GENOTYPIC DETECTION OF SOME PSEUDOMONAS AERUGINOSA VIRULENCE GENES AMONG DIFFERENT CLINICAL ISOLATES

Iman Salah Naga and Shahad Abdulwahab Abdulrazzaq(D), Dalia Metwally Ragab (1)

(1) Department of Microbiology, Medical Research Institute, University of Alexandria
(2) Bachelor of clinical analysis, Almamoun University, Iraq

ABSTRACT

Background: Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting virtually all tissues via myriad of virulence factors and possess diversity of antibiotic resistance mechanisms. This aim of this study was molecular identification of genus and species level of P. aeruginosa by detection of oprI, oprL genes respectively by PCR. Determination of antibiotic resistance profile among isolates, as well as, detection of virulence genes toxA, plcH, plcN and LasB by PCR. Identification and antimicrobial susceptibility testing of fifty P. aeruginosa isolates obtained from various clinical specimens were performed on VITEK-2 Compact system. PCR was used for the molecular confirmation of genus and species level of P. aeruginosa by detection of oprI, oprL genes, as well as, the molecular detection of virulence genes toxA, plcH, plcN and LasB. The highest prevalence of resistance was detected against 3rd and 4th generation cephalosporins (80%). Resistance to other β-lactam antibiotics including piperacillin-tazobactam, monobactams, imipenem and meropenem was 62%, 70%, 64% and 62% respectively. Resistance to aminoglycoside antibiotics ranged from 44% to amikacin and 56% to tobramycin. High level of resistance (68%) to fluoroquinolones was detected. All isolates were susceptible to lipopolypeptide antibiotics, colistin and polymixin. Using multiplex PCR, all strains were confirmed molecularly as P. aeruginosa via detection of oprI, oprL genes in the 50 (100%) isolates, plcH and plcN were detected in all 50 (100%) of strains toxA in 46 (92%) and LasB in 50 (100%) of strains each in separate PCR reactions. This investigation is a comprehensive report of virulence factors and antibiotic resistance properties of P. aeruginosa isolated from Egyptian human clinical samples. Our results revealed that all tox A, LasB, plcH, plcN genes are predominant in human infections. Antibiotic resistance was high. Hence, judicious use of antibiotics is required by clinicians.

INTRODUCTION

Pseudomonas aeruginosa is a motile, non-fermenting, Gram-negative organism belonging to the family Pseudomonadaceae.1) It is an opportunistic pathogen capable of infecting virtually all tissues, and responsible for hospital acquired infections.2) Immunosuppressed patients, burn patients, mechanically ventilated patients, leukemic and cystic fibrosis patients are particularly susceptible to P. aeruginosa infections.3) The critical traits contributing to the pathogenic potential of P. aeruginosa are the production of a myriad of virulence factors, formation of biofilms and antibiotic resistance.4) These factors enable successful infection and colonization across a wide range of environments. This arsenal includes; elastase, phospholipase C (PLC)(5), alkaline protease, exotoxin A, exoenzyme S, pyocyanin, pyoverdin, hydrogen cyanide, as well as cell-associated factors, such as alginate, lipopolysaccharide, flagella, pili.6) Exotoxin A is the most toxic substance in P. aeruginosa, it catalyzes the ADP ribosylation of the eukaryotic elongation factor 2 (eEF-2), and thus significantly affects the protein synthesis of the host cells.7)

LasB elastase, a zinc metalloprotease encoded by the lasB gene, has an elastolytic activity on lung tissue.8) In addition, the phospholipids contained in pulmonary surfactants may be hydrolysed by PLC-H and PLC-N, two phospholipases C encoded by plcH and plcN respectively.9) The outer membrane proteins of P. aeruginosa, OprI and OprL, play important roles in the interaction of the bacterium with the environment as well as the inherent resistance of P. aeruginosa to antibiotics.10) As these proteins are found only in this organism, they could be a reliable factor for rapid molecular identification of P. aeruginosa in clinical samples.12) Although conventional microbiological methods for identifying P. aeruginosa from clinical samples are reliable,
they require several days to be completed. Rapid detection of isolates is very important for consequent treatment decision of patients. PCR has the potential for identifying microbial species rapidly by amplification of sequences unique to a particular organism. (60)

Therefore, this study aimed for molecular identification of genus and species level of P. aeruginosa by detection of oprI, oprL genes respectively by PCR. Determination of antibiotic resistance profile among isolates, as well as, detection of virulence genes toxA, plcH, plcN and LasB by PCR.

