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Molecular Investigation of Outer Membrane Channel Genes Among Multidrug Resistance Clinical *Pseudomonas Aeruginosa* Isolates

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Abstract

Background: Multidrug resistance *Pseudomonas aeruginosa* (MDRPA) is most important issue in healthcare setting. It can secrete many virulence effector proteins via its secretion system type (T1SS-T6SS). They are using them as conductor for delivering the effector proteins outside to begins harmful effect on host cell increasing pathogenicity, competition against other microorganism and nutrient acquisition.
Methods: The study include investigation of 50 isolates of MDRPA for transport secretion system and resistance for antibiotics. Molecular diagnosis using *P. aeruginosa* specific primer pairs, investigation of *AprF, HasF, XcpQ, HxcQ, PscC, CdrB, CupB3,* and *Hcp* using specific primer pairs by PCR were also performed.
Results: The results revealed high resistance to beta lactam antibiotics (78% for ceftazidime, 78% for cefepime and 46% for piperacillin) can indicate possessing of isolates for beta lactamases and this confirmed by dropping resistance to piperacillin to 16% when combined with tazobactam. Also, the results shown the ability of MDRPA for pyocyanin biosynthesis using the system of genes.
Conclusions: The current study conclude that all isolates of *P. aeruginosa* were highly virulent due to their possessing of all transport secretion system to deliver different effector proteins with possible harmful effects of these proteins.

Keywords: Drug resistance, MDR, Efflux pump, *Pseudomonas aeruginosa*.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a nosocomial opportunistic pathogen that can dominate all niches (1). The transport of proteins from the cytoplasm into different compartments of the cell, the environment, and/or other bacteria or eukaryotic cells is an important function of prokaryotic cells, a process known as protein secretion. Protein secretion systems are required for bacterial development and are used in a variety of functions. Bacterial pathogens use secretion systems to influence their hosts and generate a replicative niche (2). Exoenzymes secreted by *P. aeruginosa* cause damage to host tissue by disrupting normal cytoskeletal structure, depolymerization of actin filaments, and cleavage of immunoglobulin G (IgG) and A (IgA), and all these processes lead to invasion, dissemination, and the development of chronic infections (3). Small molecules, proteins, and DNA are transported into the extracellular space or target cells via the secretion system. The structural and molecular characteristics of Gram-negative bacteria's six secretion systems (types I–VI) (4,5). Proteins can be secreted using one of two methods. One method is a one-step technique in which proteins from bacteria's cytoplasm are transported and supplied straight into the host cell through the cell membrane. Another involves a two-step

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process in which proteins are first carried out of the inner cell membrane, then into the periplasm, and finally into the host cell through the outer cell membrane (6).

Pseudomonas aeruginosa has many secretion systems. There are two types of T1SS in *p. aeruginosa*, T1SS-Apr and T1SS-Has (7,8). The secretion system of T1SS-Apr called AprDEF with outer membrane protein called AprF (PA1248) which act as outer channel for secretion system who exploit to secret important alkaline protease called AprA(PA1249) and AprX(PA1245). T1SS-Has secretion system called HasDEF with HasF (PA3404) as outer channel used to secret HasAp (PA3403) (a Heme acquisition Protein) (9,10). *P. aeruginosa* strain PAO1 possesses two complete and non-redundant T2SS, referred to as the Xcp and Hxc systems (11). Xcp T2SS plays an important role in bacterial virulence by its capacity to deliver a large panel of toxins and degradative enzymes into the surrounding environment (12). T2SS-Hxc secretion system called HxcP–HxcZ, with HxcQ (PA0685) as outer channel used to secret LapA (PA0688) (Low-molecular weight alkaline phosphatase) (13,14). The type III secretion system (T3SS) is a bacterial nanomachine that resembles a syringe on the bacterial surface. The T3SS 'needle' delivers translocon proteins into eukaryotic cell membranes, subsequently allowing injection of bacterial effectors into the cytosol (15). T3SS- PscC (PA1716) as outer channel secretion system, used to secret ExoS (PA3841) (GTPase activating protein (GAP) domain and adenosine diphosphate ribosyltransferase domain (ADPRT)) and ExoT (PA0044) (GTPase activating protein (GAP) domain and adenosine diphosphate ribosyl transferase domain (ADPRT)) and ExoY (PA2191) (Adenylate cyclase) (16) and ExoU (PA14_51530) (Patatin-like, phospholipase) (17). Type IV pili (T4P) filamentous surface appendages, sophisticated biological nanomachines (18). The T4SSs functionally encompass two major subfamilies, the conjugation systems, and the effector translocators. The conjugation systems are responsible for interbacterial transfer of antibiotic resistance genes, virulence determinants, and genes encoding other traits of potential benefit to the bacterial host. The effector translocators are used by many Gram-negative pathogens for delivery of potentially hundreds of virulence proteins termed effectors to eukaryotic cells during infection (19). T5SS secretion system called CdrB with CdrB (PA4624) as outer channel used to secret CdrA (PA4625) (Adhesin) (20) and P-usher secretion system called CupB3 with CupB3 (PA4084) as outer membrane channel used to secret CupB5 (PA4082) (Putative adhesin) which involved in the assembly of fimbriae at the bacterial cell surface (21). Hemolysin coregulated protein (Hcp), a ring-shaped hexamer secreted by all characterized T6SSs, binds specifically to cognate effector molecules (22). Hcp protein is a core component of the T6SS tail tube and acts as an exported receptor and a chaperone of effectors (23). Resistance to antibiotics may be either intrinsic or acquired (24,25). The prominence of multidrug resistant *P. aeruginosa* is growing in the world, limiting the therapeutic options (26,27). Despite the emergence of modern antibacterial agents with anti-Pseudomonal activity, *P. aeruginosa* continues to be the leading cause of life-threatening infections in hospitals (28,29). Even though increase of multi-drug resistant *Pseudomonas aeruginosa* strains, which are intractable to be treated, some available antibiotics still able to dominate pseudomonal infections with a reasonable percentage of success, for example, colistin sulfate and quinolones (ciprofloxacin and levofloxacin) (30,31). The current study was conducted to investigate outer membrane genes for transport secretion system among multidrug resistance *P. aeruginosa* isolates.

