Resistance to Antimicrobial Agents, Chemical Disinfectants and Distribution of Effectors Proteins of Type Three Secretion System in *Pseudomonas aeruginosa* Isolated From Burn and Hospital Environments in South East of Iran

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Abstract- *Pseudomonas aeruginosa* (*P. aeruginosa*) is a common bacteria associated with burn infections and resistance to a wide range of disinfectants and antimicrobial agents which is able to produce different virulence factors. In this study, the susceptibility of *P. aeruginosa* isolates from the burn (burn=57) and hospital environment (HE=19) to antimicrobial agents and chemical disinfectants was determined by disc and well diffusion agar method, respectively. The results showed 100% sensitivity to polymyxin B, while sensitivity to other agents was low and ranged from 40.8% for imipenem and amikacin to 6.6% for ceftizoxime. Among the disinfectant used, the mean diameter of inhibition zones (DIZ) was higher for Deconex, while nitrofurazone had the lowest DIZ. In most cases, the HE isolates were significantly more susceptible to disinfectants and antimicrobial agents compared to burn isolates ($P \leq 0.01$). The genes for the exoenzyme T, Y, U, and S were detected in 100%, 89.8%, 43.4%, and 48.7% of the isolates, respectively. The prevalence of *exo* U and *exo*Y was significantly higher in the burn isolates compared to HE isolates ($P=0.001$). The results of this study indicate a significantly higher level of resistance against the majority of the antimicrobial agents in the burn isolates compared to HE isolates, which was significantly higher than the environmental isolates. The prevalence of T3SS effectors proteins and their pattern were also different in the burn, and the HE isolates, indicating a divergence in pathogenicity of the burn isolates from those of the environmental isolates.

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Introduction

P. aeruginosa is amongst the most common causes of opportunistic infections responsible for the production of different types of infections in susceptible hosts, such as burn victims, wearers of contact lenses, patients with cystic fibrosis, or immune-compromised individuals (1,2). These bacteria have the ability to disseminate in different wards in the hospitals due to their low metabolic requirements, resistance to chemical disinfectants, and intrinsic or acquired resistance to antimicrobial agents (2,3). In recent years, most *P. aeruginosa* from clinical samples have seen to be multidrug-resistant (MDR) (3,4). This bacteria is able to produce a wide spectrum of different virulence factors such as those associated with the cell, namely exopolysaccharide, flagella, and type IV pili together with a variety of secreted factors such as elastase, protease, phospholipase, and effector proteins of type three secretion system (T3SS) (4). TT3S is a sec-independent system that can eject the effectors' protein directly into the cytoplasmic compartment of the eukaryotic cells. In vitro, it can damage the macrophage and epithelial cells and, therefore, is thought to play an important role in the pathogenicity of acute infections caused by such bacteria (5). Four effector proteins identified up to now are exoenzyme S (*exoS*), U (*exoU*), Y (*exoY*), and T (*exoT*). These exoenzymes have different functions such as cytotoxicity and phosphatase two activities; adenylate cyclase activates, GTPase activating protein activities, and ADP- ribosylase activities. The

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action of these effector proteins together makes the cell more prone to further infection and colonization with *P. aeruginosa* or other pathogens (5,6). The presence or absence of the various combination of the effector proteins is reported to be important in successful colonization, the evolution of genome for survival in different environments, overexpression of some efflux pumps, and resistance to some antibacterial agents (3,7).

The occurrence of *P. aeruginosa* in the environment is due to several factors, mainly the ability to utilize many natural compounds from the environment as its source of energy. The physical environment of hospitals could be an important source of infection in which the bacteria could be transmitted, such as contaminated floors, beds, and bed rails, as well as sinks (8). Thus, there is a big concern regarding the origin of clinical samples and their relation with the isolates from the environment. The clinical isolates of *P. aeruginosa* are reported to be similar in respect to chemotaxonomy, function, and genotype; however, genetic diversity among different environmental isolates of *P. aeruginosa* was also reported (9,10). Multidrug resistance is a well-known character of *P. aeruginosa*; therefore, the analysis of antimicrobial resistance patterns along with prevalence determination of exoenzymes gene of T3SS would be helpful in the distribution and is more likely to reflect the difference between the clinical and environmental samples. The aim of this study was to determine the prevalence of effector proteins of type 3 secretion system (T3SS), antibacterial resistance pattern, and the effect of various commonly used disinfections on *P. aeruginosa* isolated from the burn patients and comparisons of it with that of close environment.