SUBJECTS AND METHODS:

A total of fifty P. aeruginosa isolates were obtained from various clinical specimens after the approval of ethical committee of the Medical Research Institute, Alexandria University.

Bacterial isolation, identification and storage:
All specimens were cultured routinely on blood and MacConkey’s agar. After overnight incubation, Gram negative, non-lactose fermenting oxidase positive colonies were further identified using VITEK-2 Compact system (GN-13 card) (bioMerieux, France). Bacterial isolates were stored in Luria Bertani (13) broth containing 30% glycerol and tubes stored at -80°C. For bacterial revival, one loopful was streaked over blood agar and incubated at 37°C.

Antimicrobial Susceptibility testing of P. aeruginosa isolates:
Antimicrobial susceptibility testing was performed on VITEK-2 Compact system (bioMerieux, France) according to manufacturer’s instructions. The AST-NO21 cards and software were used for analysis.

Polymerase chain reaction (PCR):
PCR was used for the molecular confirmation of genus and species level of P. aeruginosa by detection of oprI, oprL genes, as well as, the molecular detection of virulence genes toxA, plcH, plcN and LasB.

DNA extraction was performed by boiling method. (14) Briefly, 2 to 3 colonies from overnight cultured were suspended in TE buffer containing 0.1% triton X100. Bacterial suspensions were incubated in a boiling water bath for 15 minutes followed by rapid cooling on ice. After centrifugation for 15 minutes at 14,000 rpm in a microfuge, the supernatant was used as a source for bacterial DNA.

PCR was performed in 25 μl final volume containing 12.5 μl hot start PCR master mix MyTaqTM HS Red Mix (BioLine, London, UK), 10 pmole of each primer (all primers were purchased from Thermo Fisher Scientific, California., USA) (Table 1) and 0.5 μl bacterial DNA. A negative control was prepared by the addition of the same contents to the tube with water instead of the extract. All PCR reactions were performed on Veriti thermal cycler (Applied Biosystems, California, USA). oprI/oprL and plcH/plcN were detected in 2 multiplex PCR reactions, while toxA and lasB were detected using singleplex PCR.

The reactions were performed according to the following thermal profile, initial denaturation 95°C for 3 minutes followed by 40 cycles of 95°C for 30 seconds, annealing for 15 second and extension at 72°C for 1 min/kb followed by final extension at 72°C for 5 minutes. PCR products were separated by gel electrophoresis on 2% agarose gel containing 0.5 μg/ml ethidium bromide.

RESULTS:
The present study included 50 P. aeruginosa isolates obtained from various clinical specimens. The strains were isolated from 27 (54%) females and 23 (46%) males. Twenty (40%) isolates were from respiratory tract infections, 15 (30%) from wound infections, 8 (16%) from bloodstream infections, and 7 (14%) from urine samples.

Molecular diagnosis of P. aeruginosa:
Using multiplex PCR, all strains included in the current study were confirmed molecularly as P. aeruginosa via detection of oprI, oprL genes in the 50 (100%) isolates.

Molecular detection of P. aeruginosa virulence genes:
Using multiplex PCR, plcH and plcN were detected in all 50 (100%) of strains and toxA in 46 (92%) and LasB in 50 (100%) of strains each in separate PCR reactions. ToxA gene was not detected in 2 samples from bloodstream infections, 1 from respiratory tract infection and 1 from wound infection.

Table (2) shows the antimicrobial susceptibility testing of the 50 P. aeruginosa isolates included in this study. The highest prevalence of resistance was detected against 3rd and 4th generation cephalosporins (80%). Resistance to other β-lactam antibiotics including piperacillin-tazobactam, monobactams, imipenem and meropenem was 62%, 70%, 64% and 62% respectively. Resistance to aminoglycoside antibiotics ranged from 44% to amikacin and 56% to tobramycin. High level of resistance (68%) to florosquinolones was detected. All isolates were susceptible to lipopolypeptide antibiotics, colistin and polymixin.

