Original Article

TIPS Trends in Pharmaceutical Sciences 2022: 8(1): 51-56.

PCR based identification of exotoxin A-producing *Pseudomonas aeroginosa* isolated from burn wound infection

Gholamreza Morshedi[1], Vahid Ghanbarinejad[2], Mina Zarei[2], Abazar Pournajaf[3], Hossein Hassanian-Moghaddam[4,5], Vahid Hadi[6],Abbas Isvand[7], Mohammad Reza Saadati[7], Homayoun Maleki[8], Majid Keshavarzi[9*]

[1]Department of Pharmacology and Toxicology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran.
[2]Department of Pharmacology and Toxicology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.
[3]Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran.
[4]Social Determinants of Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[5]Department of Clinical Toxicology, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[6]Department of Biochemistry and Nutrition, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran.
[7]Department of Food Quality Control Laboratory, Army Health Center of Excellence (NEZAJA), Tehran, Iran.
[8]Department of Education and Research, Army Health Center of Excellence (NEZAJA), Tehran, Iran.
[9]Department of Environmental Health Engineering, School of Public Health, Sabzevar University of Medical Sciences, Sabzevar, Iran.

Abstract

Exotoxin A (*exoA*) is a major pathogenic factor in *Pseudomonas aeruginosa*. This non-fermenting gram-negative bacillus causes a wide range of infections. *P. aeruginosa* is a major cause of hospital-acquired infections. With this view, this study was aimed to identify *P. aeruginosa* strains carrying the *exoA* gene isolated from burn wounds. As part of the cross-sectional study, 110 samples of burns were collected in several hospitals. After identification of bacterial strains by biochemical and microbiological tests, genomic DNA was extracted. PCR reaction was performed for screening for *exoA* gene. After examining DNA extracted using quantitative (Nano drop with $OD_{260/280}$=1.8-2nm) and qualitative (electrophoresis on the 0.7% gel agarose) methods, 75.4% (n; 83 of 110) isolates carried the *exoA* gene. The high prevalence of the *exoA* gene in *P. aeruginosa* strains indicate the important role of this exoenzyme in the spread of infection in burn patients. Therefore, it is recommended that people with burn infections be screened for the *exoA* gene.

Keywords: Exotoxin A, *Pseudomonas aeruginosa*, Burn Wound.

Please cite this article as: Gholamreza Morshedi, Vahid Ghanbarinejad, Mina Zarei, Abazar Pournajaf, Hossein Hassanian-Moghaddam, Vahid Hadi,Abbas Isvand, Mohammad Reza Saadati, Homayoun Maleki, Majid Keshavarzi. PCR based identification of exotoxin A-producing *Pseudomonas aeroginosa* isolated from burn wound infection. Trends in Pharmaceutical Sciences. 2022;8(1):51-56 .doi: 10.30476/TIPS.2021.93218.1121

Corresponding Author: Majid Keshavarzi, Department of Environmental Health Engineering, School of Public Health, Sabzevar University of Medical Sciences, Sabzevar, Iran.
Email: keshavarzi_Majid@yahoo.com

Received: 08/12/2021; Accepted: 01/03/2022

1. Introduction

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen that is widely distributed throughout the environment (1). It is a ubiquitous organism, especially in environments such as soil, food, water, and hospital settings (2). *P. aeruginosa* is the second most common pathogen in surgery and the third most frequent cause of hospital infections after *Escherichia coli* and *Staphylococcus aureus*, which represent approximately 10% of hospital infections (3).

P. aeruginosa is a typical hospital-acquired causative agent of respiratory and urinary tract infections, dermatitis, bacteremia, soft tissue infections, bony and articular infections, gastrointestinal and systemic infections, predominantly in patients with intense burns, bed ulcers, and in patients with cancer or AIDS who are immunosuppressed (4, 5). Burn sores are local cell-mediated reactions (6, 7). A variety of cellular and extracellular virulence factors, such as exotoxin A, flagella and elastases, are implicated in the pathogenesis of *P. aeruginosa*. Also, cell damage to the skin can reverse humoral pyocyanin, lipopolysaccharide, pili, and phospholipase (8).

