ORIGINAL ARTICLE

Evaluation of cloning efficiency of PilF and PilQ genes of Pseudomonas aeruginosa in Escherichia coli using TA cloning method

ZOHREH NASRABADI[1], MITRA SALEHI[2], KUMARS AMINI[3], AHMAD MAJD[4]
[1]*Department of Biology, Faculty of Science, Islamic Azad University, North Tehran branch, Tehran, Iran*
[2]*Department of Microbiology, Faculty of Science, Islamic Azad University, North Tehran branch, Tehran, Iran*
[3]*Department of Microbiology, Faculty of Agriculture, Islamic Azad University, Saveh branch, Saveh, Iran*
[4]*Department of Biology, Faculty of Science, Islamic Azad University, North Tehran branch, Tehran, Iran*
Corresponding author: Mitra Salehi, Ph.D Department of Microbiology, Faculty of Science, Islamic Azad University, North Tehran branch. Email: mitra_salehi_microbiology@yahoo.com

ABSTRACT

Pseudomonas aeruginosa is one of the most important pathogens that causes many complications and deaths in the world every year. Many species of Pseudomonas aeruginosa have pili that are involved in the pathogenesis of the organism. The aim of this study was to clone PilF and PilQ genes of Pseudomonas aeruginosa in Escherichia coli and express it by real time PCR. Materials and Methods: After production of PCR product by Taq DNA polymerase and its cloning in PTG19-T cloning vector, then the vector was transduced to XL1-Blue host. After transformation, evaluation of the extracted RNA and amplification was performed by real-time PCR. Results: The analysis showed that the transfection of PilF and PilQ genes in PTG-T19 plasmid and Escherichia coli XL1-blue strain leads to high mRNA expression of these genes, which indicates the success of the proposed transducing method. TA cloning is a rapid and efficient method for transfecting the aforementioned genes as compared to conventional cloning methods. Due to the important role of PilF and PilQ genes, they can be used as a good candidate for developing the recombinant vaccines against infections caused by Pseudomonas aeruginosa.
Keywords: Cloning, Pseudomonas aeruginosa, PilF, PilQ, Antibiotic resistance

INTRODUCTION

Pseudomonas is a gram-negative opportunistic pathogen known to be an important animal and human pathogen [1, 2]. This pathogen causes infections in urinary tract, respiratory system, inflammation of the skin, bacteremia, bone and joint infections, gastrointestinal infections, various systemic infections, particularly in patients with severe burns, people with cystic fibrosis, and patients with cancer and AIDS whose immune systems are suppressed [3]. Today, this bacterium is the leading cause of death in people with burns [4]. It is also one of the most common pathogens isolated from bacterial keratitis due to contact lenses [5, 6]. Pseudomonas aeruginosa is the second most common pathogen in surgery and the third most common cause of nosocomial infections after Escherichia coli and Staphylococcus aureus [7-9].

Another challenging issue is the resistance of this bacterium to many antibiotics [10]. Studies show that people with cystic fibrosis are usually unable to overcome and eliminate infections caused by Pseudomonas aeruginosa, even with the help of chemical treatment with antimicrobials [11-13]. Identifying genes involved in pathogenesis and producing vaccines against it can greatly alleviate the problems of resistance. Type IV pili are one of the effective proteins in the pathogenesis of Pseudomonas aeruginosa, in the formation of which many genes are involved. Their key components include the inner membrane (Pilf) and outer membrane (PilQ) [14, 15] . In the past, pilin encoding gene (PilA) was isolated from Pseudomonas aeruginosa PAO1 strain by PCR and cloned into expression vector and recombinant vector was transformed into E. coli BL21 [16]. However, PilF and PilQ genes are the most important pili genes, which have an important role in pathogenesis, and have been considered by many researchers [17, 18] . These genes can be considered as a good candidate for the production of an effective recombinant vaccine. In the present study, real time PCR method was used for identification and expression. This method is similar to other biochemical methods in terms of cost but can be implemented in a much shorter time and this method seems to be the optimal choice for the diagnosis of Pseudomonas aeruginosa and non-Pseudomonas aeruginosa gram-negative isolates [19].

