Iranian Journal of Microbiology
Volume 8 Number 1 (February 2016) 62-69

ORIGINAL ARTICLE

Emergence of colistin resistant *Pseudomonas aeruginosa* at Tabriz hospitals, Iran

Hamid Reza Goli[1,3], Mohammad Reza Nahaei[1,3], Mohammad Ahangarzadeh Rezaee[2,3], Alka Hasani[2,3], Hossein Samadi Kafil[1,3], Mohammad Aghazadeh[1,3*]

[1]Drug Applied Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Infectious Diseases and Tropical Medicine Research Center , Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Department of Medical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

Received: June 2015**, Accepted:** December 2016

ABSTRACT

Background and Objectives: The prevalence of multidrug resistant *Pseudomonas aeruginosa* is the main reason of new drugs resurgence such as colistin. The main objectives of this study were to determine the antibiotic resistance pattern and the rate of colistin resistance along with its correlation with overexpression of MexAB-OprM and MexXY-OprM efflux pumps among *P. aeruginosa* isolates.

Materials and Methods: Hundred clinical isolates were collected from 100 patients during 6 months in 2014. Susceptibility to the eight antibiotics was investigated using Kirby-Bauer and agar dilution methods. The Quantitative Real-time PCR was used to determine the expression levels of efflux genes.

Results: Resistance rates to various antibiotics were as follows: ticarcillin (73%), ciprofloxacin (65%), aztreonam (60%), ceftazidime (55%), gentamicin (55%), imipenem (49%), piperacillin/tazobactam (34%) and colistin (2%). In disk diffusion method, only two isolates were non susceptible to colistin, however in agar dilution method the two isolates were confirmed as resistant and two others were intermediate resistant. Sixty eight (68%) isolates were multi-drug resistant and 10 isolates were susceptible to all tested antibiotics. Both colistin resistant isolates showed overexpression of both efflux pumps, but two intermediate resistant isolates exhibited reduction of efflux genes expression.

Conclusions: Emergence of colistin resistance is increasing in *P. aeruginosa* indicating great challenge in the treatment of infections caused by MDR strains of this organism in Iran. ParRS may promote either induced or constitutive resistance to colistin through the activation of distinct mechanisms such as MDR efflux pumps, and LPS modification.

Keywords: *Pseudomonas aeruginosa*, Multi drug resistant, Colistin, MexAB-OprM, MexXY-OprM

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous environmental bacterium and one of the major causes of nosocomial (third leading cause) infections (1).

This bacterium can cause urinary tract, surgical site, bloodstream, wound and other types of infections (2-5). Treatment of infections caused by this organism is becoming more difficult due to the constant increase of drug resistance and its emergence as multidrug resistant (MDR) pathogen (3). In patients infected with *P. aeruginosa* resistant to carbapenems,

*Corresponding author: Mohammad Aghazadeh, Ph.D
Address: Drug Applied Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.
Tel: +989143134820
E-mail: aghazadehm@tbzmed.ac.ir