Materials and Methods

Ethical Approval

informed consent was obtained from all human adult participants or parents or legal guardians of minors. The project was approved by scientific committee and Bioethics

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committee under project no. 325 on 29 December 2020.

Ethical approval

The study has been approved by the Ethics Committee of Kashan University of Medical Sciences, Iran and all experiments were reviewed and approved following the current European Union Directive (2010/63/EU) guideline on the protection of animals used for scientific purposes.

Bacterial isolates

Fifty *P. aeruginosa* isolated were obtained from different specimens and subjected for primary identification test using Pseudomonaschromogenic agar (Condalab/Spain) and confirmed using genus specific (for Pseudomonas spp.) and species specific (for *P. aeruginosa*) (Table 1).

Antibiotic susceptibility Assay

It was performed using 14 antibiotics agent according to CLSI-2019 (32).

Polymerase Chain Reaction

DNA was extracted according to manufactures instructions (IntronBio/Korea). The primers were dissolved according to manufacturer instructions (Macrogen/Korea). The primer pairs and PCR conditions were demonstrated in (Table 1).

Results

Isolation findings showed a high percentage of *P. aeruginosa* among UTIs patients 18(36%), lower respiratory tract infection patients 13(26%) wounds and burn infections 9(18%), otitis media 5(10%), bacteremia 2(4%), vaginosis 2(4%) and 1(2%) for meningitis (Table 2).

Results of resistance for 14 antibiotics according to CLSI revealed that 39(78%) of *P. aeruginosa* isolates were resistant to ceftazidime (CAZ) and cefepime (FEP), 23(46%) for piperacillin (PRL), 15(30%) for gentamycin (CN), 14(28%) for ciprofloxacin (CIP), 13(26%) for tobramycin (TOB), 12(24%) for Aztreonam (ATM),11(22%) for amikacin (AK), 10(20%) for ofloxacine (OFX), 9(18%) for levofloxacin (LEV), 8(16%) for piperacillin-tazobactam (PTZ), 7(14%) for netilmicine, imipenem (IPM) and meropenem (MEM) (Fig. 1).

Outer membrane channels of Transport secretion systems presence were investigated via detection of *AprF, HasF, XcpQ, HxcQ, PscC, CdrB, CupB, HcP* genes which encode for outer membrane channels. The results revealed that 93% of *p. aeruginosa* isolated have T1SS-T6SS genes (Figs. 2 and 3). Table 3 shows high prevalence of coexistence secretion system channel genes among *p. aeruginosa* which act as conductor for delivering the effector proteins outside to begins harmful effect on host cell increasing pathogenicity and for competition against other microorganism, nutrient acquisition. If one or more channel blocked this will lead to determine and decrease the number of secreted proteins consequentially the bacterium became less virulence.