Despite many advances in antimicrobial therapy, the mortality rate from Pseudomonas aeruginosa bacteremia is alarmingly high [20]. Therefore, the treatment of infections caused by Pseudomonas aeruginosa is of special importance. In various studies, pili have been identified as one of the most likely causes of disease and attention to the genes involved in them has been suggested as antigenic structures for developing vaccines [21-23]. According to the above points, the importance of cloning these genes and their role in developing vaccines, the aim of this study was to transfer *PilF* and *PilQ* genes to Escherichia coli XL1-blue strain and express it by real time PCR.

MATERIALS AND METHODS

Bacterial strains and materials: This study was collected in Pasargad Research Laboratory (Tehran, Iran) in 60 clinical strains of P. aeruginosa from 95 patients with burn injuries in Kerman Hospital. Bacteria were identified based on biochemical methods. Was maintained. E. coli XL-1blue strain was used as a host for cloning and expression and

pTG19 vector synthesized by Biomatik Company was used.

All enzymes for digestion of nucleotide fragments and DNA ligase were provided from NEB Company (USA). Anti-His monoclonal antibody was provided from Invitrogen Company (USA). Ni2 + -NTA agarose was purchased from Kiagen (USA). The culture media were provided from Merck Company (Germany).

Biochemical tests: Catalase and oxidase test was used to examine the presence of catalase and oxidase in microorganisms, MR-VP culture medium to examine acetone, SIM medium to examine indole production from tryptophan and TSI culture with phenol content to examine acid production from three sugars: glucose, lactose and sucrose.

Gene extraction from Pseudomonas: Strains were cultured in LB medium (Merck, Germany) at 37 ° C and using a genomic extraction kit (Genetic Storage Center, Iran), all DNA was extracted from Pseudomonas aeruginosa samples using a DNA extraction kit according to the manufacturer's recommendation. DNA samples were evaluated using spectrophotometry (Nanodrop ND-1000 (Thermo Scientific, USA)) at a wavelength of 260 nm. In addition, absorption at 280/250 nm was measured to evaluate DNA purity. To design the primer, the nucleotide sequences of the PilQ and PilF genes were first obtained from the NCBI site, and the oligonucleotide primers were then designed using the Oligo7 program and then synthesized by Macrogene. Sequences of primers used for PCR regarding pilF gene of genomic DNA were amplified using the forward primer with the sequence (ATACGAATTCTAGGGAGGAGCGGATCTAC) and the reverse sequence (AAGCATAAGCGGCCGCAAATTTAAACCATGGTCATCT CGTCACACCC) and for the PilQ gene the forward primer with the sequence (AATACTGCAGAGGAGGAAAGCGATGCCCAAAG) and the reverse sequence (CCGTAAGCTTCTTGTGGAAGAACTCTATGGG) at C-terminal with hexahistidine. PCR reaction was performed using a kit (Invitrogen, San Diego, CA). For this purpose, an initial activation step for 3 minutes at 95 ° C and a denaturation step at 54 ° C for 30 seconds, a combined annealing / expansion step for 1 minute at 72 ° C and a final expansion at 72 ° C were performed for 10 minutes. Subsequently, the PCR product was analyzed using 1% agarose gel electrophoresis. The genes were purified using a gel purification kit (Sinaclon, Iran) according to the manufacturer's protocol.

Cloning: TA Cloning method (Sinaclon Kit, Iran) was used to clone PilQ and PilF genes and the cloning steps were performed according to the kit. According to the instructions, the Sinaclon (Miniprep) (EX6111) plasmid extraction kit was used to digest DNA from recombinant clones and ensure the quality of the cut plasmid DNA. The DNA fragment extracted from the recombinant clones was purified using the plasmid extraction kit (Sinaclon, Iran) and amplified and sequenced using M13 sequencing primers.

Real-time PCR expression: Real-time PCR was used to examine the mRNA expression of PilQ and PilF genes of Pseudomonas aeruginosa in E. coli XL-1blue strain. For this purpose, PCR test was performed on a final volume of 20 µl of reaction mixture containing 10 µl of SYBR Green master mix, 2 µl of cDNA product, 0.5 µl of each forward and reverse primer (10 pm) and 7 µl of nuclease-free water. PCR reaction conditions an initial activation step of 30 seconds at 95 ° C followed by 40 cycles including a 15 second denaturation step at 95 ° C and a combined annealing / expansion step of 60 seconds at 60 ° C were performed. Melting curves for each single PCR product primer were investigated and analyzed. And quantitative expression values were calculated based on 2-ΔΔct.