Table (3) shows the distribution of antimicrobial resistance among clinical samples. Resistance showed lower prevalence in urinary tract infections in comparison to other sites of infection. Additionally, resistance to 3rd and 4th generation cephalosporins recoded the highest prevalence among all infection sites.
Table (1): Primers used for the PCR reactions

| Primer | Nucleotide Sequence | Annealing temperature °C | Amplicon size (bp) | Reference |
|--------|---------------------|---------------------------|--------------------|-----------|
| oprI- F | ATGAACAAACGTTCTGAAATTCTCTGCT | 55 | 249 | (15) |
| oprI-R | CTTGCGGCTGGCTTTTTCAG | | | |
| oprL- F | ATGGAAATGCTGAAATTCGG | 55 | 504 | (16) |
| oprL-R | CTTTCTCAGCTGACGCGACG | | | |
| plcH- F | GAAGCCATGGGCTACTTCA | 55 | 307 | |
| plcH-R | AGAGTGACGAGGAGCGGTAG | | | |
| plcN- F | GTTATCGCAACCACGCCCTAC | 55 | 466 | (17) |
| plcN-R | AGGTCGAACACCTGGAACAC | | | |
| toxA- F | GGTAACCAGCTCAGCCACAT | 56 | 352 | |
| toxA-R | TGATGTCCAGGTCATGCTTC | | | |
| lasB- F | GGAATGAAACGAAGCGTTCTC | 55 | 300 | |
| lasB-R | GGTCCAGTATGATCGCGTTTG | | | |

Table (3): Antimicrobial susceptibility testing of the 50 P.aeruginosa isolates

| Antimicrobial agent                   | Sensitive | Intermediate | Resistant |
|--------------------------------------|-----------|--------------|-----------|
|                                      | No        | %            | No        | %            | No        | %            |
| Piperacillin-Tazobactam              | 15        | 30           | 4         | 8            | 31        | 62           |
| Ceftazidime                          | 8         | 16           | 2         | 4            | 40        | 80           |
| Cefpime                              | 9         | 18           | 1         | 2            | 40        | 80           |
| Aztreonam                            | 11        | 22           | 4         | 8            | 35        | 70           |
| Imipenem                             | 18        | 36           | 0         | 0            | 32        | 64           |
| Meropenem                            | 19        | 38           | 0         | 0            | 31        | 62           |
| Gentamicin                           | 22        | 44           | 2         | 4            | 26        | 52           |
| Tobramycin                           | 21        | 42           | 1         | 2            | 28        | 56           |
| Amikacin                             | 27        | 54           | 1         | 2            | 22        | 44           |
| Ciprofloxacin                        | 16        | 32           | 0         | 0            | 34        | 68           |
| Levofloxacin                         | 16        | 32           | 0         | 0            | 34        | 68           |
| Colistin                             | 50        | 100          | 0         | 0            | 0         | 0            |
| Polymixin                            | 50        | 100          | 0         | 0            | 0         | 0            |

Table (3): Distribution of antimicrobial resistance among clinical samples

| Antimicrobial agent                   | Respiratory tract | Wound swab | Blood | Urine | Total |
|--------------------------------------|-------------------|------------|-------|-------|-------|
|                                      | N=20   | %          | N= 15 | %     | N= 8  | %     | N=7  | %     | N= 50 | %     |
| Piperacillin-Tazobactam              | 15     | 75         | 7     | 47    | 6     | 75    | 3     | 43    | 31    | 62    |
| Ceftazidime                          | 18     | 90         | 10    | 68    | 7     | 87.5  | 5     | 71    | 40    | 80    |
| Cefpime                              | 18     | 90         | 10    | 68    | 7     | 87.5  | 5     | 71    | 40    | 80    |
| Aztreonam                            | 16     | 80         | 10    | 68    | 5     | 87.5  | 4     | 57    | 35    | 70    |
| Imipenem                             | 15     | 75         | 8     | 53    | 6     | 75    | 3     | 43    | 32    | 64    |
| Meropenem                            | 15     | 75         | 8     | 53    | 5     | 62.5  | 3     | 43    | 31    | 62    |
| Gentamicin                           | 13     | 65         | 6     | 40    | 4     | 50    | 3     | 43    | 26    | 52    |
| Tobramycin                           | 14     | 70         | 6     | 40    | 5     | 62.5  | 3     | 43    | 28    | 56    |
| Amikacin                             | 11     | 55         | 6     | 40    | 3     | 37.5  | 2     | 21    | 22    | 44    |
| Ciprofloxacin                        | 16     | 80         | 9     | 60    | 6     | 75    | 3     | 43    | 34    | 68    |
| Levofloxacin                         | 16     | 80         | 9     | 60    | 6     | 75    | 3     | 43    | 34    | 68    |
| Colistin                             | 0      | 0          | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
| Polymixin                            | 0      | 0          | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
**DISCUSSION:**