fluoroquinolones and aminoglycosides (MDR), antibiotics of choice are restricted for treatment (2). The lack of rapid progress in identification and designing of newer antibiotics has led to the revival of the old antibiotics (such as polymyxins) for the treatment of infections caused by this bacterium (1, 5). Polymyxins are polypeptide antibiotics which consist of five chemically different compounds (polymyxins A–E), which only polymyxin B and polymyxin E (colistin) have been used in clinical practice (1). Colistin has an excellent antibacterial activity mainly against Gram-negative bacteria such as *P. aeruginosa, Escherichia coli, Enterobacter* spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., and *Acinetobacter baumannii,* but not against *Burkholderia, Serratia* and *Proteus* spp. (6). Action of cationic colistin is concentration dependent. The mechanism of its action is binding to anionic lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria. This can cause increase of cell permeability and cell death by cell lysis (1, 6). The main side effects of colistin are nephrotoxicity and neurotoxicity (2, 6). Although, colistin resistance mechanisms have not been completely understood, but there are several possible mechanisms. These include alteration of the bacterial outer membrane, reduction of the specific outer membrane protein levels, reduction of Mg^{2+} and Ca^{2+} contents in cell envelope, efflux pumps (such as MexAB-OprM and MexXY-OprM), lipid alterations and increase of the outer membrane protein H1 levels (1, 4). In general, two main mechanisms of resistance to colistin in Gram-negative bacteria are mutation and adaptation (6). Reduction of Mg^{2+} and Ca^{2+} contents is an adaptive resistance mechanism that is controlled by the two-component regulators *pho*P-*pho*Q and *pmr*A-*pmr*B (4). Resistance caused by mutation (such as efflux pumps overexpression) is inherited, low-level and antibiotic presence independent, whereas, resistance caused by adaptation is the opposite (6). The MexAB-OprM and the MexXY-OprM efflux pumps are expressed constitutively in wild-type cells, contribute to intrinsic multidrug resistance, and harbor clinical importance, in *P. aeruginosa* (7, 8). Almost complete cross-resistance exists between colistin and polymyxin B (6). Emergence of colistin resistant Gram-negative bacteria such as *P. aeruginosa* is of concern and colistin resistant pathogens may be encountered in clinical practice (1).

The present study was conducted to assess the frequency of resistance to colistin and compare between disk diffusion and MIC methods along with role of efflux pump overexpression in resistance to this antibiotic in *P. aeruginosa* clinical isolates.

MATERIALS AND METHODS

Bacterial isolates and media. One-hundred non-repetitive clinical isolates of *P. aeruginosa* were obtained from four university teaching and treatment hospitals of Tabriz (Imam Reza, Sina, Pediatric hospital and Shahid Madani) during January to June 2014. The isolates were identified by conventional microbiological methods (9) and were stored in tryptone soy broth (Merck Co., Darmstadt, Germany) containing 30% glycerol (Merck) at -70 °C for further analysis.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (10). The tested antibiotics (Mast Diagnostics Group Ltd, Merseyside, UK) and their concentrations were as follows: ticarcillin (75 µg/ml), piperacillin/tazobactam (100/10 µg/ml), ceftazidime (30 µg/ml), aztreonam (30 µg/ml), imipenem (10 µg/ml), colistin sulfate (10 µg/ml), ciprofloxacin (5 µg/ml), and gentamicin (10 µg/ml). Colistin sulfate salt powder (Sigma-Aldrich co, St. Louis, MO, ≥ 15000 U/mg) was used in agar dilution test to determining minimum inhibitory concentration (MIC).

Pseudomonas aeruginosa ATCC 27853 was used as the control strain in antimicrobial susceptibility testing.

MIC_{50} and MIC_{90} calculation. The concentration of each antimicrobial agent, that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the strains, was calculated for colistin (11).

The formula of geometric means was used as follows:

$$MIC_{50} = \frac{(M < 50) + (n - X) \times [(M > 50) - (M < 50)]}{Y}$$

Where M < 50 is the MIC of the highest cumulative percentage below 50%, M > 50 is the MIC of the lowest cumulative percentage above 50%; n is 50% of the number of organisms tested, X is the number of organisms in the group at M <50, and Y is the

number of organism in the group at M >50.

Total RNA extraction and cDNA synthesis. Total RNA was extracted from *P. aeruginosa* isolates using the total RNA extraction kit (SinaClon Co., Tehran, Iran) and then was treated with RNase-free DNase I (SinaClon) according to the manufacturer's instructions. RNA concentration and its purity were determined by NanoDrop spectrophotometer (ND-1000, Wilmington, USA). Five microgram of DNA-free RNA was used for synthesis of cDNA by reverse transcription using M-mulv reverse transcriptase. Reverse transcriptase was inactivated by incubation at 70 °C for 10 min. The cDNAs were stored at -20 °C until use.

