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A multiresistant clone of *Pseudomonas aeruginosa* sequence type 773 spreading in a burn unit in Orumieh, Iran

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Herein, we describe the phenotypic and genotypic characterization of a multiresistant clone of *Pseudomonas aeruginosa* disseminating in a burn unit in Orumieh, Iran. A total of 58 isolates of *P. aeruginosa* were collected during August 2007 and June 2008. Minimum inhibitory concentrations (MICs) of *P. aeruginosa* isolates were determined against 11 antimicrobial agents by E test. Serotyping, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) were used for studying the clonal relationship among the isolates. Antibiotic susceptibility testing revealed that most of the isolates were multidrug resistant and colistin was the antibiotic with the highest activity. *Pseudomonas aeruginosa* isolates fell into nine different serotypes, and O10 and O11 were the most common. PFGE analyses showed 12 different genotypes and 68.1% of isolates showed more than 80% similarity, indicating possible clonal relatedness. These isolates were found to belong to the same sequence type, ST773. This sequence type has earlier been reported from China, and a double locus variant of this ST has been found earlier in France in a PER-1 extended-spectrum β-lactamase-producing *P. aeruginosa*.

Key words: PFGE; multidrug resistance; serotyping; sequence type; MLST.

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Pseudomonas aeruginosa is an important nosocomial pathogen responsible for a broad spectrum of infections, particularly in burn and intensive care units (ICUs). Multiple mechanisms of antimicrobial resistance have been described in *P. aeruginosa* including AmpC and class A β-lactamases, metallo-β-lactamases, the expression of several efflux pumps, and alterations of porins (OprD). Interplay of these mechanisms has led to the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains (1).

Multidrug-resistant *P. aeruginosa* infections are difficult to treat, and the increasing prevalence of such organisms is a global health problem (2). The MDR *P. aeruginosa* is usually defined as non-susceptible to at least three

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drugs in the following classes: antipseudomonal penicillins, cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones (2). Multidrug-resistant *P. aeruginosa* has frequently been reported as the cause of nosocomial outbreaks in burn units or as colonizers of the wounds in burn patients (3).

Pseudomonas aeruginosa infections are associated with increased costs and length of stay in ICU units among hospitalized patients (2). Epidemiological typing is essential for understanding the routes of bacterial transmission in the hospital and to determine the source of infection, which can help to effectively control nosocomial infections by preventing spread of these strains (4)

In recent years, molecular techniques have received increased attention as means of analyzing epidemiologic relatedness (5). Several typing methods have been used for epidemiological studies of *P. aeruginosa* strains including pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism, random amplified polymorphic DNA, and arbitrarily primed PCR (3). Currently, PFGE is often considered as a "gold standard" of molecular typing methods for bacterial pathogens (5). Lately, multilocus sequence typing (MLST) has emerged as standard methodology for phylogenetic studies (6).

In this study, we describe antibiotic susceptibility patterns of *P. aeruginosa* strains isolated in a burn unit in Imam Hospital of Orumieh, Iran. Further, we investigate their epidemiologic relationships by serotyping, PFGE, and MLST.

MATERIALS AND METHODS

Bacterial isolates

During August 2007 until June 2008, a total of 58 non-repetitive and consecutive isolates of *P. aeruginosa* were collected from a burn unit of Imam Hospital (reference burn unit of West- Azerbaijan, Iran) of Orumieh, Iran. A questionnaire was used for recording patients' demographic, laboratory, and clinical data. Identification of isolated bacteria was performed by using standard microbiological tests such as oxidase test, growth at 42 °C, O/F test, and growth on Cetrimide agar. Bacterial isolates were recovered from different clinical samples such as urine, sputum, wound, and blood cultures.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of *P. aeruginosa* isolates were determined against 11 antimicrobial agents; imipenem, meropenem, azetronam, cefepime, ceftazidime, amikacin, gentamicin, ciprofloxacin, piperacillin/tazobactam, tobramycin, colistin by Etest (bioMérieux, Solna, Sweden), according to the recommendations of the manufacturer. *P. aeruginosa* ATCC 27853 was used for quality control of antimicrobial susceptibility testing. EUCAST breakpoints were used for interpretation.