Exotoxin A is the highest important virulence factor of most *P. aeruginosa* strains. This toxin is intended for highly toxic mammalian cells (9, 10). The *toxA* gene is located in the genetic sequence of the *P. aeruginosa* chromosome, which is responsible for regulating exotoxin A synthesis (9, 11). *ExoA* is a significant member of the Type Il secretor system(*T2SS*), which inhibits the ADP-ribosylation protein synthesis of the eukaryotic elongation factor 2 (12-15). In addition, *exoA* converts nicotinamide dinucleotide to adenosine diphosphate ribosylate (16). *ExoA* has three components: Part I binds to host cellular receptors and establishes endocytosis, Part II transports the toxin into the cell cytoplasm, and Part III catalyzes adenosine diphosphate ribosylation of elongation factor 2 (17). In epidemiologic studies, the dissemination of resistant and extremely virulent pathogens is also the chief problem worldwide. The current study aims to investigate the *exoA* gene among the clinically *P. aeruginosa* strains isolated from wound burn infections.

2. Materials and methods

2.1. Study plan and clinical samples.

In the cross-cutting study, a complete of 110 wounds burned samples were collected from several teaching hospitals (Tehran, Iran) during a period of one year from April 2015 to April 2016. Bacterial isolates were identified using biochemical and microbiological tests such as gram staining, oxidase, O/F test, Triple Sugar Iron Agar (TSI), and Sulfide-Indole-Motility (9), pyocyanin pigment production, growth at 42 ℃ and citrate utilization. All isolates were kept in brain heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) containing 15% (v/v) glycerol (Merck Co., Germany) at -70 °C for further use. *P. aeruginosa*strain ATCC 27853 was used as quality control in this work.

2.2. DNA extraction

Genomic DNA was extracted from *P. aeruginosa* pure colonies using a G- spin Genomic DNA Extraction Kit (Fermentas, Germany) and then kept at -20 °C until used. The concentration and quality of the template DNA were evaluated by a Nano Drop™ 1000 Spectrophotometer (Thermos Scientific, Wilmington, DE, USA). DNA isolates with a concentration of 0.1 ng/µl were used as the templates for polymerase chain reaction (PCR) assay.

2.3. Polymerase Chain Reaction

The oligonucleotide primer sequences used for amplification of the *exoA* gene are listed in Table 1. The PCR reaction was carried out in a total volume of 25 µl, including 1.0 µl of bacterial DNA; 2 µl of 10×PCR buffer; 1.5 mmol/l of $MgCl_2$; 0.7 µl (each) of dATP, dGTP, dCTP, and dTTP; 0.5 µl of each primer; 1.0 µl of Taq DNA

Table 1. Primers used to identify *exoA* genes in *P. aeruginosa*.

Gene	Primer sequences (5'→3')	PCR-products (bp)
exoA	F=5′- TGCTGCACTACTCCATGGTC-3′	396 bp
	R=5′- ATCGGTACCAGCCAGTTCAG-3′	

Table 2. Percent of female and male participants in the study (*P. aeruginosa* positive).

Group	N	Percentage %
Female	42	66.67
Male	21	33.33
Total	63	19.62

Table 3. The distribution of gene detected from *P. aeruginosa.*

Group	N	Percentage %
exoA	83	75.4
no-exoA	27	24.59
Total	110	100

polymerase (5 U/µl; Amplicon Co., Denmark); and 15.7 µl of ddH2O. The target gene was amplified in a Techne TC-512 thermocycler (Eppendorf, Hamburg-Nord, Germany), as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation for 30 s at 94 °C, annealing for 40 s at 55 °C, and extension for 45 s at 72 °C, as well as a final extension for 5 min at 72 °C. The amplified PCR products were visualized by standard gel electrophoresis in a 1.5% agarose gel painted by ethidium bromide (5 µg/mL) for 30 min. The gels were recorded under ultraviolet light using the Gel-Doc.

2.4. Statistical Analysis

SPSS software version 23 (SPSS, Inc., Chicago, IL, USA) was employed for statistical analysis. Descriptive statistics and Pearson's chi-square tests were used to assess the relationship between the presence of the *exo-A* gene and gender. The statistical significance was defined as lower than 0.05 (p<0.005).