Quantitative real-time PCR (qRT-PCR). Polymerase chain reactions were conducted in duplicate runs using the SYBR premix EX TaqII, Tli RNaseH plus (Takara Bio Inc.) by a Rotor Gene Real-Time PCR machine (Corbett Research, Sydney, Australia; Model RG 3000). The specific primers were used for *MexB, MexY* and *rpsL* genes as described previously (12-14). The house keeping gene *rpsL* was used as the normalizing gene. The PCR cycling condition for the amplification of the *MexB* gene was as follows: 95 °C for 5 min and 45 cycles of 20 s at 95 °C, 10 s at 68 °C and 15 s at 72 °C with 3Mm in $MgCl_2$ concentration, while for the amplification of the *MexY* gene, 95 °C for 5 min and 45 cycles of 15 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C was used, and the amplification of the *rpsL* gene was carried out as: 95 °C for 5 min and 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 30 s at 72 °C. Results are presented as ratios of gene expression between the target gene and the reference gene (*rpsL*), obtained according to a relative quantification method as described previously (14, 15). The results represent relative expression levels for target genes in isolates compared to the PAO1 wild-type strain. An isolate was considered as hyperproducer of mRNA for *MexB* if the cDNA level was ≥3 × PAO1 and for *MexY* if the cDNA level was ≥10 × PAO1 (12).

RESULTS

The isolates were collected from 93 inpatients and 7 outpatients, 50% of them were male. Of the total isolates, 28 were obtained from patients being referred from hospitals of other cities in Northwest of Iran to Tabriz. The patients' age ranged from one day to 91 years (Mean=35.98±29.61 Years). The source of the isolates included: urine, wound, blood, respiratory samples, middle ear secretion, peritoneal fluid, stool and cerebral shunt.

In disk diffusion susceptibility tests, the isolates were resistant to ticarcillin (73%), ciprofloxacin (65%), aztreonam (60%), ceftazidime (55%), gentamicin (55%), imipenem (49%), piperacillin/tazobactam (34%) and colistin sulfate (2%) (Fig. 1). The resistance rates of *P. aeruginosa* isolates to all antibiotics, except colistin, were slightly higher amongst burned patients (Table 1). On average, most resistance rate to tested antibiotics showed in 31-50 year-old group patients, but two colistin resistant isolates were obtained from two patients in > 50 year-old group. Table 1 also depicts the rate of resistance to antibiotics and its correlation with the source of isolates. Both of our colistin resistant strains were isolated from respiratory specimens. One of them was resistant to all tested antibiotics except piperacillin-tazobactam, while the second one was only resistant to colistin and gentamicin. Both isolates produced yellow pigment on Mueller-Hinton agar and were obtained at different times in 2013 at different hospitals. Overall, ticarcillin showed the lowest antipseudomonal activity and colistin had the highest activity regardless of patients' setting (inpatients versus outpatients or ICU patients versus Non-ICU patients), patients' age and type of specimen. Sixty eight percent of our isolates were multidrug resistant (MDR), while the percentage of isolates resistant to 3, 4, 5, 6 and 7 drugs was different (Fig. 1).

Table 2 shows the number (%) of isolates with different MIC ranges against colistin and their correlation with ward of hospitals that isolates were collected. Two colistin susceptible isolates in disk diffusion method, were intermediate resistant in the MIC test, while the MIC range of two colistin resistant isolates was 128 µg/ml and both MIC_{50} and MIC_{90} for colistin in this study were recorded as 2 µg/ml.

Fig. 2 shows the correlation between overexpression of MDR efflux pumps and ranges of MIC against colistin in clinical isolates of *P. aeruginosa*. Both colistin intermediate resistant isolates showed reduction in expression of *MexB* and *MexY* genes, while both resistant isolates exhibited overexpression of these efflux genes (ranging from 3.5-52 fold more than PAO1 wild-type strain).

