Study of flagellin profiling in multidrug resistant *Pseudomonas aeruginosa* (MDRPA) isolated from burn wound infections, Tehran, Iran

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ABSTRACT

Nosocomial infections of multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) are a growing concern in hospitalized patients in burn centers. The aim of this study was to investigate the flagellin profiling and antibiotic susceptibility of *P. aeruginosa* isolated from burn wound infections. During 8 month study, 73 clinically *P. aeruginosa* isolates collected from patients hospitalized in burn ward. *P. aeruginosa* isolates were identified using standard laboratory procedures. In vitro susceptibility of clinical isolates of *P. aeruginosa* to 6 antimicrobial agents were investigated by Clinical and Laboratory Standards Institute (CLSI 2012) Kirby-Bauer disk diffusion assay. The frequency of different type of flagellin was investigated by using specific primers and by PCR method. The resistance rates of our isolates to 6 tested antimicrobial agents were relatively high. Imipenem has good activity while tobramycin and ciprofloxacin do not have any effect on *P. aeruginosa* isolates. Of 73 isolates 59 (80.8%) were multidrug resistant. Twenty eight of 73 isolates were resistant to all antibiotics. Agarose gel electrophoresis of chromosomal DNA exhibited that 59 isolates (80.8%) of *P. aeruginosa* had type A flagellin while only 14 isolates (19.2%) had type b flagellin. Given the antibiotic failure treatment, it appears that alternative ways such as immunity to prevent of these infections could be informative. Our survey of flagellin profiling of multidrug-resistant *P. aeruginosa* isolates exhibited high frequency of type a flagellin as a major virulence factor has important role of immunity against infections caused by MDRPA. Functional surveillance of multidrug-resistant *P. aeruginosa* in order to prevention of resistance dissemination is necessary.

Keywords: *Pseudomonas aeruginosa*; Multidrug-Resistant; Flagellum.

INTRODUCTION

Pseudomonas aeruginosa as a nosocomial pathogen is the major cause of infection in burn centers, especially in Iran [1]. This bacterium is responsible for a broad spectrum disease that can be ranged from urinary or wound infections to bacteremia, endocarditis, multi-organ failure and death [2]. Overall, *P. aeruginosa* infections are associated with high mortality and morbidity rates in burn patients of developing countries. Increase exposure to antibiotics, indiscriminate use of them in the treatment of burn infections and development of intrinsic and acquired resistance mechanisms has promoted the rapid development of multiple resistances among *P. aeruginosa* isolates [1-3]. Nosocomial infections of multidrug-resistant P. *aeruginosa* (MDRPA) are a growing concern in hospitalized patients in burn centers [4]. Remarkable capacity of this bacterium in resistance to many drugs have commonly been

reported by several investigators in burn units. Widespread multi resistance among P. *aeruginosa* not only leading to increased economic cost, but also can with directly threatens the life of the patient [5, 6]. Hence immunity against infections caused by MDRPA is a very important. Among the cell surface structures of *P. aeruginosa*, there is a polar flagellum that is responsible for pathogenicity in mucosa, motility, attachment of bacteria to host cells and initial development of a biofilm, and activation of the host inflammatory response via Toll-like receptor 5 (TLR5) [7, 8]. Overall, *P. aeruginosa* isolates express a primary protein component of the flagellar filament that can be classified into two serotypes, types A (A1, A2) and B [9]. The A type flagellins are heterogeneous and have variable molecular weight from 45-50 kDa, while the B type flagellins are homogeneous and are essentially conserved in sequence and have an approximately molecular weight of 53 kDa [10]. Several studies suggesting that *P. aeruginosa* flagellin as a critical proinflammatory determinant appears to be relevant to colonization and the expression of proinflammatory mediators by monocytes. Importance of flagella as target antigens for vaccination in prevention of the acquisition of *P. aeruginosa* infection both in vivo and in vitro investigations has been well established [11]. Considering lack of information about flagellin profiling of MDRPA and need to continuous surveillance to prevent the further spread of resistant isolates and also help to physicians for prescription effective treatment protocol this study was performed.