3. Results

In total, 110 non-duplicative clinically *P. aeruginosa* isolates were collected from 75.5% (n; 83) male and 65.5% (n; 72) female. The average age of the patients studied was 58.5±18.2 years, with a range between 16 and 85 years (Table 2). Molecular distribution showed that 75.4% (n;83) of isolates were carried the exoA gene (Table 3). The results showed that there was no meaningful relationship between the genus and the frequency of the *exoA* gene (*P* value=0.1). Multiplex PCR reaction with specific primers for the *exoA* gene showed that a high percentage of the samples contained the *exoA* gene (Figure 1).

4. Discussion

Invasive burn wound infection is an important cause of septicemia, septic shock and multiple malfunction syndrome (MODS) in persons suffering from severe burns (18, 19). Burn injury causes a mechanical disturbance in the skin's defense mechanism. The skin's natural barrier against microorganisms is replaced by a protein-rich moist, bloodless scar, which ultimately increases microbial growth. The gram-positive microbial population is not increased immediately after burn, but over a period of 7-10 days after burn, gram-negative replace gram-positive organisms (20).

P. Aeruginosa is a general destructive gram-negative organism. This is an opportunistic pathogen that causes rigid infections (21, 22).

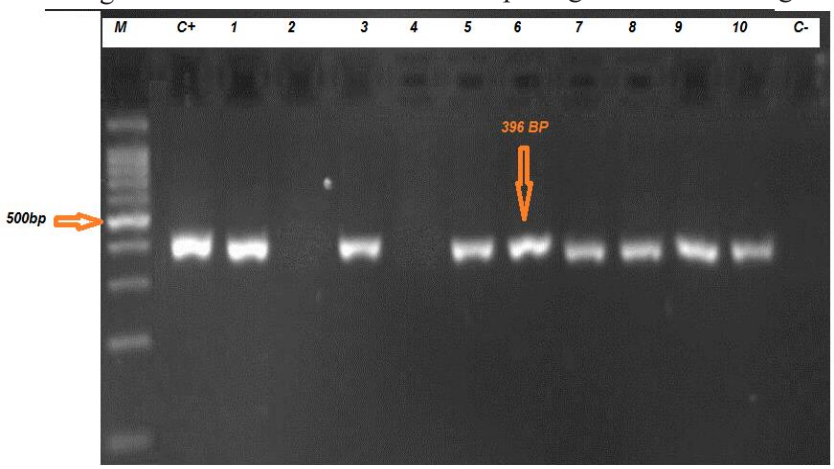

Figure 1. Agarose gel electrophoresis of PCR amplification (m): 100 bp DNA size marker (Sinaclon), Positive control (*P. aeruginosa* ATCC) Lane 1-10; template DNA and negative control; distilled water.

P. aeruginosa is a popular opportunistic and substantial agent in nosocomial infections as intense burns and Cystic fibrosis patients. This bacterium can motivate 30% of nosocomial infections due to its antibiotic resistance (23-25).

In the current study, we have used PCR-based diagnostic protocol for the identification of the *exoA* gene from *P. aeruginosa* isolated from wound burn infections. PCR showed that 75.4% of isolates were carried the *exo-A* gene.

ExoA is a major virulence factor produced by this organism. *ExoA* was first discovered by Liu and associates (26-28). Since then, *ExoA* has been toxic to a large variety of mammalian cells *in vitro* and is deadly to many animal species (29, 30). *ExoA* is a protein toxin that inhibits polypeptide synthesis by ribosylating the elongation factor ADP 2 and diphtheria toxin, which leads to cellular mortality (31, 32). Its cytotoxic activity covers a broad range of mammalian cells. It has been demonstrated that *exoA* inhibits the growth of human granulocytes and macrophages (33) and inhibits the production of interleukin-1 by peritoneal macrophages (20). These data demonstrate that *exoA*, as one of the most important virulence factors, can play a role in the physiopathology of sepsis of *P. aeruginosa* and death in burned patients (20, 21, 34, 35).

The *exoA* gene is more prevalent in wounds, burns, otitis media infection and, milk samples than others genes, and it is very important for *P. aeruginosa* because it hints to control damage in tissue and hence bacterial attack (36).

