genes in *A. baumannii* and *E. cloacae* isolates with percentage of 20 and 60% respectively. bla_{TEM} genes were predominant in *E. coli*, *K. pneumoniaea* and *S. maltophilia* isolates with percentage of 50, 33 and 33%, respectively. bla_{SHV} genes were predominant in *E. cloacae* with percentage of 60%. Similar findings were found in Indian study (Gupta, 2007), from a total of 94 isolates, 50 (n = 47), 14.89 (n = 14) and 11.70 (n = 11) ESβL rates for bla_{TEM}, bla_{SHV} and bla_{CTX-M} type beta lactamases, respectively. bla_{TEM} and bla_{CTX-M} type ESβL

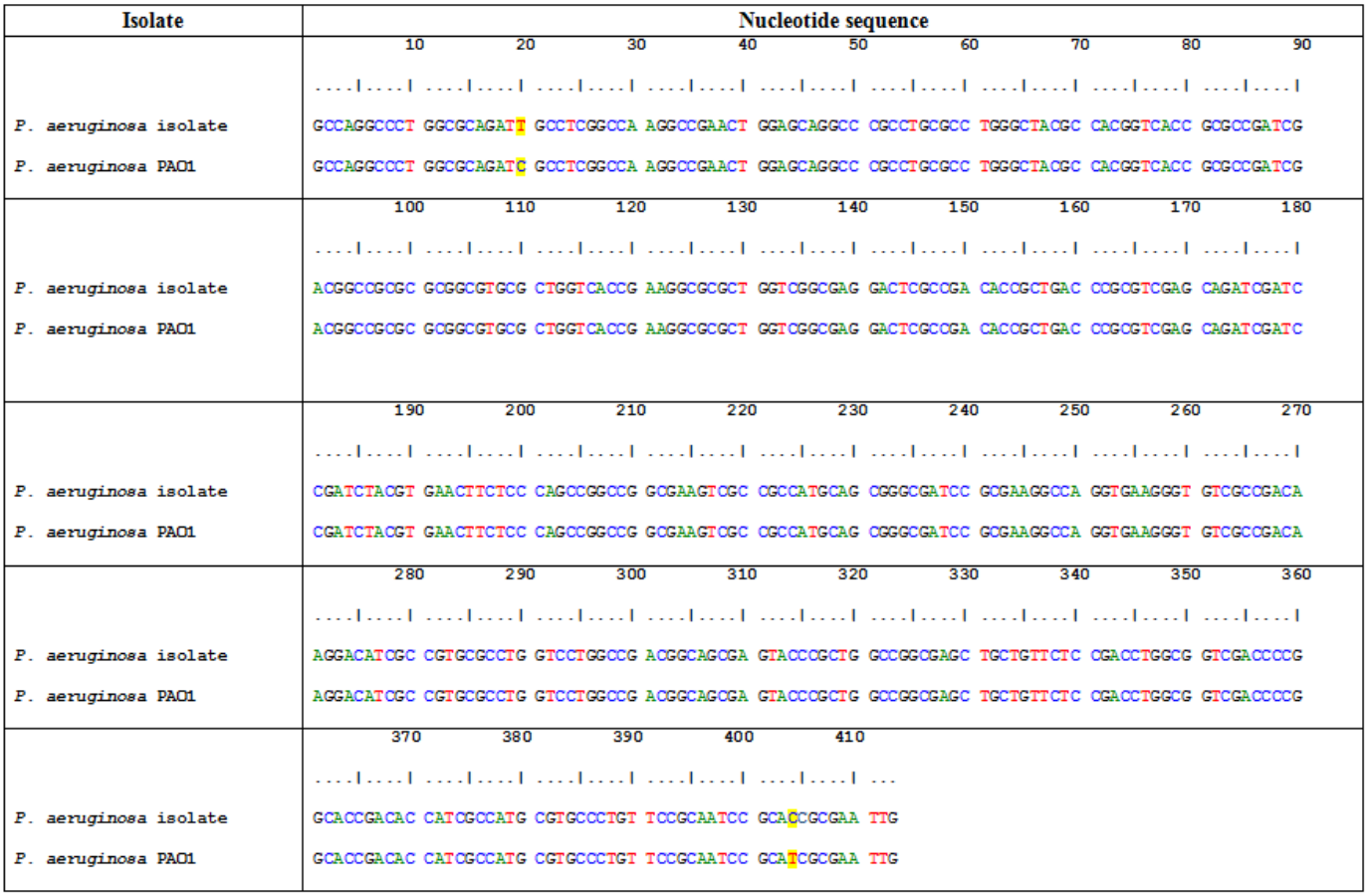

Figure 6. Multiple DNA sequence alignment of MexX gene isolated from *P. aeruginosa* tested isolate and retrieved sequences from Genbank.

were observed in 72.72 and 22.72% of *E. coli* isolates, respectively. Also, the present study revealed that bla_{IMP} gene was predominant in *A. baumannii* isolates with percentage of 27%, followed by bla_{VIM} gene 11%. bla_{VIM} gene was predominant in *P. aeruginosa* isolates with the percentage of 44%, followed by bla_{IMP} gene 18%. Both bla_{IMP} and bla_{VIM} genes were found together in *E. coli* isolates with the percentage of 33% followed by 11% of each alone. This was in accordance with Nordman and Poirel (2002), were a total of 8 pseudomonas isolates carried bla_{VIM} -type gene, these data demonstrate that bla_{VIM}-type gene are the most prevalent MβLs among clinical specimens of *P. aeruginosa*. *ACC* gene was predominant in *A. baumannii* and *P. aeruginosa* isolates with percentage of 52 and 36%, respectively, followed by *DHA-1*, *DHA-2* genes with 13 and 6% respectively. This result differs significantly from the findings of several studies were the isolation numbers of *ACC* enzymes were still significantly lower than those of *CIT (CMY)*, *FOX* and *DHA* (Philippon et al., 2002). *AdeJ* was detected in *A. baumannii* with percentage of 29.2%, while *MexX* gene was predominant in *P. aeruginosa* isolates with percentage of 46% followed by *MexE*, 3.8%. This differ from the findings of some biological observations made during a study where the basal expression level of *MexX* is much lower than that of *MexA* but that both efflux pumps are over-expressed 4 to 8 times in resistant strains, suggesting that a lower quantity of MexXY-OprM than MexAB-OprM protein may be needed for effective transport of the corresponding substrates (Llanes et al., 2004). Second, over-expression of *MexX* in clinical isolates is systematically associated with that of *MexA*. This may be related to the fact that MexXY uses OprM as a porin (Masuda et al., 2000).

Conclusion

The study figured out the most common genes responsible for the expression of β-lactamase enzymes and efflux pump system in Gram negative respiratory tract isolates. The study also revealed that, isolates

harboring more than one gene from the same class have higher resistance pattern towards antimicrobial agents than those harboring only one; also, isolates having microevolutionary changes in their nucleotide composition of the detected genes have higher resistance pattern towards antimicrobial agents than those where all bases are conserved.

Conflict of interests

The authors did not declare any conflict of interests.

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