*P. aeruginosa* as an opportunistic pathogen has different virulence factors which aid the bacteria to colonize different niches in their host and the bacteria are a leading cause of nosocomial and community-acquired infections worldwide. (19) At present, culturing bacteria remains the most commonly applied method for detecting *P.*
aeruginosa, but this method is time-consuming and susceptible to inconsistent results due to sample contamination. In addition, this method has low sensitivity. Because of the importance of early diagnosis and detection, researchers have developed various assays, each with their own advantages and disadvantages. The developmental trend shows that direct detection of P. aeruginosa in clinical samples will shorten diagnostic turnaround time and reduce the risk of contamination.\(^{(20)}\)

Identification of P. aeruginosa has traditionally relied on phenotypic methods. This still is the most accurate standard when dealing with typical isolates of P. aeruginosa. Moreover, biochemical testing takes long time to perform and requires extensive hands-on work by the technologist, both for setup and for ongoing evaluation. Molecular methods have been reported to be superior to the phenotypic methods for identification of P. aeruginosa\(^{(12, 21)}\) by designing a multiplex PCR assay based on oprI and oprL genes for molecular detection of P. aeruginosa showed that the specificity and sensitivity of the PCR assay were 74 and 100\%, respectively. The main advantage of multiplex PCR is its ability to simultaneously amplify multiple PCR products in a single reaction, thereby enabling multiplex detection and significantly reducing the detection cost and time requirements\(^{(22)}\). Lavenir et al\(^{(23)}\) also noted that all of P. aeruginosa strains contained the oprI and oprL genes. Similarly El-Shehstawi et al\(^{(24)}\) in their study reported that all of the 30 isolates were remarkably positive for both oprI and oprL genes. This is consistent with this study result as both genes oprI, oprL were detected in all 50 (100\%) of samples.

P. aeruginosa is capable of inducing different types of infections in humans. In the present study, twenty (40\%) P. aeruginosa isolates were obtained from respiratory tract infections, 15 (30\%) from wound infections, 8 (16\%) from bloodstream infections, and 7 (14\%) from urine tract infections.

Viedma et al\(^{(25)}\) in their study showed that out of 183 patients, 36 (19.7\%) suffered from lower respiratory tract infection, 30 (16.4\%) patients with urinary tract infection, 28 (15.3\%) patients with bloodstream infection and 22 (12\%) patients with intra-abdominal infection. The high incidences of P. aeruginosa in respiratory infections have been previously reported in cystic fibrosis patients.\(^{(26-28)}\)

The treatment of P. aeruginosa infections continues to be a significant challenge. The antibiotic resistance in P. aeruginosa is multifactorial in that it can occur through innate, acquired or adaptive mechanisms. The diversity of antibiotic resistance mechanisms contributes to the development of multidrug-resistant strains and makes conventional antibiotics ineffective for the treatment of infections by P. aeruginosa.\(^{(29)}\)

In this study, antimicrobial resistance showed lower prevalence in urinary tract infections in comparison to respiratory, bloodstream and wound infections which may be due to community-acquired origin of infection. Additionally, resistance to 3\textsuperscript{rd} and 4\textsuperscript{th} generation cephalosporins recorded the highest prevalence among all infection sites.

Fazeli et al\(^{(30)}\) in 2014 stated that their bacterial isolates were multidrug resistant. Resistance to gentamicin and ciprofloxacin was reported in 39.21\% and 21.56\% of their bacterial strains isolated from hospital infections. In another study by Fazeli et al\(^{(31)}\) including hospital acquired infections, hospital means and personnel’s specimen, they showed that 29\% and 32.2\% of the P. aeruginosa strains were resistant to ciprofloxacin and gentamicin, which was much lower than our results. Lower resistance rates were reported in Latin America 26.8\%\(^{(32)}\) and 10\%-32\% in Europe\(^{(33, 34)}\).

In a study of Viedma et al\(^{(25)}\), which was conducted from 2007 to 2010, all the isolates were only susceptible to colistin (100\%) and amikacin (75\%). In our study colistin was also sensitive in all 100\% of isolates.

The antibiotic resistant pattern found by Akingbade et al\(^{(35)}\) investigation showed that P. aeruginosa, had low resistant to ceftazidime (20\%), gentamicin (26.4\%), levofloxacin (30.9\%), ceftriaxone (34.5\%), and ciprofloxacin (35.5\%), which was also much lower than our results. Similar results have been reported by Lim et al\(^{(36)}\) from Malaysia and Smith et al\(^{(37)}\) from Nigeria.