Discussion

Our results may be totally agreeing with previous studies whose found that dominance of *P. aeruginosa* among UTIs, RTIs and wound-burn infections (34). Implication of *P. aeruginosa* in UTIs may be as nosocomial pathogen resulted from placing and removing of indwelling urinary catheters (35). Admission to an intensive care unit (ICU) raises the risk of MDR *P. aeruginosa* infection in critically ill pneumonia patients (36). *P. aeruginosa* is a significant cause of nosocomial infections in burn centers, which may be due to a high frequency of antibiotic resistance and the potential to form biofilms (37). *P. aeruginosa* shows resistance to a wide range of antibiotics, comprises aminoglycosides, quinolones and β-lactams. The resistance may be intrinsic (low outer membrane permeability, coding for efflux pumps and the making of antibiotic-inactivating enzymes), acquired (either horizontal transport of resistance genes or mutational alteration) and adaptive (involves formation of biofilm which provide as a diffusion barrier to edge antibiotic access to the bacterial cells) resistance (38).

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Table 1. Primer pairs and PCR conditions.

Primer name	5' to 3' sequence	Product (bp)	PCR conditions	Reference
Pseudomonas Spp.	F: GACGGGTGAGTAATGCCTA R: CACTGGTGTTCCTTCCTATA	618	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 56 ºC, 30 sec Step 4: 72 ºC, 70 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	(33)
P. aeruginosa	F: GGGGGATCTTCGGACCTCA R: TCCTTAGAGTGCCCACCCG	956	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 61 ºC, 30 sec Step 4: 72 ºC, 100 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	(33)
T1SS AprF	F: CAAGTCCGGTTCGGAGAACA R: CGTATCGGTCTTCGACAGGG	544	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 60.3 ºC, 30 sec Step 4: 72 ºC, 60 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study
T1SS HasF	F: CTATCTGATGGCAGCGGTGA R: ATCAATGACCTGCACAGCCA	333	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 58.3 ºC, 30 sec Step 4: 72 ºC, 40 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study
T2SS XcpQ	F: GGTCAACGCTCTCGAAGACA R: GATGTGCGGAGTGACCTTGA	487	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 59.3 ºC, 30 sec Step 4: 72 ºC, 50 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study
T2SS HxcQ	F: GAAGACGACTCCAGCGAGTT R: CGAGGAGGATGCTGGTATCG	544	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 60.3 ºC, 30 sec Step 4: 72 ºC, 60 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study
T3SS PscC	F: GTGGTGACTCTCGGCGATAC R: GCTCTGGTTCGACAACTCGT	396	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 60.3 ºC,30 sec Step 4: 72 ºC,40 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study
T5SS CdrB	F: GTCCACGTCGAGGTTGTAGG R: GCAACGCCTGACCTATGACT	433	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 66 ºC, 30 sec Step 4: 72 ºC, 50 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study
CupB3	F: GTTGCGCTACGCTGGTAATG R: ATCCTCTGCCCGAAGGTTTG	349	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 59.3 ºC, 30 sec Step 4: 72 ºC, 40 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study
T6SS hcp	F: ACGTCAAGGGTGAGTCCAAG R: GGACACCAGGACTTCCTTCAG	293	Step 1: 95 ºC, 2 min. Step 2: 95 ºC, 30 sec Step 3: 60.5 ºC, 30 sec Step 4: 72 ºC, 30 sec Step 6: 72 ºC, 5 min. Step 7: 4 ºC, forever	This study

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Table 2. Distribution of *P. aeruginosa* isolates among Diseases.

Disease	Specimen	Bacterial Isolate	
		No.	%
UTIs	Midstream urine	18	36%
RTIs	Bronchoalveolar lavage	13	26%
Wound and burn infections	Wound burn swab	9	18%
Otitis Media	Ear swab	5	10%
Bacteremia	Blood stream	2	4%
Vaginosis	High vaginal swab	2	4%
Meningitis	CSF	1	2%
Total		**50**	**100%**

Table 3. Coexistence of secretion system among isolates.

