

## Original Article

## Detection of Efflux MexAB-associated Multidrug Resistant (MDR) *Pseudomonas aeruginosa* Isolated from Patients in Torbat Heydarie, Northeast Iran

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### Abstract

**Background:** *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the leading causes of hospital acquired infections. Infections with *P. aeruginosa* are often hard to treat because of existence of different mechanisms of antibiotic resistance changes in permeability of drugs and activity multidrug efflux pumps. The aim of current study was to determine the antibiotic resistance pattern of *P. aeruginosa* and existence of efflux pump MexAB genes using PCR technique. **Materials and Methods:** 506 isolates cultured from different clinical specimens of patients hospitalized at Nohom Dey and Razi hospitals of Torbat Heydarie (northeast Iran) were collected and used in this study. Isolates were identified using conventional bacteriology and their susceptibility to different antibiotics were assessed using agar disk diffusion method. The PCR assay was used to detect efflux pump MexAB genes. **Results:** From 506 isolates, 50 were identified as *P. aeruginosa* and these were isolated from isolated from blood, tracheal, burn, and wound. Incidence of *P. aeruginosa* was greater in males than females, wound infections had the highest number of occurrence and patients between 30-50 years were the most infected age group. In total, 60.86% of strains were multidrug resistant (MDR). The PCR technique revealed that most of the *P. aeruginosa* isolates and all the MDR strains contained MexA and MexB genes. **Conclusion:** The emergence of MDR microorganisms poses serious therapeutic problems for patients. Determining bacterial resistance mechanisms is complex. In this way, efflux systems were responsible for antibiotic resistance and played an important role in the MDR phenotype among *P. aeruginosa* isolates.

**Keywords:** Multi-drug resistance, Efflux pump, *Pseudomonas aeruginosa*, Nosocomial infections.

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### Introduction

Multidrug resistance (MDR) in bacteria, including *Pseudomonas aeruginosa*, has been related to intrinsic drug resistance, mutations, low permeability of outer membrane, horizontal gene transfer of plasmids, transposons, bacteriophages and active efflux pumps (1-5). *P. aeruginosa* is a gram-negative bacterium and opportunistic human pathogen which is associated with burn wounds and

lung infections in patients with cystic fibrosis (3,6,7). The emergence of high prevalence nosocomial infections produced by *P. aeruginosa* has resulted in major therapeutic challenges to treatment, and is a cause of morbidity and mortality worldwide (8,9). The basic antibiotics used to treat *P. aeruginosa* infections are quinolones, cephalosporins, aminoglycosides and polymyxins (5,10). However, the emergence of drug resistant *P. aeruginosa* become a challenging public

health problem worldwide (10). Efflux pumps play a significant role in antibiotic resistance and were firstly described as an important component of resistance to tetracycline in *Escherichia coli* (10,11). Efflux pumps in bacteria have been categorized into five types (10). The efflux pump MexA-MexB-OprM is able to remove various dyes, detergents, and drugs including tetracyclines,  $\beta$ -lactams, fluoroquinolones, macrolides, novobiocin, sulfonamides, and trimethoprim (3,4,9). The MexAB-OprM genes, encoded MexAB-OprM pump, consist of inner membrane-associated complexes, MexA and MexB, and OprM, an outer membrane component. MexAB-OprM production may lead to MDR in *P. aeruginosa* isolates (12). The aim of the current study was to determine the MDR rate in *P. aeruginosa* isolated from cases of infections referred to hospitals in Torbat Heydarie, and to investigate the existence of efflux pump MexAB genes as a possible mechanism of resistance using PCR.

## Methods

**Sample collection.** This study was carried out over a period of one year. Totally, 506 different clinical, referred to Nohom Dey and Razi hospitals in Torbat Heydarie were processed for bacterial culture (Table 1). Patients' data such as gender and age were recorded for all cases. This study was in accordance with the guidelines in the Declaration of Helsinki for the ethical treatment of human subjects

***P. aeruginosa* isolation.** Specimens were inoculated on Nutrient agar (Merck, Germany) and incubated at 37°C for 24 hours. The bacterial colonies were examined the morphology, and discrete colonies sub-cultured to obtain pure cultures. Isolates were identified as *P. aeruginosa* using standard biochemical tests (13). *P. aeruginosa* PTCC 1394 was served as control strain in the experiments.