Pathogenicity of P. aeruginosa is clearly multifactorial. LasB is one of the most important proteases of P. aeruginosa.\(^{(38)}\) Probably in the early stages of infection, LasB causes a direct damage to the lung. Moreover, bleeding is often seen in the lungs of patients with pulmonary infections by P. aeruginosa due to the breakdown of elastin in the walls of the blood vessels.\(^{(39)}\) In this study, lasB gene was detected in all isolates. Nikbin et al\(^{(40)}\) in their study stated that all isolates examined harbored lasB gene. This finding is in agreement with previous reports by Nicas et al\(^{(41)}\) and Lomholt et al\(^{(38)}\). Mutation of lasB gene reduces markedly P. aeruginosa invasion. Prevalence of the lasB gene in all clinical isolates implies the importance of LasB factor to survival of P. aeruginosa in various conditions.\(^{(42)}\)

Toxin A has distinct role in hindrance of wound contraction and remodeling.\(^{(43)}\) Hummel and Unger\(^{(44)}\) established the first PCR method that specifically detected P. aeruginosa based on the toxin A gene and evaluated the effectiveness of this method for rapid P. aeruginosa detection in mechanically ventilated patients. The results showed that the PCR method targeting toxin A gene detected 57 positive samples out of 364 total samples, whereas the conventional culture method only detected 36 positive samples, indicating that the toxin A gene-based PCR method had higher sensitivity. Using PCR, we detected toxinA gene in 100\% of our isolates.

Phospholipase C plays a role in the lysis of target cells (pulmonary atelectasis) and is involved in acute and
chronic infection.\(^{45}\) Most of the 151 isolates in a study performed by Elogne et al.\(^{40}\) detected the virulence genes; \(pplcH\) in 84.1% and \(lasB\) in 72.8%. The \(pplcH\) gene was found in all types of infections with prevalence ranging from 66.7% to 84.6%. The highest prevalence was found in sepsis with 84.6% followed by lung infections with 81.5% and then urinary tract infections in 78.8%. This frequency at the urinary level is close to that of Heidary et al.\(^{70.4}\%\) in 2016 in Iran.\(^{46}\) Elastase causes the destruction of tissues containing elastin, tissue necrosis and haemorrhage.\(^{10}\) The \(lasB\) gene was found in isolates from all infectious sites with variable rates. Prevalence were high and ranged from 57.1% to 81.5%. The highest rate was found at the pulmonary level (81.5%). These results are close to those of Mitov I. et al.\(^{47}\) with \(pplcH\) in (91.6) and \(lasB\) in (100%).\(^{47}\) Similarly, in this study \(pplcH\) and \(lasB\) were detected in all 100% of isolates.

On the other hand, the PCR results of virulence factor in the study by Al-Dahmoshi et al.\(^{48}\) showed that \(exoA\) was present only among 12(46.15%), \(oprL\) was 11(42.3%), \(oprI\) was 22(84.61%) and \(lasB\) was 18(69.23%).

\(ExoA\) responsible for toxigenesis trait of \(P.\ aeruginosa\) while invasiveness achieved by \(LasB\) and so the coexistence of \(ExoA\) and \(LasB\) let both of mechanism of infection available and increase the degree of wound worseness.\(^{49-52}\) Coexistence of more than one virulence factor within the same isolate were recorded and the results displayed that 8/26 have all five virulence factors, 4/26 have four virulence factors, 1/26 have three virulence factors and 5/26 have only two virulence factors\(^{48}\). In this study, 46/50 samples had 4 virulence factors and only 4/50 had only 3 virulence factors, namely, \(pplcH\), \(plcN\) and \(lasB\).

Nikbin et al. in 2012 stated that all isolates carried \(oprI\), \(oprL\) and \(lasB\) genes.\(^{40}\) The presence of \(ExoA\), \(OprL\), \(OprI\), \(LasI\) and \(LasB\) among \(P.\ aeruginosa\) isolates suppose their linking with different levels of intrinsic virulence and pathogenicity.\(^{48, 53}\)

In summary, the study provides an insight into the phenotypic and genotypic characteristics of \(P.\ aeruginosa\) emerged in Egypt. Our finding highlighted a high rate of resistance to antibiotics. In order to reduce the risk of spread of highly resistant strains, we suggest the need to establish a periodic surveillance system, to enhance infection prevention and control measures in healthcare setting, and to increase awareness among physicians and the general public about the rational use of antibiotics. Future work should target mechanisms of resistance and genetic diversity of \(P.\ aeruginosa\) strains to draw more far-reaching conclusions.