Serotyping

Serotyping of *P. aeruginosa* isolates on the basis of O-antigens was performed by the slide agglutination method according to manufacturer's protocol with polyvalent and monovalent antisera for *P. aeruginosa* O-antigens (Bio-Rad Laboratories, Marnes-La-Coquette, France). In brief, a drop of polyclonal antibodies was placed on a glass slide and a single colony of bacteria was added, mixed gently, and after 1–2 min, presence of agglutination indicated a positive result. Then, based on the polyclonal antibodies reactions, the related monoclonal antibodies were used for determination the type of O-polysaccharide of LPS. Bacterial isolates that agglutinated by multiple antibodies (polyagglutinable) or had not any reaction (non-agglutinable) were designated as non-typeable isolates.

Pulsed-field gel electrophoresis

Genotyping of *P. aeruginosa* was performed by PFGE as described by Yetkin et al. (4) and the ARPAC *P. aeruginosa* typing database with slight modifications. In brief, five colonies of overnight culture of *P. aeruginosa* isolates were suspended in 250 μL of SE-buffer (75 mM NaCl, 25 mM EDTA Na2, pH = 8.6) and pre-heated at 54 °C. Then, 250 μL of 1% low melting point (LMP) agarose (SeaKem Gold agarose) was added and mixed gently by pipetting, 85 μL of this mixture immediately transferred into plug molds. The agarose plugs were placed in 5 mL of cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosyl) and 25 μL of proteinase K was added to each tube and incubated at 54 °C overnight. The plugs were washed three times by distilled water and twice with TE buffer (10 mM Tris:1 mM EDTA, pH 8.0), then DNA digestion was carried out using *Spe*I restriction enzyme (New England Biolabs, Hirts, UK) for 16–18 h at 36 °C according to the manufacturer's instructions.

DNA fragments were separated in 1.2% agarose (SeaKem Gold agarose) in a CHEF-Mapper (Bio-Rad, Hercules, CA, USA) in 0.5 × Tris-Borate

EDTA (TBE) running buffer at 12 °C for 30 h. The electrophoresis parameters were as follows; the initial switch time was 1 s and final switch time was 50 s, voltage gradient 6 V/cm, angle 120° and ramp shape was linear. A lambda ladder PFGE marker (New England Biolabs) was used as a size marker, also *P. aeruginosa* ATCC 27853 was run in every six lanes of PFGE to allow normalization of gels.

Gels were stained with ethidium bromide and banding patterns were visualized by ultraviolet transillumination and photographed, pictures were saved as a tagged image file format. The banding patterns were analyzed using the GelCompare software (version 3; Applied Maths, Sint-Martens-Latem, Belgium) and dendrograms were constructed by use of the unweighted pair group method with Dice coefficient and a position tolerance of 1%. Dice co-efficient of ≥ 0.80 was considered cutoff for possible clonal relatedness (7).

Multilocus sequence typing

Twenty-seven isolates of *P. aeruginosa* were subjected to MLST as representative isolates of distinct PFGE patterns. MLST was performed according to Curran et al. (8). Seven housekeeping genes; *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA,* and *trpE* were amplified, and PCR products were purified using the Jetquick spin column technique (Genomed GMBH, Löhne, Germany). The purified PCR products were used as templates for the sequencing reactions. Sequencing reaction was carried out on each DNA strand using BigDye Terminator Ready Reaction Mix v3.1 and internal forward and reverse sequence primers according to Curran et al. (8).

Nucleotide sequences were determined for both strands using ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The results were analyzed with BioNumerics software for assignment of allelic numbers and sequence types (STs) were obtained by comparing the results with existing alleles in the MLST database (http://pubmlst.org/paeruginosa). *P. aeruginosa* isolates with five or more identical alleles were considered as part of the same clonal complex (7).