In the current study, from the 83 *P. aeruginosa* isolates, the *exoA* gene was detected in 83 (75.4%) PCR isolates. As well, Vasil and colleagues reported *exoA* production in close to 90% of 111 isolates of *P. aeruginosa* by analyzing for ADP ribosyl transferase activity (37, 38). Khan and Cerniglia introduced that the *exoA* gene was found in 93% of *P. aeruginosa* through PCR (26). Amini examined the catalytic locus of the *P. aeruginosa exoA* gene and showed that 90% of the isolated bacteria had *exoA* gene, while in the present study 93.67% of the isolated bacteria had this gene (39). Some other studies have identified *P. aeruginosa* strains mediated by the *exoA* gene by PCR and reported the sensitivity of this method is the reliable diagnosis, which is similar to our results (11, 24, 27, 40). Hummel examined the sensitivity of the PCR-mediated *exoA* gene for the diagnosis of *P. aeruginosa* and reported the high sensitivity and speed of PCR in diagnosis (41, 42).

All of the above research and our findings suggest that *exoA* may play an important role in the onset of *P. aeruginosa* and its lethality. *ExoA* contributes to the dissemination of *P. aeruginosa* within the body of burned patients and its horizontal spread within the burned skin. In addition, it is responsible for the general virulence of *P. aeruginosa*. The results of this study may put us on the line for controlling serious wound burn infection caused by *P. aeruginosa* organisms and any sequelae or complications arising from it. In addition to these findings, we reported the application of PCR procedures that can be used promptly and specifically to detect *P. aeruginosa* strains.

5. Conclusion

This study demonstrated that *P. aeruginosa* carrying the *exo-A* gene was the most prevalent in burn units. Therefore, due to the role of this enzyme in the development and spread of infection, especially in burned patients, attention to these strains and appropriate action for burn patients infected with these organisms, such as adequate treatment and, isolation is inevitable.

Acknowledgments

The authors of this investigation would like to thank the staff of Shahid Motahari Burn and Reconstruction Center (Tehran, Iran) for providing clinical specimens.

Conflict of Interest

The authors have no conflict of interest.

References

1. Rumbaugh KP, Griswold JA, Hamood AN. Pseudomonas aeruginosa strains obtained from patients with tracheal, urinary tract and wound infection: variations in virulence factors and virulence genes. *J Hosp Infect.* 1999 Nov;43(3):211-8.
2. Sadikot RT, Blackwell TS, Christman JW, Prince AS. Pathogen-host interactions in Pseudo-

monas aeruginosa pneumonia. *Am J Respir Crit Care Med*. 2005 Jun 1;171(11):1209-23. doi: 10.1164/rccm.200408-1044SO.

3. O'Carroll MR, Syrmis MW, Wainwright CE, Greer RM, Mitchell P, Coulter C, Sloots TP, Nissen MD, Bell SC. Clonal strains of Pseudomonas aeruginosa in paediatric and adult cystic fibrosis units. *Eur Respir J.* 2004 Jul;24(1):101-6. doi: 10.1183/09031936.04.00122903.

4. Moore NM, Flaws ML. Epidemiology and pathogenesis of Pseudomonas aeruginosa infections. *Clin Lab Sci.* 2011 Winter;24(1):43-6. PMID: 21404964.

5. Jalili M, Ehrampoush MH, Zandi H, Ebrahimi AA, Mokhtari M, Samaei MR, Abbasi F. Risk assessment and disease burden of legionella presence in cooling towers of Iran's central hospitals. *Environ Sci Pollut Res Int.* 2021 Dec;28(46):65945-65951. doi: 10.1007/s11356-021-14791-9.

6. Sedlak-Weinstein E, Cripps AW, Kyd JM, Foxwell AR. Pseudomonas aeruginosa: the potential to immunise against infection. *Expert Opin Biol Ther*. 2005 Jul;5(7):967-82. doi: 10.1517/14712598.5.7.967. PMID: 16018741.

7. Grimwood K, Kyd JM, Owen SJ, Massa HM, Cripps AW. Vaccination against respiratory Pseudomonas aeruginosa infection. *Hum Vaccin Immunother.* 2015;11(1):14-20. doi: 10.4161/hv.34296. Epub 2014 Nov 1.

8. Faezi S, Safarloo M, Amirmozafari N, Nikokar I, Siadat SD, Holder IA, Mahdavi M. Protective efficacy of Pseudomonas aeruginosa type-A flagellin in the murine burn wound model of infection. *APMIS*. 2014 Feb;122(2):115-27.