Further studies will be helpful in revealing the correlation between the drug resistance pattern and virulence factor expression.

This investigation is a comprehensive report of virulence factors and antibiotic resistance properties of \(P.\ aeruginosa\) isolated from Egyptian human clinical samples. Our results revealed that all \(tox\ A\), \(lasB\), \(pplcH\), \(plcN\) genes are predominant in human infections.

REFERENCES:

1. Lyczak JB, Cannon CL, Pier GB. Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. Microbes Infect. 2000; 2(9):1051-60.
2. Van Delden C, Iglewski BH. Cell-to-cell signaling and Pseudomonas aeruginosa infections. Emerg Infect Dis. 1998; (4):551-60.
3. Fegan M, Francis P, Hayward AC, Davis GH, Fuerst JA. Phenotypic conversion of Pseudomonas aeruginosa in cystic fibrosis. J Clin Microbiol. 1990;28(6):1143-6.
4. Balasubramanian D, Schneper L, Kumari H, Mathiee K. A dynamic and intricate regulatory network determines Pseudomonas aeruginosa virulence. Nucleic Acids Res. 2013;41(1):1-20.
5. Newman JW, Floyd RV, Fothergill JL. The contribution of Pseudomonas aeruginosa virulence factors and host factors in the establishment of urinary tract infections. FEMS Microbiol Lett. 2017; 364(15).
6. Wagner V, Filiatrault M, Ricardo K, Iglewski B. Pseudomonas aeruginosa virulence and pathogenesis issues. Pseudomonas; genomics and molecular biology/ edited by Pierre Cornelis. 2008.
7. Rawat S, B P. Prevalence and characterization of virulence properties of pseudomonas aeruginosa from clinical samples and hospital environment in dehradun. Int J Biol Pharm Res. 2015;6:491-9.
8. Wolf P, Elsasser-Beile U. Pseudomonas exotoxin A: from virulence factor to anti-cancer agent. Int J Med Microbiol. 2009; 299(3):161-76.
9. Benie CK, Dadie A, Guessend N, N’Gessou-Koudio NA, Kouame ND, N’Golo D C, et al. Characterization of Virulence Potential of Pseudomonas Aeruginosa Isolated from Bovine Meat, Fresh Fish, and Smoked Fish. Eur J Microbiol Immunol (Bp). 2017;7(1):55-64.
10. Eloigne CK, N’Guetta AKA, Yeo A, David CNg, Guessend N, Anné JC, et al. Prevalence of Pseudomonas aeruginosa’s Virulence Genes Isolated from Human Infection in Abidjan, Côte d’Ivoire. Microbiology Research Journal International, 2018; 25(1):1-8.
11. Nikaido H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science. 1994; 264(5157):382-8.
12. De Vos D, Lim A, Jr., Pirnay JP, Stuelens M, Vandenvelde C, Duimslaguer L, et al. Direct detection and identification of Pseudomonas aeruginosa in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, oprl and oprL. J Clin Microbiol. 1997; 35(6):1295-9.
13. Japoni A, Alborzi A, Kalani M, Nasiri J, Hayati M, Farshad S. Susceptibility patterns and cross-resistance of antibiotics against Pseudomonas aeruginosa isolated from burn patients in the South of Iran. Burns. 2006; 32(3):343-7.
14. Yang JL, Wang MS, Cheng AC, Pan KC, Li CF, Deng SX. A simple and rapid method for extracting bacterial DNA from intestinal microflora for ERIC-PCR detection. World J Gastroenterol. 2008; 14(18):2872-6.

15. De Vos D, Lim A, Jr, De Vos P, Sarniguet A, Kersters K, Cornelis P. Detection of the outer membrane lipoprotein I and its gene in fluorescent and non-fluorescent pseudomonads: implications for taxonomy and diagnosis. J Gen Microbiol. 1993; 139(9):2215-23.

16. Lim A, Jr, De Vos D, Brauns M, Mossialos D, Gaballa A, Qing D, et al. Molecular and immunological characterization of OprL, the 18 kDa outer-membrane peptidoglcan-associated lipoprotein (PAL) of Pseudomonas aeruginosa. Microbiology. 1997; 143 (Pt 5):1709-16.

17. Stover CK, Pham QX, Erwin AL, Mizoguchi SD, Warrench P, Hickey MJ, et al. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature. 2000; 406(6799):959-64.