Materials and Methods

Identification and isolation of clinical and environmental isolates of *P. aeruginosa*

From July 2012 to September 2013, a total of 76 isolates of *P. aeruginosa* were collected from burn wound infections (n=57) and hospital environment (HE) (n=19, from the operating room, sinks, bed rails, sheets, and floor). Only one positive sample from each location was considered for further studies. The isolates were identified by standard biochemical tests and were kept in TSB medium with 40% glycerol at -70º C.

Antibacterial susceptibility testing

The activities of 10 antibacterial agents against the bacterial isolates were determined by the disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2010). The following discs were used in this study: ciprofloxacin (CIP: 5 µg); cefepime (CPM: 30 µg); imipenem (IPM: 10 µg); ceftizoxime (CZX: 30 µg); gentamicin (GM: 10 µg); amikacin (Ak: 30 µg); piperacillin/tazobactam (PTZ: 100/10 µg); ceftazidime (CAZ: 30 µg) and polymyxin B (PB: 300 U). All the discs except polymyxin B, which was obtained from Mast chemical company, England, were obtained from HiMedia, India. The standard strain of *P. aeruginosa* ATCC 27853 was used as the quality control for susceptibility testing.

Determination of the inhibitory activity of chemical disinfectant against *P aeruginosa*

Isolates of *P. aeruginosa* were tested by agar well diffusion against the following disinfectants:

-Deconex 2%, and Decosept solution (Borer Chemie AG, Switzerland);

-Dettol 5% (Reckitt Benckiser, United Arab Emirates);

-Nitrofurazone powder 2% and Silver sulfadiazine 1% (Ubichem, China);

-Povidone-iodine 10% and Peranacid M1 (Behvarzane, Iran);

- Sodium hypochlorite 10% bleach (Behdad, Iran).

The bacterial inoculums were briefly adjusted to 0.5 Mcfarland standard and were uniformly spread over the surface of the Mueller-Hinton agar using sterile cotton swabs. Chemical disinfectant (200 µl) were poured into agar wells (8 mm diameter holes cut in the agar gel, 20 mm apart from one another). The plates were incubated at 35º C overnight, and the diameter of the inhibition zone (DIZ) around the wells was measured and recorded (3).

DNA extraction and amplification for detection of effector proteins encoding genes of T3SS

DNA was extracted by the boiling method, according to Pitout *et al.*, (11). From a 24-hour growth medium, 3-5 colonies were transferred into 200 µl of sterile distilled water. The bacterial suspension was boiled in a water bath at 95º C for 20 minutes. The suspension was centrifuged afterward at 8000 xg for 7 minutes. The supernatant was qualified by NanoDrop (NanoDrop ND-1000) and was used as the template for the PCR assay with a Gradient Thermo block (Biometra, Germany). The PCR assay was performed according to Strateva *et al*., (12) with some modifications. Briefly, the assay was conducted in a total volume of 25 µl containing 1x reaction buffer, Taq DNA polymerase (1.5 U), $MgCl_2$ (2.5 mM), deoxyribonucleoside triphosphate (0.2 mM), 0.2 µl of

each primer obtained from Generay Biotech, Shanghai, Co. Ltd. (Table 1). All other materials were obtained from SinaClon, Iran. For DNA amplification the following protocol was used: initial denaturation at 95° C for 5 min, followed by 30 cycles of denaturation at 95° C for 1 min, annealing at 54° C for 1 min, with an extension at 72° C for 1 min, followed by a final extension at 72° C for 7 min. PCR products were separated in 1.2% agarose gel. The gels were stained with ethidium bromide (10 µg/ml) for 30 min and were observed by a UV gel document. A 100 bp DNA ladder (Fermentas, Lithuania) was used as a size marker.