RESULTS

Bacterial approval and extraction: Pseudomonas was identified from the collected strains by differential and microbial biochemical tests. The extracted DNA concentration was determined to be 200 µg/mL. After extraction using the kit, the concentration of the desired genome was examined by gel electrophoresis (Figure 1).

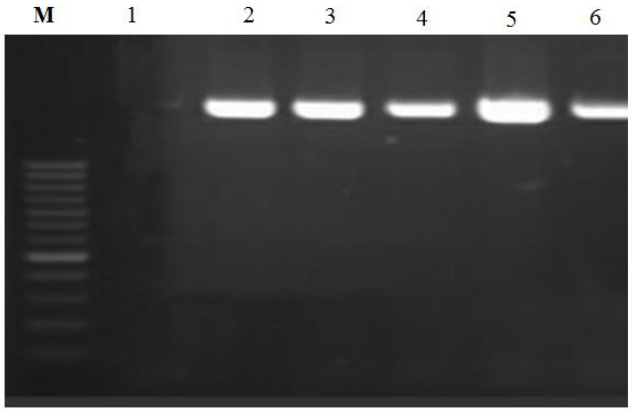

Figure 1: Approval of genome quality after extraction using gel electrophoresis method. M Molecular marker. 1. Negative control. 2 to 6. Genomic DNA

The results of amplification of PilF and PilQ genes obtained from 60 strains of Pseudomonas aeruginosa by PCR using electrophoresis showed a size of approximately 1203 bp for *PilF* gene and 579 bp for *PilQ*. These results revealed that only 3% of the 60 Pseudomonas aeruginosa strains lacked the *PilF* and *PilQ* genes (Figure 2). Therefore, these genes can be a good candidate for vaccine selection against infections caused by Pseudomonas aeruginosa.

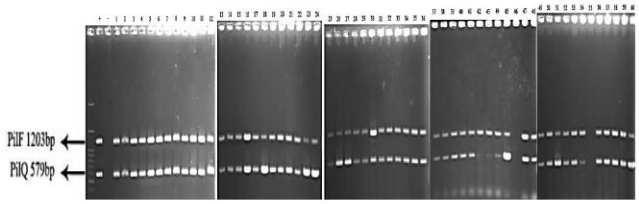

Figure 2. Image of *PilF* and *PilQ* gene amplification results from 60 Pseudomonas aeruginosa strains

Cloning: Cloning of *PilF* and *PilQ* genes was performed using the TA cloning kit according to the manufacturer's instructions, and the steps of binding the vector and

genomic DNA and transferring the recombinant vector into the host of Escherichia coli XL1-blue strain were successfully performed. At this stage, the transferred cells were cultured in a plate containing ampicillin, X-gal and IPTG and grown as blue-white colonies (Figure 3).

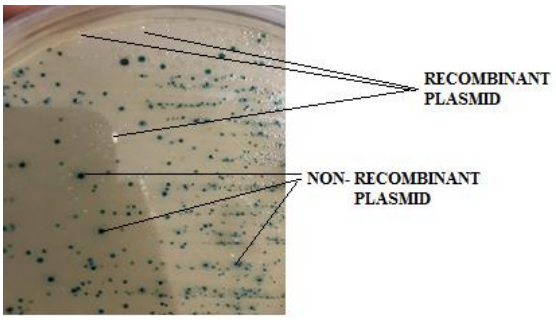

Figure 3. Plate of recombinant white blue colonies. Blue/white screening colonies are shown in both white and blue. White colonies, recombinant colonies and blue colonies lack recombinant plasmids

Recombinant plasmid synthesis was successfully performed in Escherichia coli XL-1 blue strain as an expression host. The DNA fragment extracted from the recombinant clones was amplified and sequenced using M13 sequencing primers. *PilQ* gene and *PilF* gene fragments after cloning can be seen in Figures 4A and 4B.