MATERIALS AND METHODS

Bacterial strains

The present descriptive study was performed on burn cases who were hospitalized in several burn centers. A total of 73 clinically significant *P. aeruginosa* isolates were recovered from burn wound infections of hospitalized patients during 8 month's study. The repetitive isolates from the same patients were excluded. Samples were obtained from the depth of the lesion by swab. All the samples were transported to the laboratory and were processed immediately. All isolates were identified as *P. aeruginosa* by using standard microbiological tests such as; Gram stain, oxidase test, catalase test, argenine dihydrolase, ornithine decarboxylase, growth at 42°C, growth on cetrimide agar medium (Liofilchem, Italy), O/F (Oxidation-Fermentation) test and pigment production [12]. Samples confirmed as a *P. aeruginosa* isolates were stored in Tryptic Soy Broth (TSB; Merck, Germany) containing 20% glycerol at -70°C and were subjected to further investigation.

Antimicrobial susceptibility testing

To evaluate antimicrobial susceptibility of isolates Kirby-Bauer's Disk diffusion method was done according Clinical Laboratory and Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) criteria [13]. The following antimicrobial agents were used in this study: ceftazidime (CAZ 30µg), amikacin (AK 30µg), gentamicin (GM 10µg), tobramycin (TN 10µg), imipenem (IMI 10µg) and ciprofloxacin (CIP 5µg). Antibiotic disks used in this research were supplied by MAST Laboratories Ltd (Bootle, Merseyside, UK). *P. aeruginosa* ATCC 27853 was used as the control strain in antimicrobial susceptibility testing.

Serological assay

Bacteria were grown for 16 to 18 hours at 37 °C on Tripticase Soye Agar media plates (TSA, Merck, Germany). For serological typing of flagellin, the suspension of bacteria in PBS slowly vortex and held at room temperature for 15 min. The suspension of bacteria was mixed with an equal volume of diluted polyclonal antibody and agglutination was viewed by light microscopy [14].

DNA extraction and PCR assay

Genomic DNA was extracted from bacteria on nutrient agar medium by using QIAamp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer's procedure. The presence of flagellin genes was detected by PCR and the primers were designed as follows: Forward primer: 5´-TTAGCGCAGCAGGCTCAGAAC-3´and reverse primer: 5´-ATGGCCTTGACCGTCAACAC-3´. Primers were synthesized by TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany). Specificity of the primer pairs was evaluated by using Basic local Alignment Search Tool (BLAST)

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) and also Gene Runner software (Hastings Software, Hastings- on-Hudson, NY, USA). The PCR reactions for detection flagellin genes were done a total volume of 25 µL. The reaction mixture contained 1x buffer(10 mM Tris-HCl, 50 mM KCl), 1.5 mM MgCl2, 200µM dNTPs, 0.5µM of primers, 20 pmol of each primer, 1 U of Takara Taq (Takara Shuzo Co., Ltd., Shiga, Japan) and 3 µL of the template DNA. PCR conditions for amplification of 1200 bp and 1400bp fragments of the type a and b flagellin was done by thermocycler (AG 22331; Eppendorf, Hamburg, Germany) as follows: initial denaturation for 5 min at 94ºC, 35 cycles of 1min at 94 ºC (denaturation), 1 min at 68 ºC(annealing), and 90s at 72 ºC(extension); and a final extension cycle of 5 min at 72 ºC. Amplified fragments (expected size 1200bp for type a flagellin and 1400 bp for type b felagellin) were separated by 1% agarose gel electrophoresis at 80V for 2h. Finally, fragments were stained by ethidium bromide and detected under UV light. *P. aeruginosa* 8821M strain as control positive of type a, *P. aeruginosa* PAO1 strain as control positive of type b flagellin and for negative control *Staphylococcus aureus* was used.

Statistical analysis

All samples were analyzed using with SPSS software for Windows, version 11.0 (SPSS Inc., Chicago, IL).

RESULTS

During the 8 months, a total of 73 samples of *P. aeruginosa* were recovered from adult male and female patients that were hospitalized in burn centers. The age range of the patients was from 12 to 68 years with a median of 39.2. Forty nine hundreds (67%) were female and 24(32%) were male.