Table 1. Primers used in this study for the detection of the type III effector proteins-encoding genes. Annealing temperatures used were 60, 55, 60, and 58ºC for *exoS,exoT,exoU* and *exoY* respectively

Gene	Primer sequence (5' to 3')	Amplicon (bp)
exoS	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	504
exoT	CAATCATCTCAGCAGAACCC TGTCGTAGAGGATCTCCTG	1159
exoU	GGGAATACTTTCCGGGAAGTT CGATCTCGCTGCTAATGTGTT	428
exoY	TATCGACGGTCATCGTCAGGT TTGATGCACTCGACCAGCAAG	1035

Results

Susceptibility of clinical and environmental isolates of *P. aeruginosa* to antimicrobial agents

Hundred percent of the isolates were susceptible to polymyxin B, followed by 40.8% susceptibility for AK and IPM. CZX had the lowest activity (6.6%). For all the antibacterial agents tested except for CZX and PTZ, the HE isolates were more susceptible than the burn isolates, and the differences were statistically significant (Table 2). Multiple drug resistance (MDR) was referred to as isolates resistance to CIP, GM (or AK), as well as being resistant to β-lactam antibacterial agents. In this respect, 51 (67.1%) of the isolates (burn=71.9%, HE=63.15%) had the MDR phenotype; the difference between the two groups was not significant.

Table 2. *In vitro* susceptibility of *P. aeruginosa* against selected antibacterial agents

Antimicrobial Disks Abbreviations	Number (%) isolates susceptible			*P*
	Burn (n=57)	Environment (n=19)	Total (n=76)	
Ciprofloxacin; CIP	14 (24.6)	11 (57.9)	25(32.9)	0.009
Cefepim; CPM	6 (10.5)	7 (36.8)	13(17.1)	0.01
Imipenem; IPM	17 (29.8)	14 (73.7)	31(40.8)	0.001
Ceftizoxime; CZX	4 (7.01)	1 (5.26)	5 (6.6)	0.09
Gentamicin; GM	15 (26.3)	11 (57.9)	26(34.2)	0.014
Amikacin; AK	19(33.3)	12 (63.15)	31(40. 8)	0.02
Pip/taz*; PTZ	9 (15.8)	6 (31.57)	15(19.7)	0.1
Ceftazidime; CAZ	13 (22.8)	11 (57.9)	24(31.58)	0.006
polymyxinB; PB	57 (100)	19 (100)	-	-

*Pip/taz: pipracillin/tazobactam

Inhibitory activity of chemical disinfectants on the burn and environmental isolates of *P. aeruginosa*

The highest mean of the DIZ against *P. aeruginosa* isolates in both burn and HE isolates were found for Deconex, Decocept, and pavidion iodine (Table 3). For most of the other agents tested, the mean DIZ for burn isolates was lower than that of the HE isolates, and there was a significant difference in the Silver sulfadiazine and Sodium hypochlorite ($P \leq 0.01$) case. The lowest mean for DIZ was observed for peranacide M1, Dettol, and nitrofurazone, and their DIZ was significantly lower than that of Deconex, Decocept, and pavidion iodine ($P \geq 0.003$).

Frequency and distribution of type lll effector protein-encoding genes in burn and environmental isolates of *P. aeruginosa*

The PCR results for the amplification of the effector proteins of T3SS revealed that all the burn and HE isolates carried the *exo*T gene. Prevalence of other exoenzymes in burn and HE isolates was *exoS*: 43.85% and 42%*; exoU*: 56.1% and 26.32%; and *exoY*: 94.74%

and 73.7% respectively. The difference in the isolates harboring *exoU* and *exoY* in burn and HE isolates was significant ($P \leq 0.02$).

Table 3. Inhibition zone diameter of chemical disinfectants against clinical (n=57) and environmental (n=19) isolates of *P. aeruginosa*

Chemical disinfectant	Inhibition zone diameter in mm (mean ±SD)		*P*
	Burn (n=57)	Environment (n=19)	
Deconex (2%)	22.75 ±1.9	23.26 ±2.8	0.38
Decocept solution (undiluted)	18.32 ±6.2	18.05 ±6.07	0.87
Dettol (5%)	2.37 ±5.9	5.47 ±7.5	0.09
Silver sulfadiazine (1%)	7.21 ±9.7	14.11 ±11.35	0.01
Nitrofurazone powder (2%)	1.11 ±0.4	0.0± 0.0	0.24
Povidone iodine (10%)	19.12 ±6.5	21.32 ±0.3	0.24
Peranacid M1 (1%)	0.46 ±2.4	1.58 ±6.82	0.29
Sodium hypochlorite (10%)	13.18 ±9.89	19.95 ±7.85	0.009