Table 1. Resistance rate of isolates according to hospital wards and source of isolates

	percentage of resistance to							
	ticarcillin	Piperacillin	ceftazidime	aztreonam	imipenem	colistin	gentamicin	ciprofloxacin
Hospital Wards (No.)								
ICU (41)	78	39	80.4	85.3	58.5	4.8	41.4	70.7
Burn (15)	100	60	80	0	80	0	66.6	93.3
Oncology (1)	0	0	0	100	0	0	0	0
Urology (2)	100	50	50	0	0	0	50	100
Surgery (5)	40	20	40	100	40	0	40	40
CCU (4)	100	25	75	83.3	75	0	75	100
ENT (2)	0	0	0	62.5	0	0	0	0
Internal (5)	60	40	20	40	20	0	60	60
Infectious (8)	75	25	62.5	0	37.5	0	25	37.5
Neurology (6)	83.3	16.6	83.3	75	33.3	0	83.3	83.3
Trauma (2)	100	50	100	40	100	0	100	100
Neonatal (1)	100	0	0	50	0	0	0	0
Transplantation (1)	100	0	100	0	0	0	100	100
Outpatients (7)	0	0	0	93.3	0	0	0	0
Specimen type (No.)								
Urine (31)	51.6	19.3	38.7	35.4	19.3	0	35.4	41.9
Wound (20)	90	50	50	80	60	0	60	75
Blood (13)	84.6	61.5	53.8	53.8	76.9	0	53.8	76.9
Lower Respiratory (27)	88.8	29.6	85.1	81.4	66.6	7.4	77.7	85.1
Upper respiratory (4)	25	0	25	25	25	0	25	25
Middle Ear secretion (2)	50	50	0	50	50	0	50	50
Peritoneal fluid (1)	100	100	100	100	100	0	100	100
Stool (1)	100	0	100	100	0	0	100	100
Cerebral shunt (1)	0	0	0	0	0	0	0	0

Abbreviations: ICU: Intensive Care Unit, CCU: Coronary Care Unit, ENT: Ear Nose & Throat.

Table 2. Results of agar dilution test against colistin in *P. aeruginosa* isolates

	Number of isolates with MIC range (µg/ml) of										
	0.1	0.5	1	2	4	8	16	32	64	128	256
Total	4	1	12	79	2	0	0	0	0	2	0
ICU patients	3	1	5	31	0	0	0	0	0	2	0
Burn patients	0	0	1	12	2	0	0	0	0	0	0
Other patients	1	0	6	36	0	0	0	0	0	0	0