The antimicrobial susceptibility test revealed that the rate of resistant to ceftazidime was in 72 isolates (98.6%), ciprofloxacin in 73 (100%), tobramycin in 73 (100%), amikacin in 71 (97.3%), gentamicin in 72 (98.6%) and imipenem in 47 (64.4%). The rates of resistance to antibiotics tested varied between 64% and 100 % (Table 1).

Table 1. Antimicrobial susceptibilities of 73 *P. aeruginosa* isolated from burn patients to 6 antimicrobial agents

Antibiotics	Antibiotic susceptibility (n=73)		
	R n (%)	I n (%)	S n (%)
amikacin	71(97.3)	0(0)	2(2.7)
gentamicin	72(98.6)	0(0)	1(1.4)
ceftazidime	72(98.6)	0(0)	1(1.4)
imipenem	47(64.4)	4(5.5)	22(30.1)
tobramycin	73(100)	0(0)	0(0)
ciprofloxacin	73(100)	0(0)	0(0)

R: resistant; I: intermediate; S: sensitive

Table 2. Different pattern of multi- drug resistance in *P. aeruginosa* strains isolated from burn patients

Antibiotic resistance profiles	Number of antibiotic resistant isolates	Antibiotics No.	Total number of resistant isolates (%)
TN-CIP-GM-CAZ-AK-IMI	28	6	28(38.4)
TN-CIP-GM-CAZ-AK TN-CIP-GM-CAZ-IMI TN-CIP-CAZ-AK-IMI	5 4 4	5	13(17.8)
TN-CIP-GM-CAZ TN-CIP-GM-IMI TN-CIP- CAZ-AK TN-CIP- CAZ-IMI TN-CIP-AK-IMI	5 4 2 3 4	4	18(24.7)
TN-CIP-GM TN-CIP-CAZ TN-CIP-AK	4 3 2	3	9(12.3)
TN-CIP	5	2	5(6.8)

Ceftazidime; CAZ, amikacin; AK, gentamicin; GM, tobramycin; TN, imipenem; IMI, ciprofloxacin; CIP

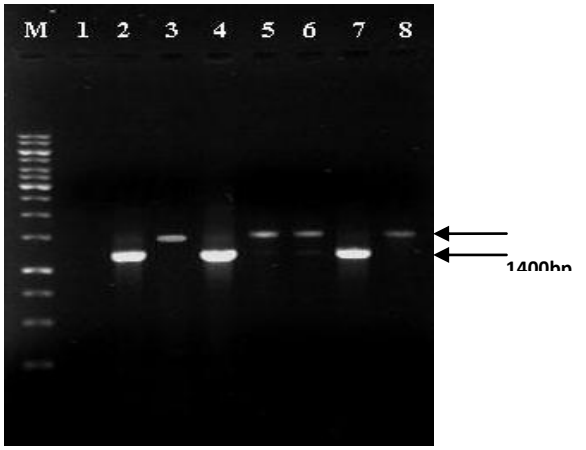

Figure 1. Gel electrophoresis results of PCR product. M: DNA Ladder (Invitrogen™)
(250 bp), Lane 1: Negative control, Lane 2: *P. aeruginosa* 8821M (standard type a), Lane 3: *P. aeruginosa* PAO1 (standard type b), Lane 4, 7: *P. aeruginosa* clinical isolates (type a), Lane 5, 6, 8: *P. aeruginosa* clinical isolates (type b).

None of the isolates tested were sensitive to all antibacterial agents. Multidrug-resistant (MDR) was defined as resistance to more than two antibiotic unrelated antibiotics. Twenty eight of 73 isolates *P. aeruginosa* were resistant to all antibiotics. Of 73 isolates 59 (80.8%) were known as MDRPA isolates. The most frequent resistance profile among our isolates was included resistance to 6(38.4%), 4(27.4), 5(17.8%), 3(12.3%) and 2(6.8%) antibiotics. Different pattern of multi-drug resistance in *P. aeruginosa* investigated in this study are summarized in table 2. Of 73 clinical isolates of *P. aeruginosa*, 59 isolates (80.8%) had type a flagellin and 14 isolates (19.2%) had type b flagellin (Figure 1).