Screening for presence of metallo-β-lactamases

Metallo-β-lactamases were screened for by PCR according to Mendes et al. (9). DNA sequencing of the obtained PCR products of VIM- and IMP-positive strains was carried out on both strands with the same methodology as described for MLST. Experimentally determined sequences were analyzed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and following alignment subjected to BLAST searches (http://blast.ncbi.nlm.nih.gov)

RESULTS

Phenotypic methods

During the study period, a total of 58 clinical isolates of *P. aeruginosa* were collected from the burn unit of Imam Hospital in Orumieh, Iran. Thirty-one (53.4%) of the isolates were derived from female patients and 27 (46.6%) from male patients. The mean age of patients was 25.1 ± 18.8 years. The most common specimen sources were; wound (79.3%), urine (12.1%), blood culture (5.2%), ear (1.7%), and sputum specimen (1.7%). Table 1 shows MIC_{50}, MIC_{90} values and antibiotic susceptibility profiles of *P. aeruginosa* isolates against 11 antibiotics. According to the susceptibility testing data, 47 (81.03%) of the isolates were MDR *P. aeruginosa* (resistant to $\geq$ three antibiotics from different classes).

Table 1. MIC values of *Pseudomonas aeruginosa* isolated from the burn unit of Imam Hospital in Orumieh, Iran

Resistant, N (%)	Intermediate, N (%)	Susceptible, N (%)	MIC_{90} (mg/L)	MIC_{50} (mg/L)	Antibiotic
44 (93.6)	3 (6.4)	–	256	256	Aztreonam
44 (93.6)	–	3 (6.4)	256	256	Amikacin
46 (97.9)	–	1 (2.1)	256	256	Ceftazidime
47 (100)	–	–	256	256	Cefepime
44 (93.6)	–	3 (6.4)	32	32	Ciprofloxacin
7 (14.9)	–	40 (85.1)	4	2	Colistin
44 (93.6)	–	3 (6.4)	256	256	Gentamicin
46 (97.9)	–	1 (2.1)	32	32	Imipenem
46 (97.9)	–	1 (2.1)	32	32	Meropenem
46 (97.9)	–	1 (2.1)	32	32	Piperacillin-tazobactam
44 (93.6)	–	3 (6.4)	256	256	Tobramycin

Serotyping revealed that serotype O10 with 51.7% frequency was the most prevalent among the isolates (30/58; 51.7%). Serotype O11 was the second most prevalent serotype (12/58; 20.7%) among isolates. The other serotypes found in this study were as follows, O1 (n = 5), O4 (n = 3), O13 (n = 1), O12 (n = 1), O6 (n = 1), O7 (n = 1), O3 (n = 1). Three of the isolates were non-typeable.

Screening for metallo-β-lactamases

Five of the isolates were positive in the MBL-PCR according to Mendes. Three of the isolates featured VIM and two isolates featured IMP. The sequenced PCR products were either 382 bp (VIM) or 188 bp (IMP). Both IMP products belonged to the IMP-1 group (sequence identical to IMP-1) and the three VIM products all belonged to the VIM-1 group (sequence identical to VIM-4).

PFGE

Molecular typing of the isolates was carried out by PFGE. Cluster analysis based on dendrogram created using GelCompare software showed 12 different genotypic profiles among isolates. Of 47 isolates, 32 (68.1%) showed more than 80% of similarity (Dice coefficient) and were regarded potentially clonally related based on the criteria of relatedness proposed by Tenover et al. (7). Twenty-four (75%) isolates of this cluster were O10 serotype, and 26 (81%) of the isolates were recovered from burn wound specimens. We found four genotypes that included two isolates and the remaining seven isolates showed unique PFGE patterns. The constructed dendrogram for relative relatedness among 47 *P. aeruginosa* isolates is shown in Fig. 1.

MLST

Multilocus sequence typing of 27 representative *P. aeruginosa* isolates revealed a total of seven different STs, namely; ST773, ST235, ST970, ST972, ST207, ST967, and ST623. The most prevalent sequence type was ST773 (n = 15), of which 80% (n = 12) of the isolates belonged to O10 serotype, whereas one isolate belonged to O11 serotype, one isolate was O1, and one isolate was O3. Six isolates were sequence type 235; four of which belonged to O11 serogroup, one was O7 serotype, and one isolate was non-typeable. The isolates were not closely related according to PFGE. Three of the isolates featured VIM-1 group MBLs and one was IMP-1 group positive.