ICU, intensive care units

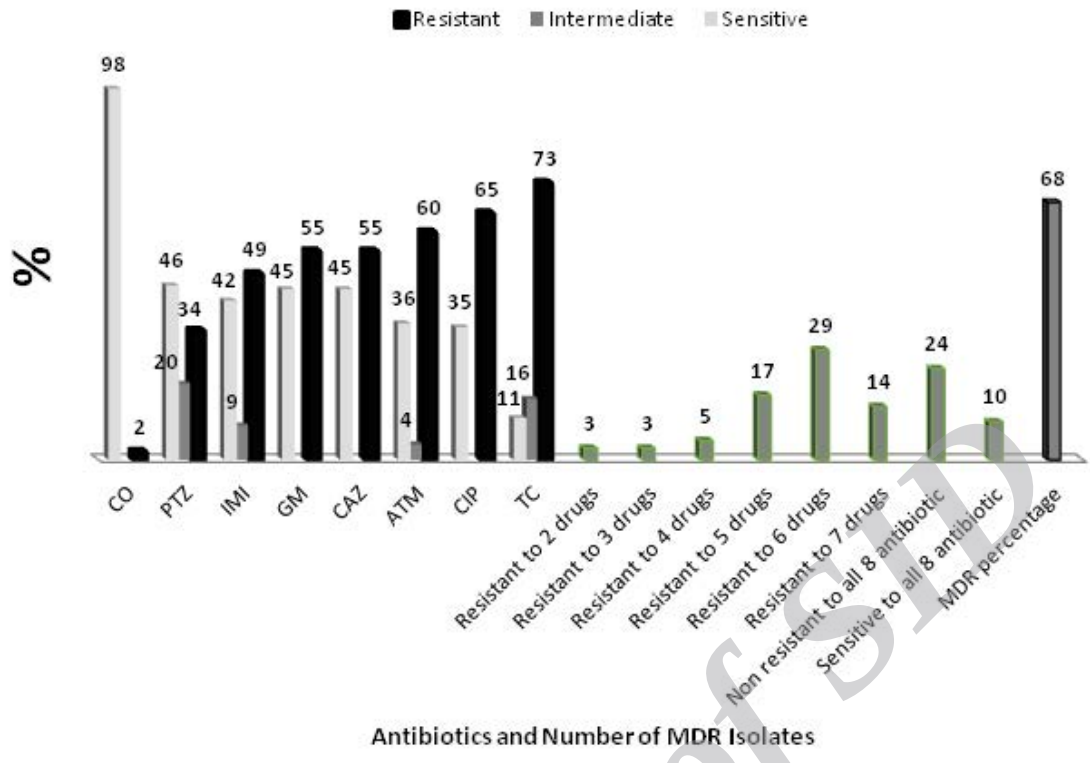

Fig 1. Results of Antimicrobial Susceptibility testing and Number of MDR Isolates
Abbreviations: TIC: Ticarcillin, PTZ: Piperacillin/Tazobactam, CAZ: Ceftazidime, AT: Aztreonam, IM: Imipenem, CO: Colistin, GM: Gentamicin and CIP: Ciprofloxacin.

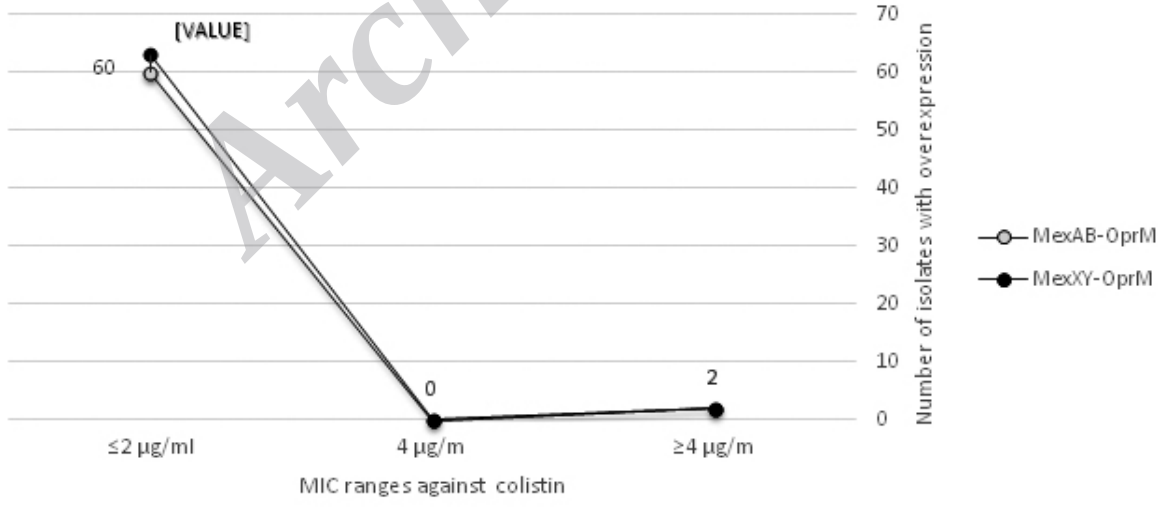

Fig. 2. Correlation between MIC ranges of colistin and overexpression of efflux pumps in *P. aeruginosa*