DISCUSSION

P. aeruginosa is a serious nosocomial pathogen and major cause of fatal infections in cystic fibrosis, hospitalized, immunocompromised and especially burn patients [15, 16]. The main causes of mortality and morbidity in burn wound infections could be associated with drug resistance and lack of proper immune response [11]. The existence of multi drug resistance and its spreading among *P. aeruginosa* isolates lead to many problems concerning the treatment of such infections [4]. Therefore continuous surveillance in order to prevent the further spread of MDRPA isolates and also inhibition of colonization in burn centers should be employed. In our study the resistance rate of *P. aeruginosa* isolates to investigated antibiotics was relatively high. All isolates were absolutely resistant to tobramycin and ciprofloxacin. In compare to performed studies in Brazil, Turkey, Thailand, United State, China and Iran (2010 and 2012) a high resistance to tobramycin and ciprofloxacin were seen in our study [4, 6, 17-19]. The lowest resistance rates were seen against imipenem (64.4%) in compare with other tested antibiotics. Previous studies that were performed in Iran revealed different resistance rate to antibiotics used to treat burn wound infected by *P. aeruginosa*.

For example, Rastegar et al In the Tohid Burn Center in Tehran showed that more than 95% of isolates were resistance to gentamicin, carbenicillin, co-trimoxazole, ceftizoxime and tetracycline while resistance to amikacin, and ciprofloxacin was 90% and 82% respectively [20]. In a study conducted by Moradian et al the rate of resistance to ciprofloxacin, ceftazidime, gentamicin, amikacin and imipenem was 38.9%, 92.6%, 55.5%, 46.2% and 53.7% respectively [18]. Another study that was done by Hadadi et al showed that 75% of isolates were resistance to imipenem and 39% to ciprofloxacin [21].

The most main factors that play crucial role in creating these differences may be as follows: Hygienic statement, difference in the type of strains, epidemiological conditions, unrestricted prescriptions of antibiotics for treatment of burn infections and ability of strains in acquisition of resistance genes [18, 22, 23].

Of 73 isolates 59 (80.8%) were multidrug resistant which is higher than rate of MDR reported in Turkey (20.9%) [19], Brazil(71%) [17], Iran(42.3%) [22] and lower than china (90.1%) [6](1-s) and Thailand (100%) [23]. Multidrug resistance in *P aeruginosa* is an increasing problem especially in developing countries [1]. The high frequency of MDRPA isolated from burn units has been confirmed by several investigators [1, 4]. Karlowsky et al. reported the increased rate of MDRPA from 7.2%

in to 9.9% during three years [24]. Previous results in Iran showed a progressive increase in MDRPA from 5.46% [4] in 2009 to 45.3% [22] in 2013. In accordance with recent data unfortunately, the results of our study disclosed that the increase of multi drug resistance among *P. aeruginosa* isolated from Iranian burn patients has reached a critical point to the extent that for treatment of these infections must be recruited multiple drug regimens.

Given the antibiotic failure in the treatment of infections caused by *P. aeruginosa* and need to multiple drug regimens, it appears that alternative ways such as immunity to prevent and treatment of these infections could be informative [25-27]. As previous mentioned, flagellin protein as a major virulence factor has important role of immunity against infections caused by MDRPA. According to the studies, more than 95 % of clinical isolates of *P. aeruginosa* are flagellated [28]. Therefore it is important to know the frequency and type of flagellin. In our study all of isolates were positive for flagellin and type a flagellin was predominant. This results is in line with other studies [29, 30].

CONCLUSION

In summary, our results demonstrated high frequency of type a flagellin their important role as a major virulence factor in *P. aeruginosa* isolated from burn patients. Imipenem was the most effective drug in vitro assessment while tobramycin and ciprofloxacin could not be effective drugs for treatment of burn infections. High frequency of MDR among *P. aeruginosa* isolated from burn patients revealed the important role of indiscriminate and unregulated use of antibiotics as selective pressure for dissemination MDR in our region. However, functional surveillance is urgently needed to prevent dissemination of MDRPA isolates in our burn centers.

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