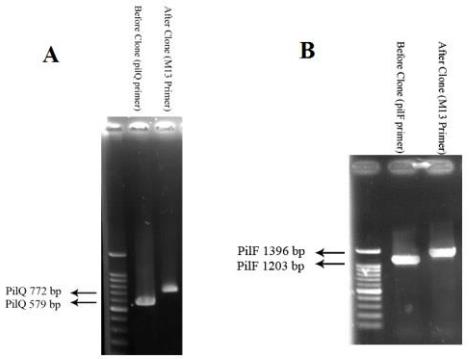

Figure 4-A) Results related to amplification of *PilQ* gene before and after cloning with M13 primer B) Image related to results of amplification of *PilF* gene before and after cloning with M13 primer

Evaluation of PilF and PilQ expression levels: As shown in Figure 5, the CT level of the 16C gene of non-cloned and cloned E. coli is higher than the CT of the PilF and PilQ genes, and this rate is higher than that of the non-cloned PilF and cloned PilQ, which means that the expression of 16 S genes is higher than genes of non-cloned PilF and cloned PilQ genes.

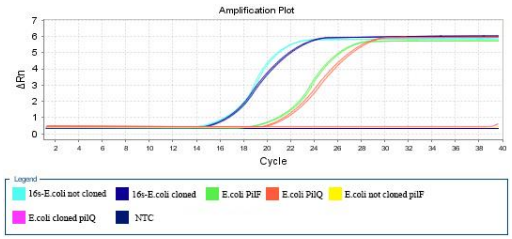

Figure 5. Gene amplification curve for PilF and PilQ genes using real-time PCR

DISCUSSION

In the present study, it was found that *PilF* and *PilQ* genes were present and amplified in all 60 strains except 2 cases (3%). This reveals the high efficiency of this study in selecting the above genes for the prevention and treatment of infections caused by Pseudomonas aeruginosa as well as a good candidate for vaccine. In this regard, Karpi et al. in 2015, while isolating the gene encoding pilin (*PilA*) from PAO1 strain of Pseudomonas aeruginosa by PCR and cloning in expression vector pET_22b, confirmed the structure of the recombinant vector by dual enzymatic digestion and sequencing [16]. As noted in a 2008 study by Koo et al., mutations in the lipidation fraction of *PilF* result in the formation of secretin in both the inner and outer membranes, which dramatically reduces susceptibility to bacteriophage and twitching motion [17]. In addition, in 2016, Faezi et al. measured the expression of $PilQ_{380-705}$-PilA binding protein with pET26b vector and the expression in Escherichia coli BL21, the activity of specific antiserum with binding protein by ELISA. They stated that the PilQ380-705-PilA binding protein appears to be a good vaccine candidate against Pseudomonas aeruginosa infection [24]. All the above research confirms that *PilF* and *PilQ* genes are good genes studied in Pseudomonas.

In 2016, Bahador et al. diagnosed, cloned, expressed and purified recombinant *flagellin B* in *Pseudomonas aeruginosa* to evaluate the performance of rabbit polyclonal antibody activity against this *flagellin B.* They isolated the rabbit *flagellin B* gene from Pseudomonas aeruginosa by PCR, cloned it into pET28a vector and then transferred it to XL1-Blue E. coli strain [14]. In our study, PTG-19 vector was used, which having M13 primer site for screening and sequencing, lacZ gene for blue/white screening and BamH1 restriction enzyme to release the desired fragment from PTG-19 vector is one of its advantages over other vectors.

Despite all efforts, the future of immunization against Pseudomonas remains challenging and there still is no effective method. Several Pseudomonas aeruginosa antigens have been identified as the treatment method, including lipopolysaccharide (LPS) O antigen, outer membrane proteins F and I, type III secretory system (PcrV) components, flagellum, pili, DNA, and completely killed cells, attenuated biomarkers, in addition to a number of non-integral outer membrane candidate protein antigens [25]. Despite the immunogenicity of these antigens, the clinical efficiency of these genes in healthy volunteers with cystic fibrosis or burn injuries is still unknown [26-28].

Research has shown that the formation of the *PilQ* channel in the outer membrane is essential for the exit of pili. And *PilF* reacts directly or indirectly with the *PilQ* monomer through the inner membrane to facilitate the passage of the periplasm to the outer membrane [17]. The present study also used these two genes to develop a good vaccine candidate in future studies due to the cooperation of these two genes with each other in pili type IV and their role in binding and pathogenesis.

In the present study, TA cloning was used and the efficiency of this method was observed. This method is one of the most convenient and easiest cloning methods, which compared to sticky-ended binding and blunt-ended binding,

TA cloning does not require restriction enzymes for the complex steps of DNA purification. The TA cloning technique can also be used for cancer therapeutic purposes through gene amplification and cloning in the expression vector to produce recombinant gene expressing the protein [29] .