18. Kawaguchi T, Ide T, Koga H, Kondo R, Miyajima I, Arinaga-Hino T, et al. Rapidly growing hepatocellular carcinoma after direct-acting antiviral treatment of chronic hepatitis C.Clin J Gastroenterol.2018;11(1):69-74.

19. de Bentzmann S, Pleiati P. The Pseudomonas aeruginosa opportunistic pathogenesis and human infections. Environ Microbiol. 2011; 13(7):1655-65.

20. Tang Y, Ali Z, Zou J, Jin G, Zhu J, Yang J, et al. Detection methods for Pseudomonas aeruginosa: history and future perspective. RSC Advances. 2017;7 (82): 51789-800.

21. Qin X, Emerson J, Stapp J, Stapp L, Abe P, Burns JL. Use of real-time PCR with multiple targets to identify Pseudomonas aeruginosa and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. J Clin Microbiol. 2003;41(9):4312-7.

22. Mahony JB, Blackhouse G, Babwah J, Smieja M, Buracool S, Chong S, et al. Cost analysis of multiplex PCR testing for diagnosing respiratory virus infections. J Clin Microbiol. 2009;47(9):2812-7.

23. Lavenir R, Jocktane D, Laurent F, Nazaret S, Cournoyer B. Improved reliability of Pseudomonas aeruginosa PCR detection by the use of the species-specific ecfX gene target. J Microbiol Methods. 2007; 70(1):20-9.

24. El-Sheshawy NM, Khattab MA, Nour MS. Genetic Identification of Pseudomonas aeruginosa Virulence Genes among Different Isolates. J Micro Biochem Technol. 2015; 7(5).

25. Viedma E, Juan C, Villa J, Barrado L, Orellana MA, Sanz F, et al. VIM-2-producing multidrug-resistant Pseudomonas aeruginosa ST175 clone, Spain. Emerg Infect Dis. 2012;18(8):1235-41.

26. West SEH, Zeng L, Lee BL, Kosorok MR, Laxova A, Rock MJ, et al. Respiratory Infections With Pseudomonas aeruginosa in Children With Cystic FibrosisEarly Detection by Serology and Assessment of Risk Factors. JAMA. 2002; 287(22):2958-67.

27. Martinez-Solano L, Macia MD, Fajardo A, Oliver A, Martinez JL. Chronic Pseudomonas aeruginosa infection in chronic obstructive pulmonary disease. Clin Infect Dis. 2008; 47(12):1526-33.

28. Tingvatn P, Smith L, Rose B, Zhu H, Conibear T, Al Nassaf K, et al. Phenotypic characterization of clonal and nonclonal Pseudomonas aeruginosa strains isolated from lungs of adults with cystic fibrosis. J Clin Microbiol. 2007;45(6):1697-704.

29. Pang Z, Raudonis R, Glick BR, Lin TJ, Cheng Z. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. Biotechnol Adv. 2019; 37(1):177-92.

30. Fazeli N, Montaz H. Virulence Gene Profiles of Multidrug-Resistant Pseudomonas aeruginosa Isolated From Iranian Hospital Infections. Iran Red Crescent Med J. 2014;16 (10):e15722.

31. Fazeli H, Akbari R, Moghim S, Narimani T, Arabestani MR, Ghoddousi AR. Pseudomonas aeruginosa infections in patients, hospital means, and personnel's specimens. J Res Med Sci. 2012; 17(4):332-7.

32. Brown PD, Izundu A. Antibiotic resistance in clinical isolates of Pseudomonas aeruginosa in Jamaica. Rev Panam Salud Publica. 2004; 16(2):125-30.

33. Bonfiglio G, Carciotto V, Russo G, Stefani S, Schito GC, Debbia E, et al. Antibiotic resistance in Pseudomonas aeruginosa: an Italian survey. J Antimicrob Chemother. 1998; 41(2):307-10.

34. Bouza E, Garcia-Garrote F, Cercenado E, Marin M, Diaz MS. Pseudomonas aeruginosa: a survey of resistance in 136 hospitals in Spain. The Spanish Pseudomonas aeruginosa Study Group. Antimicrob Agents Chemother. 1999; 43(4):981-2.

35. Akingbade O BS, Ojo D, Afolabi R, Motayo B, Okerentugba P, et al. Plasmid profile analysis of multidrug resistant Pseudomonas aeruginosa isolated from wound infections in South West, Nigeria. orb Appl Sci J. 2012;20(6):766-75.

