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Suppression of Virulence Factors in Pseudomonas Aeruginosa Clinical Isolates through Natural Compound-Mediated Quorum Sensing Inhibition

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KEYWORDS

Pseudomonas aeruginosa, virulence factors, cinnamaldehyde , salicylic acid ,Quorum sensing inhibition

ABSTRACT:

Opgective: The pathogenesis of Pseudomonas aeruginosa dependent on a number of virulence factors that help to enable for host colonization. Among the virulence factors: biofilm, pyocyanin, elastase and protease. The problem of antibiotic resistance in P. aeruginosa is highly significant due to its possession of mechanisms and enzymes that assist in resistance. Consequently, reducing resistance and the pathogenicity of these bacteria is the objective of many researchers. This study aims to utilize natural compounds and examine their effects in inhibiting certain virulence factors of P. aeruginosa (elastase and protease) by decreasing the gene expression of the regulatory system that controls the production of virulence factors and bacterial pathogenicity, without impacting bacterial growth.

Methods: samples were diagnosed and verified using the Vitek-2 system. minimum inhibitory concentration (MIC) of cinnamonaldehyde (CA) and salicylic acid (SA) against P. aeruginosa isolates was determined. The sub-MIC concentrations of CA and SA were utilized to quantify their inhibitory effect on proteases and elastase in the presence and absence of these compounds, as well as the expression levels of the LasI and RhlR genes. Quantitative real-time PCR was utilized to detect a reduction in LasI and RhlR expression.

Result: Protease mean decreased with SA to 0.05 (±0.059) and with CA to 0.08 (±0.050) from 0.43 (±0.22) to 0.05 (±0.059). Additionally, the mean elastase was decreased with SA to 0.1138 (± 0.151) and with CA to 0.2 (± 0.217). Whereas SA and CA changed the expression level of LasI from 1 to 0.3 and 1.3 folds, respectively, the expression level of RhlR was reduced from 1 to 0.5 and 0.2 folds.

Conclusion: Natural compounds CA and SA significantly reduced the phenotypic virulence factors; protease, elastase and reducing the expression of the RhlR and lasl genes in p. aeruginosa.

Introduction

Pseudomonas aeruginosa is a human pathogen that causes chronic illness in people with impaired immune systems. It also causes infections in burn victims, wound, cystic fibrosis, acute leukemia, organ transplantation, and intravenous medication delivery. Patients suffering from addiction [1].

P. aeruginosa pathogenesis is based on a number of virulence factors that contribute to host colonization. The virulence factors include biofilm, pyocyanin, elastase, protease, and rhamnolipid. [2].

P. aeruginosa uses this protease to help cause infection by overcoming or destroying lines of defense for the host to cause disease as well as control the host's environment and modify it according to the bacterial need inside the host's cells. Consequently, it is regarded as a virulence factor that bacteria utilize to engage with the host and challenge the immune system during the host's defense against the bacterium that subsequently causes illness.

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3.Since it affects the proteolysis of several host proteins, including collagen, which keeps membrane integrity intact, and elastin, which is crucial for lung elasticity and blood vessel integrity, this elastase is a major factor in pathogenesis. Studies have shown that the breakdown of these proteins by elastase leads to pulmonary fibrosis and vascular inflammation in cystic fibrosis patients [4].

Quorum sensing (QS), an intercellular communication mechanism that bacteria utilize to control population density via signaling molecules and receptors as well as to control gene expression in response to an ever-changing environment, is primarily linked to the production of these virulence factors. When the population reaches a threshold concentration, a bacterium's ability to sense information from other cells in the population and communicate with them is referred to as QS (i.e. a quorum) to control the development of a wide range of virulence factors employing small signaling molecules known as auto inducers [5].

The suppression of the QS system has been proposed as a new technique for developing anti-pathogenic agents. Quorum sensing inhibitors (QSIs), which act on bacterial virulence factors without compromising bacterial vitality, can help diminish bacterial resistance to medicines and might be used to treat or halt infections caused by P. aeruginosa. 6. Anti-virulence is a significant way of battling infectious illnesses, as there are few substances employed as anti-virulence, which are the reverse of anti-growths in their impact and do not inhibit bacterial growth, resulting in a decrease in bacterial resistance. It is well recognized that when bacteria meet growth inhibitors, they acquire resistance to such inhibitors, making treating bacterial infections challenging[7]. There are many natural compounds act QSIs distributed in plants, and they have physiological functions, among which is the reduction of phenotypic patterns controlled by the QS of Pseudomonas bacteria [8].

cinnamaldehyde (CA) a natural plant compound that is one of the main components of cinnamon, it gives it flavor and odor properties, its considered a 23 essential oil (EO) derived from medicinal plants; which are secondary metabolites of plants that do not show any function in the growth or development of plants, but they play a role for plant in protecting themselves from insects, viruses, bacteria and fungi [9].

CA has antimicrobial, antioxidant properties. It has an important effect on the formation of biofilm by P. aeruginosa, which led to the disruption of the biofilm when CA is present as an inhibitor [10]. A recent study on Pseudomonas fluorescens revealed that the application of sub-lethal concentrations of cyanamaldehyde resulted in inhibition of the phenotype based on QS, such biofilm formation, swimming and swarming motility, and extracellular proteases [11].

Salicylic Acid (SA)

It's a natural Phenolic compound present in plant extracts used as a nonsteroidal anti-inflammatory drug (NSAID), whose function is to induce a defense response against the attack of pathogenic organisms. It is a metabolite of aspirin and is responsible for the anti-inflammatory properties of this drug in humans. It inhibits the growth and formation of many virulence factors as in biofilms. It acts as an antibacterial [12] . It downregulates some virulence factors in P. aeruginosa among the virulence factors that reduce its regulation is, protease and elastase.