The relation between the presence of genes encoding the effector proteins of T3SS and resistance to chemical disinfectant and antimicrobial agents

A significant difference was observed between some disinfectant and the presence or absence of the effectors' proteins. The following disinfectants had a higher zone in *exo*enzyme negative isolates compared to *exo*enzyme positive isolates: *exoS*$^-$: with Deconex (P=0.03) and Nitrofurazone (P=0.04); *exoU*$^-$: with Silver sulfadiazine (P=0.000) and bleach (P=0.04); *exoY*$^-$: with Deconex and bleach (P=0.01). However, for some disinfectants, exoenzyme combination *exo*enzyme positive isolates compared to *exo*enzyme negative isolates, there was a significant difference in the DIZ for the following disinfectants: exoS$^+$: with Silver sulfadiazine (P=0.000), and exoU$^+$: with Povidone-iodine (P=0.04), Figure 1. Also, all susceptible isolates to CIP, GM and CAZ were *exoY* negative, and the difference between the sensitive and resistant isolates was significant ($P \leq 0.04$). However, *exoU*$^+$ isolates had a higher number of susceptible isolates to GM than the *exoU*$^-$ isolates, and the differences were significant (P=0.03). For other antibacterial agents and exoenzymes, no significant differences were found. *exo: exoenzyme*: Silver sulfadiazine (1%).

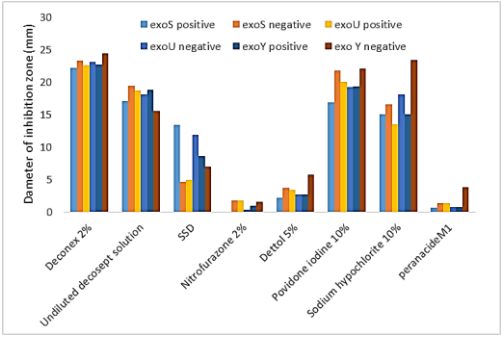

Figure 1. Mean diameter of inhibition zone (DIZ) of different chemical disinfectants against *P. aeruginosa* (n=76) isolates with or without the indicated *exo*enzymes (*exo*) of T3SS

Discussion

Owing to the presence of large genomes, *P. aeruginosa* can grow on many environmental compounds, and colonize multiple animate and inanimate surfaces (8,13). Severe burn patients with long hospital stays are at risk of infection with this opportunistic pathogen through various routes (8,14,15). Outbreaks of MDR *P. aeruginosa* strains are increasing in burn units in recent years (3). *P. aeruginosa* is an important cause of nosocomial infections in burn hospitals in Iran, and many isolates are reported to be MDR (16,17), this is in accordance with our study with 67.1% of the isolates being MDR. Resistance to antibacterial agents was high, and in many cases, was higher in burn isolates compared to HE isolates. Although the genotype of the isolates may be the same in the burn, and HE isolates, horizontal gene transfer in isolates from patients makes them more resistant than the commensal bacteria in the environment (3).

Antiseptics and disinfectants are routinely used in hospitals to prevent the environmental dissemination of bacteria, especially the MDR isolates (3,18). In this study, the disinfectants were selected based on their general use in the burn hospital unit under the study period. The highest zone of inhibition was found for Deconex and Decosept, and the least active compounds were nitrofurazone, pernacide, and Dettol. These results are in accordance with other works finding a low activity for nitrofurazone and high activity of Deconex and sodium hypochlorite (3,19). For nearly all the compounds tested, the DIZ for burn isolates was found to be lower than the HE isolates, and the difference was significant in the case of SSD and sodium hypochlorite (Table 3). Japoni *et al.*,

(20) reported a low activity for SSD against P. *aeruginosa* (95% of burn isolates were resistant) while the environmental isolates were found to be more susceptible (53.3% resistant).

According to Romão *et al.,* (21), *Pseudomonas* strains from the same clonal groups show different susceptibility to disinfectant and that the PFGE profile seems to be an invalid marker for resistance to disinfectants. The antibacterial resistance pattern was also different in the two groups with burn isolates more resistant to antibacterial agents than the environmental isolates, and the differences were significant in the case of CIP, CPM, IPM, GM, AK and CAZ (Table 2). It is possible that bacteria need a survival mechanism in the human body, while in the environment, they need other strategies for survival (8,9,12,22). This is also in accordance with other investigations showing the MDR isolates expressed fewer virulence factors than the non-MDR isolates (23,24).