36. Lim KT, Yasin RM, Yeo CC, Puthucheary SD, Balan G, Maning N, et al. Genetic fingerprinting and antimicrobial susceptibility profiles of Pseudomonas aeruginosa hospital isolates in Malaysia. J Microbiol Immunol Infect. 2009;42(3):197-209.

37. Smith S, Ganiyu O, John R, Fowora M, Akinsinde K, Odeigah P. Antimicrobial resistance and molecular typing of pseudomonas aeruginosa isolated from surgical wounds in Lagos, Nigeria. Acta Med Iran. 2012; 50 (6):433-8.

38. Lomholt JAP, Poulsen K, Kiliian M. Epidemic population structure of Pseudomonas aeruginosa: evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. Infect Immun. 2001; 69(10):6284-95.

39. N. Kapur KG, I.B. Masters, P.S. Morris, A.B. Chang. Lower airway microbiology and cellularity in children with newly diagnosed non-CF bronchiectasis. Pediatr Pulmonol. 2011;47(3):300e307.

40. Nikkin VS, Aslani MM, Sharazi F, Hashemipour M, Shahcheraghi F, Ebrahimipour GH. Molecular identification and detection of virulence genes among Pseudomonas aeruginosa isolated from different infectious origins. Iran J Microbiol. 2012; 4(3):118-23.

41. Nicas TI, Iglewski BH. Production of elastase and other exoproducts by environmental isolates of Pseudomonas aeruginosa. J Clin Microbiol. 1986;23(5):967-9.

42. Cowell BA, Twining SS, Hobden JA, Kwong MS, Fleiszg SM. Mutation of lasA and lasB reduces Pseudomonas aeruginosa infection of epithelial cells. Microbiology. 2003; 149(Pt 8):2291-9.

43. El-Din AB, El-Nagdy, M.A., Badr, R. and ELSabagh, A.M. Pseudomonas aeruginosa exotoxin A: its role in burn wound infection and wound healing. Egypt J Plast Reconstr Surg. 2008; 32:59-65.

44. Hummel A, Unger G. Detection of Pseudomonas aeruginosa in bronchial and tracheal aspirates by PCR by amplification of the exotoxin A gene. Zentralbl Hyg Umweltmed. 1998; 201(4-5):349-55.
45. Ben Haj Khalifa A, Moissenet D, Vu Thien H, Khedher M. [Virulence factors in Pseudomonas aeruginosa: mechanisms and modes of regulation]. Ann Biol Clin (Paris). 2011; 69(4):393-403.
46. Heidary Z, Bandani E, Eltekhary M, Jafari AA. Virulence Genes Profile of Multidrug Resistant Pseudomonas aeruginosa Isolated from Iranian Children with UTIs. Acta Med Iran. 2016; 54(3):201-10.
47. Mitov I, Strateva T, Markova B. Prevalence of virulence genes among bulgarian nosocomial and cystic fibrosis isolates of pseudomonas aeruginosa. Braz J Microbiol. 2010;41(3):588-95.
48. Al-Dahmoshi H. O. M, Al-Khafaji N. S, Jeyad A. A, Shareef H. K. F, A-JR. Molecular Detection of Some Virulence Traits Among Pseudomonas aeruginosa Isolates, Hilla-Iraq. Biomed Pharmacol J. 2018;11(2).
49. Tumbarello M, De Pascale G, Trecarichi EM, Spanu T, Antonicelli F, Maviglia R, et al. Clinical outcomes of Pseudomonas aeruginosa pneumonia in intensive care unit patients. Intensive Care Med. 2013; 39(4):682-92.
50. Al-Wrafy F, Brzozowska E, Gorska S, Gamian A. Pathogenic factors of Pseudomonas aeruginosa - the role of biofilm in pathogenicity and as a target for phage therapy. Postepy Hig Med Dosw (Online).2017;71(0):78-91.
51. Chatterjee M, Anju CP, Biswas L, Anil Kumar V, Gopi Mohan C, Biswas R. Antibiotic resistance in Pseudomonas aeruginosa and alternative therapeutic options. Int J Med Microbiol. 2016; 306(1):48-58.
52. Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM. Bacteriophage cocktail for the prevention of biofilm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. Antimicrob Agents Chemother. 2010; 54(1):397-404.
53. Wessel AK, Liew J, Kwon T, Marcotte EM, Whiteley M. Role of Pseudomonas aeruginosa peptidoglycan-associated outer membrane proteins in vesicle formation. J Bacteriol. 2013; 195(2):213-9.