DISCUSSION

This study showed that resistance to colistin is emerging in the Northwest of Iran while this antibiotic is available in hospitals and pharmacies of Tabriz city. It is in use for treatment of acute infections caused by bacteria resistant to other antibiotics. A research study conducted earlier in Northwest of Iran (16) reported a much higher (14.9%) resistance to colistin, although all isolates of *P. aeruginosa* were MDR and were isolated from burned patients. Moreover, 68.1% of their isolates belonged to sequence type 773 and they performed MIC of colistin by E-test method. Data from other researches available from Iran showed that all of their isolates were susceptible to colistin or polymyxin B (17-19). Nevertheless, reports from neighboring countries showed resistance to colistin varied from 0 to 31.7% (20, 21). Differences between these reports are due to differences between availability of colistin and policies related to the use of this antibiotic in hospitals of these countries. Two colistin resistant strains of our study were isolated from two separate patients. The first patient was hospitalized in neurology ICU due to cerebral hemorrhage and before performance of microbial culture, had consumed cefazolin, ciprofloxacin, ceftazidime, co-trimoxazole, gentamicin and imipenem. The second patient was hospitalized in lung ICU due to heart failure and before microbial culture, had consumed imipenem, vancomycin and clindamycin. However, both of them expired eventually. The different features of colistin resistant isolates show that probably they have different sequence type. None of the patients had consumed colistin before the microbial culture or previous hospitalizations and none of them had cation (Ca^{2+} or Mg^{2+}) deficiency. These show that the physiological resistance in these isolates has not been made.

Both colistin resistant isolates showed overexpression of MexAB-OprM and MexXY-OprM efflux pumps, while colistin is not a specific substrate of these efflux systems (7). These efflux pumps are cause of cross-resistance to different antimicrobial agents and disinfectants (7, 8), which this may be the cause of resistance to colistin in our two isolates. Two research studies confirmed that colistin is able to kill the inactive subpopulation located deep in *P. aeruginosa* biofilm, while the active subpopulation located in the upper layer lives against this antibiotic (22, 23). They have shown that the *mex*AB-*opr*M gene is expressed by the active subpopulation of *P. aeruginosa* under colistin exposure and this gene is mandatory for the development of colistin tolerance. All these efflux pumps are thought to transport and pump out polymyxins present in cells. Moreover, Poole et al. showed that overexpression of MexXY-OprM multidrug efflux system in *P. aeruginosa* is the cause of increased susceptibility to polymyxin B and polymyxin E (colistin) (24). On the other hand, the mutation of *pmr*B gene and expression change of *pmr*AB or *pho*PQ may have occurred in our colistin resistant isolates. Although, the low level of MIC observes in mutation mechanism of resistance to colistin (6), but in these isolates, it was 128 µg/ml. This shows that both mechanisms including efflux pumps overexpression and mutation of *pmr*B may occurred in our resistant isolates. This was consistent to another research study which reported that exposure of wild-type *P. aeruginosa* to colistin was resulted in increased *MexY* and repressed *opr*D via parRS two component system (25).

The standard disk diffusion for the detection of colistin resistance is not highly reliable because colistin does not have good diffusion on agar culture medium and may give unexpected results under different environmental conditions (26). In susceptibility testing of colistin (MIC), its sulphate salt form must be used, because the methanesulphonate (sodium salt form of colistin) is an inactive pro-drug which undergoes hydrolysis to colistin during incubation in vitro and potentially to changeable extents from laboratory to laboratory (27). Two studies reported general conformity in the results obtained from agar dilution and broth micro-dilution methods about testing of colistin sulfate (28, 29). However, it was suggested that results of the disk diffusion test should be confirmed with a dilution method, because the disk diffusion method used in their study revealed falsely susceptible microorganisms (28). This was compatible with our study because we found that two isolates which were susceptible to colistin in disk diffusion method, but were intermediate resistant when agar dilution method was used.