9. Dong D, Zou D, Liu H, Yang Z, Huang S, Liu N, He X, Liu W, Huang L. Rapid detection of Pseudomonas aeruginosa targeting the toxA gene in intensive care unit patients from Beijing, China. *Front Microbiol.* 2015 Oct 6;6:1100. doi: 10.3389/fmicb.2015.01100.

10. Khosravi AD, Shafie F, Abbasi Montazeri E, Rostami S. The frequency of genes encoding exotoxin A and exoenzyme S in Pseudomonas aeruginosa strains isolated from burn patients. *Burns*. 2016 Aug;42(5):1116-1120.

11. Neamah AA. Molecular Detection of virulence factor genes in Pseudomonas aeruginosa isolated from human and animals in Diwaniya province. *Kufa J Vet Med Sic*. 2017;8:218-30.

12. Rumbaugh KP, Hamood AN, Griswold JA. Analysis ofPseudomonas aeruginosaClinical Isolates for Possible Variations within the Virulence Genes Exotoxin A and Exoenzyme S. *J Surg Res.* 1999;82(1):95-105.

13. Lee VT, Smith RS, Tümmler B, Lory S. Activities of Pseudomonas aeruginosa effectors secreted by the Type III secretion system in vitro and during infection. *Infect Immun.* 2005 Mar;73(3):1695-705. doi: 10.1128/IAI.73.3.1695-1705.2005.

14. Kaszab E, Szoboszlay S, Dobolyi C, Háhn J, Pék N, Kriszt B. Antibiotic resistance profiles and virulence markers of Pseudomonas aeruginosa strains isolated from composts. *Bioresour Technol*. 2011 Jan;102(2):1543-8. doi: 10.1016/j.biortech.2010.08.027.

15. Swietnicki W, Czarny A, Antkowiak L, Zaczynska E, Kolodziejczak M, Sycz J, et al. Identification of a potent inhibitor of type II secretion system from Pseudomonas aeruginosa. *Biochem Biophys Res Commun*. 2019;513(3):688-93.

16. Amirmozafari N, Fallah Mehrabadi J, Isazadieh K, Habibi A. Molecular analysis of exotoxin A associated with antimicrobial resistance of Pseudomonas aeruginosa strains isolated from patients in Tehran hospitals. *Iran J Med Microbiol.* 2015;8(4):36-43.

17. Mashouf RY, Esmaeili R, Alikhani MY, Ghanbari M. Evaluation of exotoxin A gene and frequency of polymerase chain reaction sensitivity in detection of pseudomonas aeruginosa isolated from burn patients. *Tehran Univ Med J.* 2014;72(3):167-73.

18. Chai J, Sheng Z, Yang H, Diao L, Li L. Successful treatment of invasive burn wound infection with sepsis in patients with major burns. *Chin Med J.* 2000;113(12):1142-6.

19. Zhang P, Zou B, Liou YC, Huang C. The pathogenesis and diagnosis of sepsis post burn injury. *Burns Trauma.* 2021 Feb 4;9:tkaa047.

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

21. Gang RK, Bang RL, Sanyal SC, Mokaddas E, Lari AR. Pseudomonas aeruginosa septicaemia in burns. *Burns*. 1999 Nov;25(7):611-6.