**Drug susceptibility testing.** Antibiotic resistance patterns of *P. aeruginosa* isolates were determined using disk diffusion method on Mueller-Hinton agar according to the CLSI guidelines (14,15). The following antibiotics were used in the assay: amikacin (30 $\mu$ g), ceftriaxone (30 $\mu$ g), ciprofloxacin (5 $\mu$ g), gentamicin (10 $\mu$ g), imipenem (10 $\mu$ g), norfloxacin (10 $\mu$ g), and tetracycline (30 $\mu$ g), (Mast, UK).

**DNA extraction and PCR.** A single colony, identified as *P. aeruginosa*, was inoculated on 5 mL of brain heart infusion broth and incubated over night at 37°C, then total genomic DNA extraction of isolates was performed by boiling method (16). To confirm the presence of MexA and MexB genes PCR amplification was performed with a set of primer pairs. The primers used to target the genes were MexA (F) CTCGACCCGATCTACGTC, MexA(R) GTCTTCACCTCGACACCC, MexB (F) TGTCGAAGTTTTTCATTGAG and MexB(R) AAGGTCACGGTGATGGT. These primers amplified a specific fragment length of 503bp for MexA gene and 280bp of MexB gene. PCR was carried out in a total volume of 20 $\mu$ L and consisted of 10 pM of genomic DNA, 10 pM of each primer, and 5 $\mu$ L of PCR Master Mix (Takapouzist, Iran). The amplification program was as follows: initial denaturation at 95°C (3m), then 35 cycles of denaturation at 94°C (1m), annealing at 58°C (1m), extension at 72°C (1m), and then a final extension at 72°C (7m). The amplified PCR products and 100-bp DNA ladder as the size marker (Kiagen, Iran) were separated by agarose gel electrophoresis on 1.5% agarose followed by staining with ethidium bromide (Qiagen, Germany). After electrophoresis results were visualized using the gel documentation system (Protein Simple, USA).

**Statistical analysis.** SPSS software (SPSS Inc no. 17) was used to analyze the relationship between the resistance to antibiotics and presence of genes.

## Results

**Bacterial isolates.** Five hundred and six swab samples were collected from blood (14%), tracheal (30%), wound (38%), and burn infections (18%). Of the 506 clinical cultures, 50 (9.88%) were positive for *P. aeruginosa*. The prevalence and total distribution of *P. aeruginosa* recovered from various types of infections is given in Table 1. The prevalence of *P. aeruginosa* in study population were at the highest levels in men (66%), patients between 30-50 years (56%) and specimens from cases with wound infections (38%). Significant differences ( $P < 0.05$ ) were found in the prevalence rates of *P. aeruginosa* between age groups, male and female cases and type of infections.

**Table 1.** Total distribution of *P. aeruginosa* in the swab samples taken from various types of infections

| Different Criteria       | No. of Samples | <i>P. aeruginosa</i> |
|--------------------------|----------------|----------------------|
| <b>Type of infection</b> |                |                      |
| Wound                    | 167            | 19                   |
| Burn                     | 93             | 9                    |
| Tracheal                 | 181            | 15                   |
| Blood                    | 65             | 7                    |
| <b>Gender</b>            |                |                      |
| Male                     | 257            | 33                   |
| Female                   | 249            | 17                   |
| <b>Age group, y</b>      |                |                      |
| < 10                     | 22             | 2                    |
| 10-30                    | 80             | 5                    |
| 30-50                    | 197            | 28                   |
| 50-70                    | 189            | 13                   |
| > 70                     | 18             | 2                    |
| <b>Total</b>             | <b>506</b>     | <b>50</b>            |

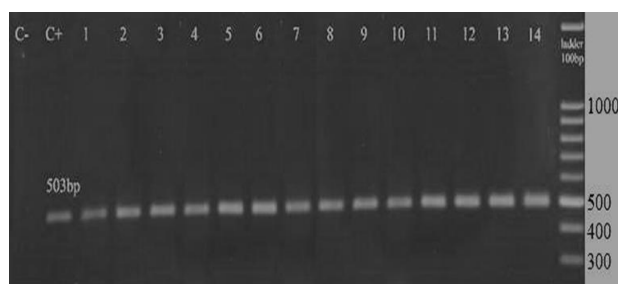
**Table 2.** Antibiotic resistance patterns of *P. aeruginosa* isolated from patients

| Antibiotic    | Sensitive (%) | Intermediate (%) | Resistant (%) |
|---------------|---------------|------------------|---------------|
| Amikacin      | 34.80         | 21.73            | 43.47         |
| Ceftriaxon    | 0             | 17.39            | 82.61         |
| Ciprofloxacin | 60.87         | 0                | 39.13         |
| Gentamicin    | 56.52         | 4.35             | 39.13         |
| Imipenem      | 60.87         | 4.35             | 34.78         |
| Norfloxacin   | 60.87         | 0                | 39.13         |
| Tetracycline  | 4.35          | 8.70             | 86.95         |