The present investigation showed that the clinical isolates of *P. aeruginosa* expressed high level of resistance to current antipseudomonal antibiotics along with gaining resistance to newer antibiotics. The causes of resistance to current antibiotics may be incorrect usage of antibiotics, lack of knowledge, lack of personal hygiene and in some cases over-the-counter usage. We found that regardless of colistin which is drug of choice for the treatment of infections caused by

MDR isolates of *P. aeruginosa*, piperacillin/tazobactam and ticarcillin were the most and the least effective antipseudomonal antibiotics. Resistance to imipenem was 49%, while until recently carbapenems were considered as the drug of choice for MDR *P. aeruginosa.* Resistance to carbapenems is the main cause of resuming the administration of colistin for the treatment of the infections caused by *P. aeruginosa*. In this study, imipenem was the third effective drug, while in other studies especially on burned patients in Iran, it was the most effective antipseudomonal antibiotic (17, 30)

CONCLUSION

Due to the different treatment strategies in various hospitals, resistance rates to colistin are different in geographical regions. It is crucial to obtain information related to colistin resistance in the world. These data can help to create the appropriate guidelines for correct and specific use of this antibiotic which can help to control colistin resistance spread. Emergence of multi-drug and colistin resistant isolates of *P. aeruginosa* is a serious problem worldwide. When new and effective antibiotics are not available, the colistin is the last chance for the treatment. Thus, antibiotic susceptibility testing against colistin should be mandatory in the microbiological laboratories of Iran and other countries. Preventable guidelines such as combined drug therapy or use of in-vitro synergy tests should also be considered to limit the resistance to colistin in *P. aeruginosa.* Collectively, our data indicate that ParRS may promote either induced or constitutive resistance to colistin through the activation of distinct mechanisms such as MDR efflux pumps, and LPS modification.

ACKNOWLEDGEMENTS

We thank the staffs of Imam Reza, Sina, Shahid Madani and Pediatric hospitals of Tabriz for assistance in collecting isolates and patients information. This study is a report of a database from PhD thesis entitled, Evaluation of correlation between MexAB-OprM and MexXY-OprM efflux system expression rate and class 1 integron prevalence to multidrug resistant (MDR) *Pseudomonas aeruginosa* isolates in Tabriz University of Medical Sciences, registered in Drug Applied Research Center of Tabriz (Grant No. 93.31).

REFERENCES

1. Dhariwal AK, Tullu MS. Colistin: re-emergence of the 'forgotten' antimicrobial agent. *J Postgrad Med* 2013; 59: 208-15.
2. Martis N, Leroy S, Blanc V. Colistin in multi-drug resistant *Pseudomonas aeruginosa* blood-stream infections: a narrative review for the clinician. *J Infect* 2014; 69: 1-12.
3. Hachem RY, Chemaly RF, Ahmar CA, Jiang Y, Boktour MR, Rjaili GA, et al. Colistin is effective in treatment of infections caused by multidrug-resistant *Pseudomonas aeruginosa* in cancer patients. *Antimicrob Agents Chemother* 2007; 51: 1905-11.
4. Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system *Par*R-*Par*S. *Antimicrob Agents Chemother* 2010; 54: 3372-3382.
5. Lee JY, Na IY, Park YK, Ko KS. Genomic variations between colistin-susceptible and -resistant *Pseudomonas aeruginosa* clinical isolates and their effects on colistin resistance. *J Antimicrob Chemother* 2014; 69: 1248-56.
6. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. *Clin Infect Dis* 2005; 40: 1333-1341.
7. Fernando DM, Kumar A. Resistance-nodulation-division multidrug efflux pumps in Gram-negative bacteria: role in virulence. *Antibiotics* 2013; 2: 163-81.
8. Guénard S, Muller C, Monlezun L, Benas P, Broutin I, Jeannot K, et al. Multiple mutations lead to MexXY-OprM-dependent aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa. Antimicrob Agents Chemother* 2014; 58: 221-228.
9. Forbes BA, Sahm D, Weissfeld A (2005). Diagnostic microbiology. St Louis: Mosby.
10. Wayne PA (2014). Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing: Twenty-fourth informational supplement, M100-S24. Clinical and Laboratory Standards Institute (CLSI); vol(34), No.1.
11. Hojabri Z, Ahangarzadeh Rezaee M, Nahaei MR, Soroush MH, Ghojazadeh M, Pirzadeh T, et al. Comparison of *in-vitro* activity of doripenem versus old carbapenems against *Pseudomonas aeruginosa* clinical isolates from both CF and Burn patients. *Adv Pharm*