Two isolates were ST970, these isolates had more than 80% similarity in PFGE analysis, both of them belonged to O4 serotype, one of them was MBL negative, whereas the other one was bla_{VIM} positive. We found one isolate with ST623 belonging to O1 serotype. The other STs which encountered in this study were ST967 and ST207; they belonged to O10 and O1 serotypes, respectively; none of which were MBL positive. In this study, we also encountered another new sequence type (ST972), in a strain belonging to serotype O13, which was bla_{IMP} positive.

In total, three STs, ST970, ST967, and ST972 were identified for the first time in this study (http://pubmlst.org/paeruginosa).

DISCUSSION

Application of different typing methods for studying clonal relationship between *P. aeruginosa* isolates have been reported previously (7). In this study, we have reported dissemination of a clone of MDR *P. aeruginosa* in a burn unit of Imam Hospital in Orumieh, Iran. Antibiotic susceptibility profiles of isolates showed a high level of resistance to most antibiotics (Table 1), colistin was the most active agent with 85.1% susceptibility. Although nephrotoxicity and neurotoxicity side effects of colistin have restricted its clinical use, this drug has been reintroduced into clinical practice during two past decades.

The prevalence of imipenem-resistant *P. aeruginosa* has been reported to vary from 3% to 62% in Tehran hospitals of Iran (10, 11). Khosravi et al. reported that 41% of *P. aeruginosa* isolates in Ahwaz, southwest of Iran were resistant to imipenem (12). The varied range of resistance to imipenem among *P. aeruginosa* isolates may be due to some factors including hospital wards (ICU or burn units) and the antibiotics administered among patients.