Isolates possess	Type of secretion system	Percentage
5 secretion system	T1SS/T2SS/T3SS/T5SS/T6SS	92%
4 secretion system	T1SS/T2SS/T5SS/T6SS	4%
3 secretion system	T1SS/T5SS/T6SS	2%
2 secretion system	T5SS/T6SS	2%

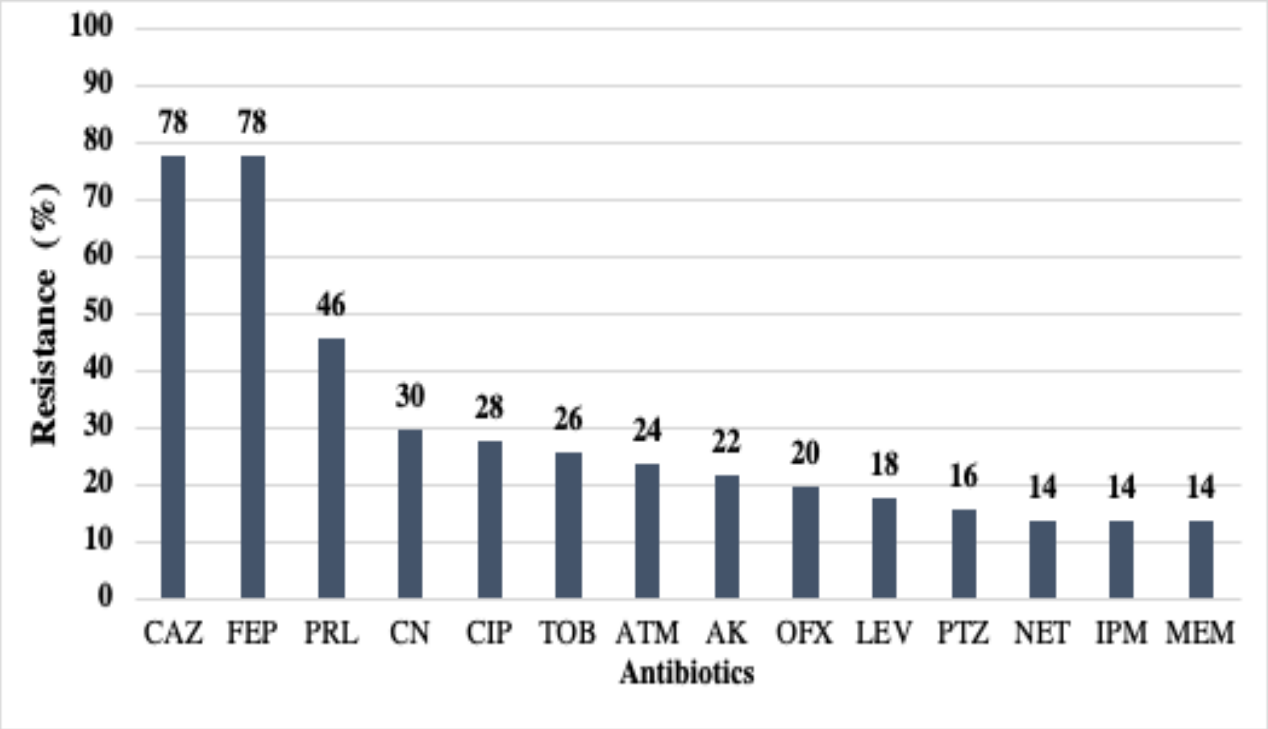

Fig. 1. Antibiotic resistance percentage of *P. aeruginosa* to 14 antibiotics (ceftazidime (CAZ), cefepime (FEP), piperacillin (PRL), gentamycin (CN), ciprofloxacin (CIP), tobramycin (TOB), Aztreonam (ATM), amikacin (AK), ofloxacine (OFX), levofloxacin (LEV), piperacillin-tazobactam (PTZ), netilmicine, imipenem (IPM) and meropenem (MEM).