**Antibiotic resistance profile.** Table 2 shows the results of antibiotic resistance patterns of *P. aeruginosa* strains to seven selected antibiotics. Isolates showed the highest levels of resistance to tetracycline (86.95%) followed by ceftriaxon (82.61%), amikacin (43.47%), ciprofloxacin, norfloxacin, and gentamicin (39.13%). The lowest rate of resistance was against imipenem (34.78%). Only 4.34 % of the isolates were susceptible to seven antibiotics. According to criteria defined for MDR (17,18), 60.86% of *P. aeruginosa* isolates were determined as MDR, which exhibited resistance to four of seven antibiotics and 30.43% of isolates were showed resistance all seven antibiotics. The MDR strains were isolated from blood, tracheal and wound

infections. One strain was from a burn infection and showed no resistance to any antibiotics.

**Presence of MexA-MexB.** The presence of efflux pump MexA and MexB genes was found in 43 (86.95%) and 41 (82.60%) of isolates respectively. Only in 8 isolates lacked MexB gene. The results were correlated with high rates of resistance to tetracycline (> 80%), ceftriaxon (> 80%), amikacin (> 40%), and quinolones and aminoglycosides (> 35%), suggesting a possible role for MexAB genes. According to the PCR results, all MDR strains recognized by susceptibility techniques (60.86%), contained MexA and MexB genes (Figs. 1 & 2).

**Figure 1.** Results of PCR amplification of MexA gene (503 bp) of *Pseudomonas aeruginosa*, c-: negative control, c+: positive control, Ladder 100bp.**Figure 2.** Results of PCR amplification of MexB gene (280 bp) of *Pseudomonas aeruginosa*, c-: negative control, c+: positive control, Ladder 100bp.

## Discussion

*P. aeruginosa* is one of the important pathogens leading a broad-spectrum of nosocomial infections worldwide (8,18). In this study, 50 samples (9.88%) were positive for *P. aeruginosa* out of 506 samples collected from Nohom Dey and Razi hospitals in Torbat Heydarie city. Siguan et al. have been reported low prevalence of *P. aeruginosa* (18.8%) in human

infections previously (19). In other investigations, prevalence rate of *P. aeruginosa* has been shown 27.7% by Ranjan et al.(20), 32% by Bhattacharjee et al.(21), 27.78% by Masaadeh and Jaran (22) and 62.6% by Khosravi et al.(10), which were higher compared to our findings. The main cause of this disagreement is that the samples of current study were not entirely taken from burn wounds. Moreover, low prevalence rate of *P. aeruginosa* (9.88%) in the clinical samples of this study may be due to health care quality is different with those of other studies. The results of the present study showed that, the highest rates of isolates of *P. aeruginosa* were found in wounds (38%), followed by tracheal (30%), burns (18%), and blood (14%) infections. Javiya et al. reported the highest number of *P. aeruginosa* isolates from urine infections (23) and in another study by Manjunath et al., 37.17% of isolates were obtained from wound specimens which is similar to our results (13). *P. aeruginosa* remains a common cause of wound infection especially in burns wounds because burns have large exposed area of dead tissue and no defense against microbial infections (13). We reported a higher prevalence of *P. aeruginosa* in men than women, which is same as Al-Hasan et al.(24) and Khan et al.(25). High prevalence in women were also reported by Mulu et al.(26) and Okon et al.(27). Our results showed that the highest rates of isolation were in the age group of 30–50 years, that was similar to Manjunath et al. (13). In another investigation, Rajat et al. showed 29% isolation rate in the age group of 31–45 years (28) which is partly in line with our finding. Chander Anil and Raza also reported 20% isolation rate in age group of 21–40 years (29). This may be due to prolonged hospitalization, prolonged antibiotic therapy and decreased immunity. The results is contrast with a another report in which a high prevalence of *P. aeruginosa* in old patients (30), and in children was reported (31).