Bull (APB) 2013; 3: 121-125.
12. Oh H, Stenhoff J, Jalal S, Wretlind B. Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa* strains. *Microb Drug Resist* 2003; 9: 323-328.
13. Fazeli H, Sadighian H, Esfahani BN, Pourmand MR. Molecular epidemiology and mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa* isolates causing burn wound infection in Iran. *J Chemother* 2014; 26: 222-228.
14. Dumas J-L, van Delden C, Perron K, Köhler T. Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. *FEMS Microbiol Lett*. 2006; 254: 217-225.
15. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* 2001; 29: e45.
16. Yousefi S, Nahaei MR, Farajnia S, Aghazadeh M, Iversen A, Edquist P, et al. A multiresistant clone of *Pseudomonas aeruginosa* sequence type 773 spreading in a burn unit in Orumieh, Iran. *APMIS : Acta pathol Microbiol et Immunol Scand* 2013; 121: 146-52.
17. Neyestanaki DK, Mirsalehian A, Rezagholizadeh F, Jabalameli F, Taherikalani M, Emaneini M. Determination of extended spectrum beta-lactamases, metallo-beta-lactamases and *Amp*C-beta-lactamases among carbapenem resistant *Pseudomonas aeruginosa* isolated from burn patients. *Burns* 2014; 40: 1556-1561.
18. Shahcheraghi F, Nikbin VS, Feizabadi MM. Identification and genetic characterization of metallo-beta-lactamase-producing strains of *Pseudomonas aeruginosa* in Tehran, Iran. *New Microbiol* 2010; 33: 243-8.
19. Sheikh AF, Rostami S, Jolodar A, Amin M. Detection of Metallo-Beta Lactamases Among Carbapenem-Resistant *Pseudomonas aeruginosa*. *Jundishapur J Microb* 2014; 7: e12289.
20. Bayram Y, Parlak M, Aypak C, Bayram I. Three-year review of bacteriological profile and antibiogram of burn wound isolates in Van, Turkey. *Int J Med Sci* 2013; 10: 19-23.
21. Gill MM, Usman J, Kaleem F, Hassan A, Khalid A, Anjum R, et al. Frequency and antibiogram of multidrug resistant *Pseudomonas aeruginosa*. *JCPSP* 2011; 21: 531-534.
22. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. *Mol Microbiol* 2008; 68: 223-240.
23. Schweizer HP. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genet Mol Res* 2003; 2: 48-62.
24. Poole K, Lau CH-F, Gilmour C, Hao Y, Lam JS. Polymyxin susceptibility in *Pseudomonas aeruginosa* linked to the MexXY-OprM multidrug efflux system. *Antimicrob Agents Chemother* 2015; 59: 7276-89.
25. Muller C, Plésiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2011; 55: 1211-21.
26. van der Heijden IM, Levin AS, De Pedri EH, Fung L, Rossi F, Duboc G, et al. Comparison of disc diffusion, Etest and broth microdilution for testing susceptibility of carbapenem-resistant *P. aeruginosa* to polymyxins. *Ann Clin Microbiol Antimicrob* 2007; 6: 8.
27. Bergen PJ, Li J, Rayner CR, Nation RL. Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2006; 50: 1953-8.
28. Gales AC, Reis AO, Jones RN. Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J Clin Microbiol* 2001; 39: 183-190.
29. Hogardt M, Schmoldt S, Gotzfried M, Adler K, Heesemann J. Pitfalls of polymyxin antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. *J Antimicrob Chemother* 2004; 54: 1057-1061.
30. Mirsalehian A, Feizabadi M, Nakhjavani FA, Jabalameli F, Goli H, Kalantari N. Detection of VEB-1, OXA-10 and PER-1 genotypes in extended-spectrum beta-lactamase-producing *Pseudomonas aeruginosa* strains isolated from burn patients. *Burns* 2010; 36: 70-74.