Although there are many reports concerning the virulence factors in the isolates obtained from patients with cystic fibrosis, there is little information about the presence of different virulence factors in other infections caused by *P. aeruginosa* and their relation with antibacterial resistance. In this study, we investigate the prevalence of type III secretion system genes in the *P. aeruginosa* isolates from burn as well as close environmental sources. Only one isolate from a given environment or patient was analyzed to prevent over-representation of certain strains. Overall, in 97.7% of the isolates, more than one exoenzyme was amplified by the PCR method (in 2.3% of the isolates, only *exoY* was detected). In a study by Strateva *et al*., (12), eight different patterns of exoenzymes were detected. In this study, six patterns were detected. The two extra patterns in Stratava reports, "*exoU*$^{+}$/*exoT*$^{+}$" and "*exoS*$^{+}$/ *exoU*$^{+}$/*exoT*$^{+}$" had a very low prevalence (2.8% and 1.2% respectively) and therefore their absence is not surprising in this study. However, the two more prevalent patterns found in Stratava's study "*exoS*$^{+}$,*exoU*$^{-}$, *exoT*$^{+}$, *exoY*$^{+}$" (47.2%) and "*exoS*$^{-}$,*exoU*$^{+}$,*exoT*$^{+}$,*exoY*$^{+}$"(21%) are somehow similar to our study with 30.3% and 36.8% prevalence respectively (Table 4).

Table 4. Distribution and the prevalence of (as percentages) of the type III effectors proteins-encoding genes and pattern among burn and environmental isolates of *P. aeruginosa*

Gene Pattern	Number (%) of isolates		
	Burn	**Environmental**	**Total**
***exoT*$^{+}$**	2 (5.1)	5 (26.3)	7 (9.2)
***exoT*$^{+}$ / *exoY*$^{+}$**	6 (15.4)	2 (10.5)	8 (10.5)
***exoT*$^{+}$ / *exoS*$^{+}$**	1 (2.6)	0 (0)	1 (1.3)
***exoT*$^{+}$ / *exoY*$^{+}$ / *exoS*$^{+}$**	16 (41)	7 (36.84)	23 (30.3)
***exoT*$^{+}$ / *exoY*$^{+}$ / *exoU*$^{+}$**	24 (61.54)	4 (21.1)	28 (36.8)
***exoT*$^{+}$ / *exoY*$^{+}$ / *exoU*$^{+}$ / *exoS*$^{+}$**	8 (20.5)	1 (5.3)	9 (11.8)
Total in each group	57(100)	19 (100)	76 (100)

exoS, exoU, exoT, exoY exoenzymes; S, T, U, and Y of the type III effector proteins-encoding genes, respectively. + mark indicates presence and – indicates absence.

There are controversial reports concerning the prevalence of certain exoenzymes in *P. aeruginosa* with certain infectious sites in the infected patients and between clinical and environmental samples and also in the case of MDR and non-MDR isolates (5,12,25,26). Our finding showed a higher prevalence of *exoY*$^{+}$, and *exoU*$^{+}$ in burn isolates compared to that of the environmental isolates, but we did not find any difference between the MDR and non-MDR isolates in this respect. We also did not find any relation between resistance to fluoroquinolone and the isolates with *exoU*$^{+}$, as was reported by Wong-Beringer *et al*., (7). However, there was some relation between susceptibility to some disinfectants and presence or absence of exoenzymes, for example, the higher sensitivity of the *exoY* ¯isolates against CIP, GM, and CAZ, and also higher levels of sensitivity to GM in *exoU* ¯isolates. In fact, *exoU*, *exoS*, and *exoY* genes are variably encoded genes that could either be present or absent in a given strain of *P.* aeruginosa (5,24). Therefore, resistance to certain chemical disinfectants or antibacterial agents could be a co-selected trait in favor of bacterial survival in different environments.

In conclusion, further studies are needed, especially with a higher number of isolates on the expression of different exoenzymes and their simultaneous expression at various conditions and to evaluate their role in *P. aeruginosa* pathogenicity and virulence in infection with

this opportunistic bacteria.

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