Due to the fact that many virulence factors are involved in Pseudomonas virulence, the use of optimal methods for the treatment of Pseudomonas infections is essential. Therefore, the use of cloning, expression, and sequencing techniques of PilQ and PilF genes as an appropriate step in the development of an effective vaccine is essential for the treatment of Pseudomonas infections.

Considering the role of *PilF* and *PilQ* genes in the pathogenicity of Pseudomonas aeruginosa, in order to improve the treatment methods against Pseudomonas aeruginosa to counteract the adverse effects of this bacterium, it is recommended to study eukaryotes and eukaryotic vectors in order to develop a good vaccine against it. Also, in order to develop a vaccine, it is better to use immunoassay methods to evaluate the quality and immunogenicity of the above genes.

CONCLUSION

Cloning and expression of *PilF* and *PilQ* genes in PTG-T19 plasmid and *E. coli* XL1-blue strain have been successfully performed. In addition, TA cloning method has a high speed and efficiency for amplification of the above genes, which is one of the advantages of this technique over conventional cloning. It is hoped that by using the above method, recombinant products with minimum impurities and maximum quality will be marketed in accordance with international standards.

REFERENCES

1. Palleroni N, Moore DPE. 12 Microbiology of Hydrocarbon-Degrading Pseudomonas. 2010.
2. Casaz P, Happel A, Keithan J, Read DL, Strain SR, Levy SB. The Pseudomonas fluorescens transcription activator AdnA is required for adhesion and motility. *Microbiology (Reading, England)*. 2001;147(Pt 2):355-61. doi:10.1099/00221287-147-2-355
3. Valerius NH, Koch C, Høiby N. Prevention of chronic Pseudomonas aeruginosa colonisation in cystic fibrosis by early treatment. *Lancet*. 1991;338(8769):725-6. doi:10.1016/0140-6736(91)91446-2
4. Le Coadic M, Simon M, Marchetti A, Ebert D, Cosson P. Daphnia magna, a host for evaluation of bacterial virulence. *Applied and environmental microbiology*. 2012;78(2):593-5. doi:10.1128/aem.06278-11
5. Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC. Global transposon mutagenesis and a minimal Mycoplasma genome. *Science*. 1999;286(5447):2165-9. doi:10.1126/science.286.5447.2165
6. Palleroni N, Kunisawa R, Contopoulou R, Doudoroff M. Nucleic acid homologies in the genus Pseudomonas. *International Journal of Systematic and Evolutionary Microbiology*. 1973;23(4):333-9.
7. Strateva T, Yordanov D. Pseudomonas aeruginosa - a phenomenon of bacterial resistance. *Journal of medical microbiology*. 2009;58(Pt 9):1133-48. doi:10.1099/jmm.0.009142-0
8. Franzetti L, Scarpellini M. Characterisation ofPseudomonas spp. isolated from foods. *Annals of microbiology*. 2007;57(1):39-47.
9. Wood LF, Ohman DE. Identification of genes in the σ^{22} regulon of Pseudomonas aeruginosa required for cell envelope homeostasis in either the planktonic or the sessile mode of growth. *mBio*. 2012;3(3). doi:10.1128/mBio.00094-12
10. Knirel YA, Kocharova NA, Shashkov AS, Kochetkov NK, Kholodkova EV, Stanislavsky ES. Somatic antigens of Pseudomonas aeruginosa. The structure of O-specific polysaccharide chains of the lipopolysaccharides from P. aeruginosa II (Sandvik) and V (IM-1, Verder-Evans). *European journal of biochemistry*. 1987;166(1):189-97. doi:10.1111/j.1432-1033.1987.tb13501.x
11. Haynes WC. Pseudomonas aeruginosa--its characterization and identification. *Journal of general microbiology*. 1951;5(5 Suppl.):939-50. doi:10.1099/00221287-5-5-939
12. Ng WW, Chau PY, Leung YK, Livermore DM. In vitro activities of Ro 17-2301 and aztreonam compared with those of other new beta-lactam antibiotics against clinical isolates of Pseudomonas aeruginosa. *Antimicrob Agents Chemother*. 1985;27(5):872-3. doi:10.1128/aac.27.5.872
13. Kumari S, Harjai K, Chhibber S. Characterization of Pseudomonas aeruginosa PAO specific bacteriophages isolated from sewage samples. *Am J Biomed Sci*. 2009;1(2):91-102.
14. Behrouz B, Amirmozafari N, Khoramabadi N, Bahroudi M, Legaee P, Mahdavi M. Cloning, Expression, and Purification of Pseudomonas aeruginosa Flagellin, and Characterization of the Elicited Anti-Flagellin Antibody. *Iranian Red Crescent medical journal*. 2016;18(6):e28271. doi:10.5812/ircmj.28271
15. Austin B, Goodfellow M. Pseudomonas mesophilica, a new species of pink bacteria isolated from leaf surfaces. *International Journal of Systematic and Evolutionary Microbiology*. 1979;29(4):373-8.
16. Korpi F, Behrouz B, Motameifar M, Irajian G. Cloning, expression and purification of Pseudomonas aeruginosa pilin protein in the prokaryotic host. *Iranian Journal of Medical Microbiology*. 2016;9(4):55-62.
17. Koo J, Tammam S, Ku SY, Sampaleanu LM, Burrows LL, Howell PL. PilF is an outer membrane lipoprotein required for multimerization and localization of the Pseudomonas aeruginosa Type IV pilus secretin. *J Bacteriol*. 2008;190(21):6961-9. doi:10.1128/jb.00996-08
18. Persat A, Inclan YF, Engel JN, Stone HA, Gitai Z. Type IV pili mechanochemically regulate virulence factors in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America*. 2015;112(24):7563-8. doi:10.1073/pnas.1502025112
19. 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. *Journal of clinical microbiology*. 2003;41(9):4312-7. doi:10.1128/jcm.41.9.4312-4317.2003
20. Fothergill JL, Neill DR, Loman N, Winstanley C, Kadioglu A. Pseudomonas aeruginosa adaptation in the nasopharyngeal reservoir leads to migration and persistence in the lungs. *Nat Commun*. 2014;5:4780. doi:10.1038/ncomms5780
21. Faezi S, Bahrmand AR, Siadat SD, Nikokar I, Sardari S, Mahdavi M. Construction, expression, purification and characterization of secretin domain of PilQ and triple PilA-related disulfide loop peptides fusion protein from Pseudomonas aeruginosa. *Iran J Basic Med Sci*. 2017;20(5):458-66. doi:10.22038/ijbms.2017.8667
22. Bitter W, Koster M, Latijnhouwers M, de Cock H, Tommassen J. Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer