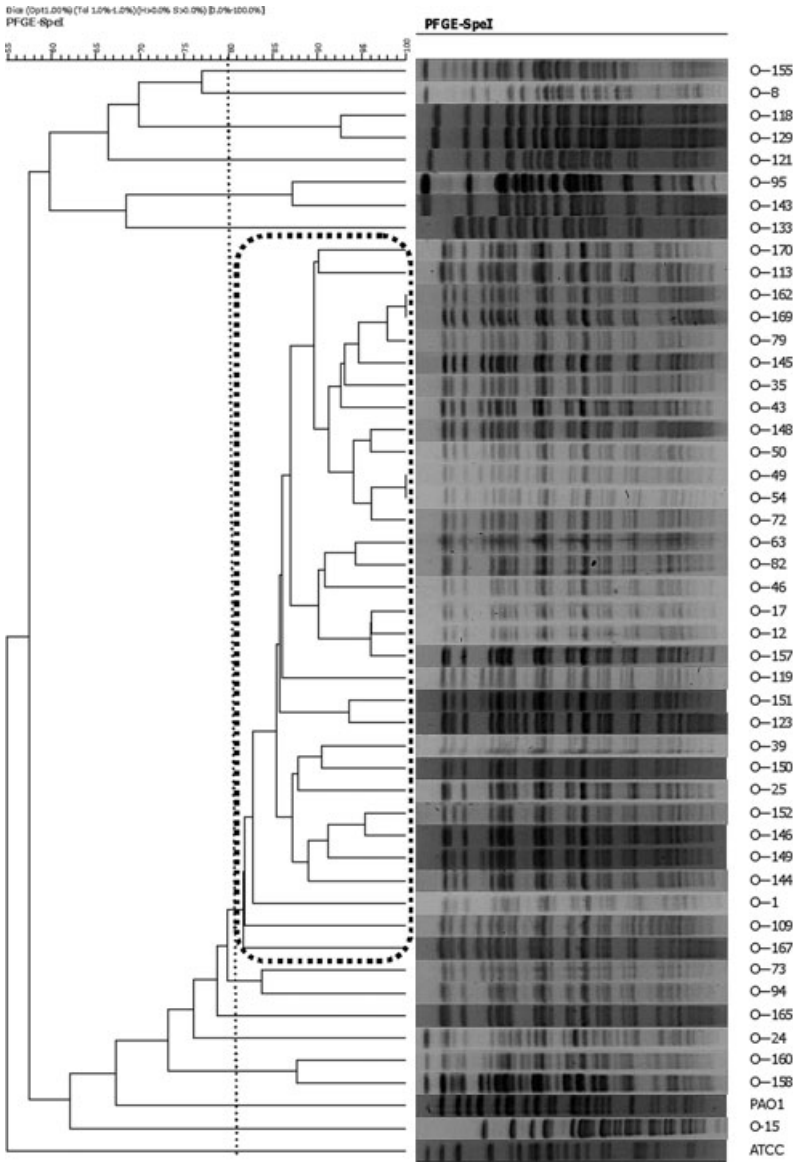

Fig. 1. UPGMA dendrogram for percentage of similarity among PFGE profiles of *Spe*I-digested DNA from 47 *Pseudomonas aeruginosa* isolates from a burn unit of Imam Hospital of Orumieh, Iran. A large strains cluster with $\geq 80\%$ similarity is encircled. Representative isolates of this cluster (n = 12) belonged to ST773. ATCC = *P. aeruginosa* ATCC 27853.

In this study, serotyping revealed nine different serotypes, the dominant serotypes among our isolates were O10 (51.7%), followed by O11 (20.7%). Also in a study at Motahhari Hospital in Tehran, O11 serotype was dominant (13). Certain clones of the O11 serotype of *P. aeruginosa* have worldwide distribution and are usually reported to be associated with multidrug resistance, although there are some variations in different parts of the world (3, 14, 15). In this study, the O10 serotype of *P. aeruginosa* emerged as the most prevalent serovar associated with MDR phenotype, which is a new finding in this area.

PFGE analyses showed 12 different genotypes among the *P. aeruginosa* isolates which is in agreement with other studies showing high discriminatory power for the PFGE method. The predominant cluster in PFGE method comprised 32 (68.1%) isolates with

more than 80% similarity, which were considered to be possibly clonally related. The majority of isolates (75%) in this cluster belonged to the O10 serotype.

MLST was performed on 27 representative isolates based on PFGE dendrogram. Majority of isolates (n = 15) belonged to ST773, this sequence type has been reported earlier in the MLST database in a sputum isolate from China in 2006. No more information is currently available in the MLST database regarding this isolate, and the finding has to our knowledge not been published. Furthermore, a double-locus variant of this sequence type was described in a PER-1 extended-spectrum β-lactamase producing *P. aeruginosa* from France, a strain that originated in Turkey, which is a neighboring country of Iran (16).

The other prevalent sequence type in this study was ST235; this sequence type of *P. aeruginosa* has been described frequently in VIM-producing isolates and has already been reported from Hungary, Italy, Greece, Poland, Sweden, and Norway (7, 17, 18). This sequence was also found in an IMP-1-producing isolate from Japan (19). Furthermore, ST235 was recently described as common among MDR isolates from countries north and south of the Mediterranean basin (20). The majority of clinical isolates belonging to ST235 are associated with serotype O11 and harbor clinically important acquired β-lactamases, such as bla_{VIM} and $bla_{\text{PER-1}}$ (16, 21). In our study, all of the ST235 isolates were MDR and the majority of them belonged to O11 serotype and four of six isolates with this sequence type were MBL positive (three bla_{VIM} and one bla_{IMP}). Currently, there are 16 single locus variants of ST235 in the MLST database and some of these variants were identified in clinical isolates carrying VIM-type metallo-β-lactamases (7).

In conclusion, we describe clonal expansion of an MDR strain of *P. aeruginosa* ST773 in a burn unit in Iran. The finding emphasizes the importance of continuingly following the epidemiological relatedness of MDR *P. aeruginosa* spreading in ICUs and burn units. Most isolates belonged to the O10 serotype, contrary to the situation in southern Europe where O11 prevails.

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