Prevalence of MDR bacteria and effectiveness of several antipseudomonal drugs make the use of antibiotics to treat infectious diseases a serious problem, especially for the human pathogen *P. aeruginosa* with its ability to adapt to antibiotics and emerging a multidrug-resistant phenotype (9). This study focused mainly on screening the antibiotic

resistance patterns of *P. aeruginosa* isolates and used anti-pseudomonal drugs. According to the disk diffusion method, we found high levels of resistance to all antibiotics with the prevalence of MDR among *P. aeruginosa* isolates from Nohom Dey and Razi hospital in Torbat Heydarie. The majority of the published studies, have reported that MDR isolates exhibited resistance against at least three types of antipseudomonas drugs from a variety of antibiotic classes (10). According to our results, 60.86% of *P. aeruginosa* isolates were defined as MDR, the most and least effective antibiotics against the bacteria were imipenem (60.87%) and ceftriaxon (0%) respectively. The rate of resistance of *P. aeruginosa* isolates against ceftriaxon, ciprofloxacin and norfloxacin were 82.61%, 39.13% and 39.13% respectively. Delpano et al. reported higher levels of antimicrobial resistance compared to the present study. Their results showed 100% resistance to gentamicin, meropenem, imipenem, and ciprofloxacin, and 83% - 94% to other tested antibiotics (10). In a study in Nepal, the rate of resistance against ceftriaxone and ciprofloxacin was 50%, and 100%; there was no resistance against gentamicin and ampicillin (32). Similar results were also reported from Pakistan (33) but, Krishnakumar et al. found 50% ampicillin resistance in India (34). Indian studies showed the prevalence of resistance against gentamicin among isolates as 63%. High levels of resistance to gentamicin have also been documented from Iran and Pakistan(27,35-37). These results are in contrast with a previous study by Ahmed et al. that found 43.9% gentamicin resistance in clinical samples in Egypt (38). According to these reports, our isolates were less resistant to gentamicin (39.13%). In the present study, amikacin resistance rate was estimated as 43.47% which is higher than other resistance results, reported in Russia (25%), USA (13.1%), France (9%) Spain (9%), and Turkey (4%) (39). High rate of resistance to tetracycline (100%) has also been reported from Egypt (38). In the current study, 86.95% of isolates were resistant to tetracycline. Many studies have focused on the resistance patterns of *P. aeruginosa* isolates to carbapenems, which are still as a therapeutic choice for nosocomial infection caused by *P. aeruginosa* (10). Resistance of *P. aeruginosa* to imipenem, the most effective antibiotic at Torbat Heidaryeh hospital (34.78%) is an emerging problem

in that area. Khosraviet al. reported prevalence of MDR isolates about 41% in Ahwaz with 38% of them being resistant to carbapenems (40), which is similar to our results.

Recent studies have shown that efflux pumps play a significant role in bacterial drug resistance (6,10,18). Since MexAB-OprM efflux pump is mediated in resistance to many important antibiotics, including fluoroquinolones, tetracyclines and B-lactams (9,10), we investigated the presence of encoding genes of this pump. Genotyping investigation was performed using PCR for the existence of efflux pump MexAB genes and showed that 43 *P. aeruginosa* isolates had the MexA gene and 41 isolates had MexB gene. Auda Al-Grawi et al. obtained similar results (39). Because of the possible role of this efflux pump in drug resistance, more than 40% of all tested isolates were estimated to be resistant to tetracyclin, ceftriaxon and amikacin. Similar to our findings, Khosravi et al. reported efflux MexAB genes were detected in all MDR *P. aeruginosa* strains (10). Expression of efflux pumps in MDR bacteria, that are capable of pumping out structurally different antimicrobial agents, contributes to reduced susceptibility against drugs.

In this work, we investigated the presence of only one of the multiple mechanisms of drug resistance and confirm the presence of the MexAB efflux genes in *P. aeruginosa* isolates, which cannot reveal the existence a functional MexAB efflux pump and its mediation in resistance. This is preliminary study to analyse the genetics of efflux pump-mediated drug resistance in *P. aeruginosa*. More investigations are required to prove whether these genes are functional and expressed. Though existence of the MexAB efflux genes is a possible reason for multi-drug resistance in this bacterium, it is necessary to use the efflux pump inhibitors to identify the association between presence of these genes and antibiotic resistance.

## Conclusion

Imprudent use of broad-spectrum antibiotics may cause high rates of resistance in *P. aeruginosa* strains in our investigation. The present study demonstrated an increasing rate of MDR *P. aeruginosa* in wounds, tracheal and blood samples obtained

from two university hospitals. A strategy should be designed and implemented to limit the MDR rate. Hence, the prudent use of antibiotics is needed. Also, because of the regional variation of drug resistance and susceptibility pattern in each hospital, it is important that each hospital using the local data on resistance pattern develops their own antimicrobial policy. In the present study, Efflux MexAB genes were detected in all MDR strains. Understanding the exact mechanisms involved in drug resistance can improve the strategies for the management and control of infection disease at the hospitals.

## Conflict of Interest

The authors declare no conflict of interests.

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