membrane of Pseudomonas aeruginosa. *Molecular microbiology*. 1998;27(1):209-19. doi:10.1046/j.1365-2958.1998.00677.x
23. Tung CJ, Chen CM, Lee PH. A correlational study on health training program participation and influences upon communities volunteers in Taipei City. *The journal of nursing research : JNR*. 2005;13(4):245-52.
24. 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;122(2):115-27. doi:10.1111/apm.12101
25. Le Moigne V, Gaillard JL, Herrmann JL. Vaccine strategies against cystic fibrosis pathogens. *Hum Vaccin Immunother*. 2016;12(3):751-6. doi:10.1080/21645515.2015.1102810
26. Sharma A, Krause A, Worgall S. Recent developments for Pseudomonas vaccines. *Human vaccines*. 2011;7(10):999-1011. doi:10.4161/hv.7.10.16369
27. Priebe GP, Goldberg JB. Vaccines for Pseudomonas aeruginosa: a long and winding road. *Expert review of vaccines*. 2014;13(4):507-19. doi:10.1586/14760584.2014.890053
28. Chang YS, Klockgether J, Tümmler B. An intragenic deletion in pilQ leads to nonpiliation of a Pseudomonas aeruginosa strain isolated from cystic fibrosis lung. *FEMS microbiology letters*. 2007;270(2):201-6. doi:10.1111/j.1574-6968.2007.00664.x
29. Souii A, Gharbi J, M'Hadheb-Gharbi MB. Molecular Analysis of RNA-RNA Interactions between 5' and 3' Untranslated Regions during the Initiation of Translation of a Cardiovirulent and a Live-Attenuated Coxsackievirus B3 Strains. *Int J Mol Sci*. 2013;14(3):4525-44. doi:10.3390/ijms14034525