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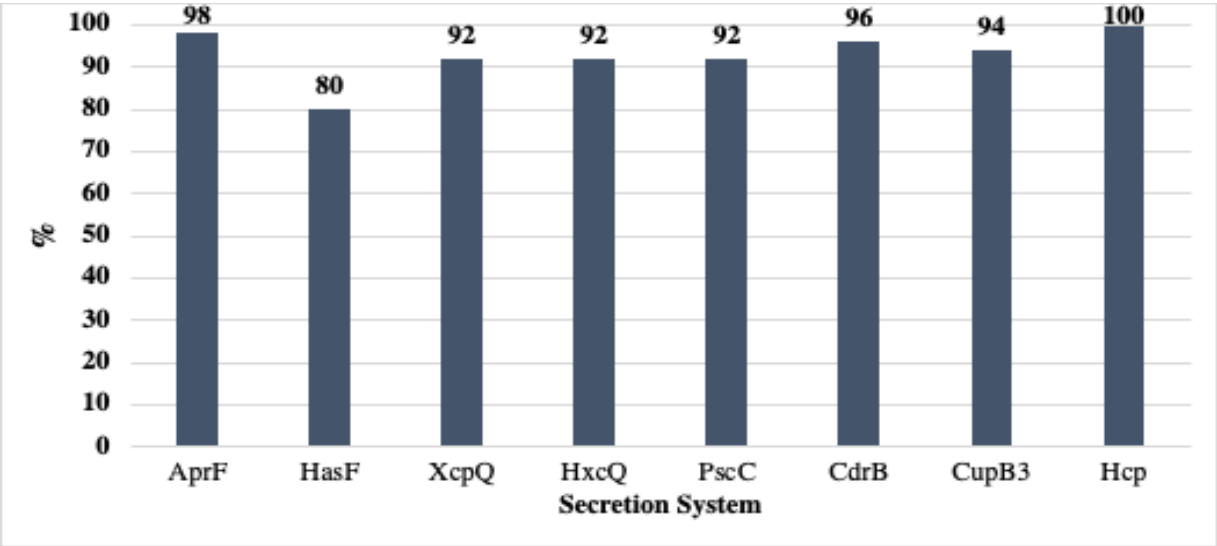

Fig. 2. Distribution of Secretion systems among 50 clinical isolates.

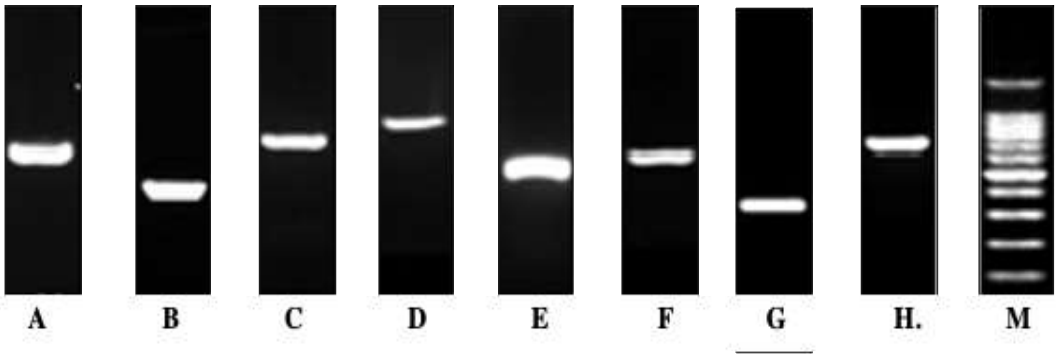

Fig. 3. 1.5% agarose gel electrophoresis of among *P. aeruginosa* isolates. A. AprF amplicon (544 bp), B. HasF amplicon (333 bp), C. XcpQ amplicon (487 bp), D. HxcQ amplicon (544 bp), E. PscC amplicon (396 bp), F. CdrB amplicon (433 bp), G. CupB3 amplicon (349 bp), H. Hcp amplicon (293 bp), and M 100 bp DNA ladder.

The results shown high resistance to beta lactams (ceftazidime (CAZ), cefepime (FEP), piperacillin (PRL) and this is mainly mediated by beta lactamases due to that when use piperacillin-tazobactam the resistance was dropped from 46% to 16%. Beta lactamases regard as intrinsic mechanism of resistance leading to inactivating of beta lactam rendering them inactive. Beta lactamase inhibitor like tazobactam (An irreversible inhibitor of a wide variety of bacterial beta-lactamases) can improve many beta lactams like piperacillin once combined with them.

Piperacillin-tazobactam is the most widely used lactamase inhibitor combination for treating *P. aeruginosa* infections (39,40). The results of current study revealed the ability of all *P. aeruginosa* isolated to produce pyocyanin making them more virulent and have great harmful consequences due to implication of pyocyanin in tissue damage, interfering with immune response and triggering proinflammatory responses (41).

Determination of different virulence genes of *P. aeruginosa* isolates suggest that they are associated with different levels of intrinsic virulence and pathogenicity. This may have different consequences on the outcome of infection (42). So, our result revealed that the isolate has high virulence mechanism to deliver the effector proteins affecting host cells and other bacteria in their milieu (43-45).

The current study concludes that all isolates of *P. aeruginosa* were highly virulent due to

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their possessing of all transport secretion system to deliver different effector proteins with possible harmful effects of these proteins.

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