22. Ciofu O, Tolker-Nielsen T. Tolerance and Resistance of Pseudomonas aeruginosa Biofilms

to Antimicrobial Agents-How P. aeruginosa Can Escape Antibiotics. *Front Microbiol.* 2019 May 3;10:913. doi: 10.3389/fmicb.2019.00913.
23. Kolak J, van Saene HK, de la Cal MA, Silvestre L, Peric M. Control of bacterial pneumonia during mechanical ventilation. *Croat Med J.* 2005 Apr;46(2):183-96.
24. Rossolini GM, Mantengoli E. Treatment and control of severe infections caused by multiresistant Pseudomonas aeruginosa. *Clin Microbiol Infect.* 2005 Jul;11 Suppl 4:17-32.
25. Zabihi T, Shahsanaei Goneirani M. Antibiotic resistance patterns in Pseudomonas aeruginosa strains isolated from patients hospitalized in different hospital wards. *Int J Med Rev.* 2017;4(2):40-2.
26. Liu PV. Extracellular toxins of Pseudomonas aeruginosa. *J Infect Dis.* 1974 Nov;130 Suppl(0):S94-9.
27. Ghanbarzadeh Corehtash Z, Khorshidi A, Firoozeh F, Akbari H, Mahmoudi Aznaveh A. Biofilm Formation and Virulence Factors Among Pseudomonas aeruginosa Isolated From Burn Patients. *Jundishapur J Microbiol.* 2015 Oct 21;8(10):e22345. doi: 10.5812/jjm.22345.
28. Badr R, Nagdy M, Sabagh A, Din A. Pseudomonas aeruginosa exotoxin a as a virulence factor in burn wound infections. *Egypt J Med Microbiol* 2008;17(1):125-33.
29. Wolf P, Elsässer-Beile U. Pseudomonas exotoxin A: from virulence factor to anti-cancer agent. *Int J Med Microbiol.* 2009 Mar;299(3):161-76. doi: 10.1016/j.ijmm.2008.08.003.
30. Pastan I. Immunotoxins containing Pseudomonas exotoxin A: a short history. *Cancer Immunol Immunother*. 2003 May;52(5):338-41.
31. Pavlovskis OR, Iglewski BH, Pollack M. Mechanism of action of Pseudomonas aeruginosa exotoxin A in experimental mouse infections: adenosine diphosphate ribosylation of elongation factor 2. *Infect Immun.* 1978 Jan;19(1):29-33.
32. Michalska M, Wolf P. Pseudomonas Exotoxin A: optimized by evolution for effective killing. *Front Microbiol*. 2015 Sep 15;6:963.
33. Stuart RK, Pollack M. Pseudomonas aeruginosa exotoxin A inhibits proliferation of human bone marrow progenitor cells in vitro. *Infect Immun.* 1982 Oct;38(1):206-11.
34. Furuya N, Hirakata Y, Tomono K, Matsumoto T, Tateda K, Kaku M, Yamaguchi K. Mortality rates amongst mice with endogenous septicaemia caused by Pseudomonas aeruginosa isolates from various clinical sources. *J Med Microbiol.* 1993 Aug;39(2):141-6.
35. Wagener BM, Hu R, Wu S, Pittet JF, Ding Q, Che P. The Role of Pseudomonas aeruginosa Virulence Factors in Cytoskeletal Dysregulation and Lung Barrier Dysfunction. *Toxins (Basel).* 2021 Nov 2;13(11):776.
36. Kadhim SR. Genetic Study of Txo A Gene in Pseudomonas aeruginosa Isolated from Burn Wound Infections. *Res J Pharm Tech.* 2020;13(11):5301-5.
37. Bjorn MJ, Vasil ML, Sadoff JC, Iglewski BH. Incidence of exotoxin production by Pseudomonas species. Infect Immun. 1977 Apr;16(1):362-6. doi: 10.1128/iai.16.1.362-366.1977.
38. Azimi S, Kafil HS, Baghi HB, Shokrian S, Najaf K, Asgharzadeh M, Yousefi M, Shahrivar F, Aghazadeh M. Presence of exoY, exoS, exoU and exoT genes, antibiotic resistance and biofilm production among Pseudomonas aeruginosa isolates in Northwest Iran. *GMS Hyg Infect Control*. 2016 Feb 22;11:Doc04.
39. Amini B, Kamali M, Zarei Mahmod Abadi A, Mortazavi Y, Ebrahim Habibi A, Bayat E, et al. Cloning of catalytic domain of exotoxin a from Pseudomonas aeruginosa. *J Adv Med Biomed Res*. 2010;18(71):24-33.
40. 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 Sep;41(9):4312-7.
41. Hummel A, Unger G. Nachweis von Pseudomonas aeruginosa aus Bronchial- und Trachealsekreten mittels PCR durch Amplifikation des Exotoxin A-Genes [Detection of Pseudomonas aeruginosa in bronchial and tracheal aspirates by PCR by amplification of the exotoxin A gene]. *Zentralbl Hyg Umweltmed.* 1998 Dec;201(4-5):349-55. German. PMID: 9916289.
42. Zarei O, Shokoohizadeh L, Hossainpour H, Alikhani MY. Molecular analysis of Pseudomonas aeruginosa isolated from clinical, environmental and cockroach sources by ERIC-PCR. *BMC Res Notes*. 2018 Sep 15;11(1):668.