Methods

Isolation and Identification of P. aeruginosa

Between September and December 2021, 102 samples were taken from the Fallujah and Ramadi Teaching Hospitals in Iraq's Anbar Province. The samples were collected from individuals suffering from burns, wounds, urinary tract infections, and sputum difficulties. These samples were subsequently taken to the Department of Biology's laboratory at the College of Science, University of Anbar. In the lab, the samples were submitted to both microscopic and biochemical analysis for diagnostic reasons.

salicylic acid (SA) and cinnamaldehyde (CA) stock solution preparing and Minimum inhibitory concentration determination

To find the minimum inhibitory concentration (MIC) of SA and CA against Pseudomonas aeruginosa, a resazurin microtiter plate assay was employed. Using 10 mg of SA, stock solutions of CA and SA were made at 1000 µg/ml by dissolving CA in DMSO (1:1). Each well of the microtiter plate had 100 µl of BHI broth. 100 µl of each of the stock solutions for CA and SA were added to the

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first row individually, and 100 µl of each was then transferred to the next row. After adjusting the turbidity of P. aeruginosa cultures to the McFarland standard, 10 µl was applied to each well. After that, plates were incubated for 24 hours at 37°C. After incubating 0.015% resazurin for two hours, the concentration without any color change was defined as the minimum inhibitory concentration (MIC). The capacity of CA and SA to suppress the activity of elastase and protease was evaluated using the sub-MIC, which is one dilution below the MIC.

Elastase assay:

To measure the amount of elastase, with or without inhibitors, 200µl of bacterial supernatant was added to 180µl of reaction in buffer containing 20 mg of elastin Congo Red (ECR) at pH 7.5 and 100 mM Tris HCL. After that, the mixture was shaken nonstop for three hours at 37°C (150 rpm). The filtrate was collected and placed in a cuvette to test the elastase activity at a density of 495 nanometers after being centrifuged at 10,000 g for 10 minutes to remove the insoluble ESR. The blank consisted of an ESR and a Tris HCL, with distilled water (devoid of the enzyme) used to replace the supernatant in the appropriate quantity[13].

Protease assay

Protease production from P. aeruginosa was quantified both with and without inhibitors. Each isolate in this test was placed in four tubes, and 1 mL of the reaction solution—which included casein as a substrate and 50 mM Tris HCL—was micropipetted into each tube. To every tube, 0.8% of Tris 41 HCL at pH 7 was added. Next, 500 µl of previously generated bacterial supernatant, both with and without inhibitors, was added to the tubes. All tubes—aside from the fourth, which is regarded as a blank—were shaken while incubating at 25°C for three hours before the termination solution (0.1M HCL) was added. and departed for ten minutes. Centrifugation at 10,000 rpm for 10 minutes at 4 ℃ was used to remove precipitated casein. Using a spectrophotometer, measure the absorbance at 440 nm by taking the supernatant and transferring it to a cuvette[14].

Gene expression:

Pseudomonas aeruginosa strains that produced every experimental virulence factor were grown on Luria Bertani medium. TRIzolTM Reagent was used to extract total RNA in accordance with the manufacturer's instructions. The WizScript RT FDmix Kit (Wizbio solution/Korea) was utilized to generate complementary DNA (cDNA) from total RNA. QRT-PCR was used to measure the expression of the RhlR QS and LasI genes. As directed, Wiz Pure TM qPCR Master Mix (SYPER) was employed in the reaction mix. cDNA synthesis was carried out during thermal cycling at 50 °C for 15 minutes. Thermo-Start activation is carried out for 60 minutes at 95 °C, then there are 40 cycles of denaturation (20 seconds at 95 °C), annealing (20 seconds at 50–60 °C), and extension (20 seconds at 72 °C). The housekeeping gene recA served as a reference for normalizing the quantified gene expressions. Melt curves verified the specificity of the reaction. Utilizing the comparative threshold cycle (CT) approach ($2^{-\Delta\Delta Ct}$) [15], the relative changes in RhlR and LasI expression were ascertained.

Table 1; primers used in this study

Primer Name		Primer sequence (3′- 5′)	Size(bp)	Annealing temperature
LasI	F	CTACAGCCTGCAGAACGACA	168	58 °
	R	ATCTGGGTCTTGGCATTGAC		
RhlR	F	AGTTGCTGACCCAGAAGCTG	181	58 °
	R	TGGATGTTCTTGTGGTGGAA		
recAs	F	GCGGTGAAAGAAGGTGATGA	120	

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	R	GTAGATGCCCTTGCCGTAAA		58 °

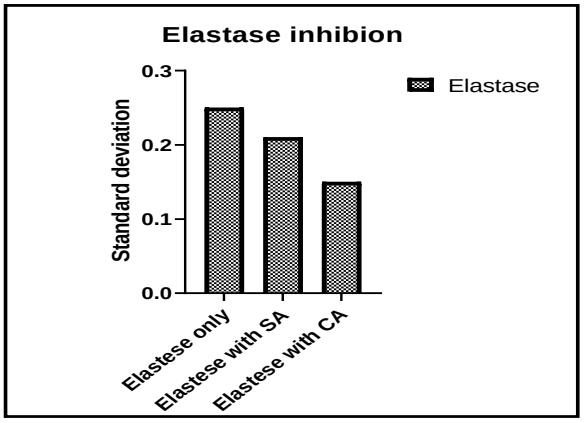

Figure1;Standard deviation values of the elastase in the presence of inhibitors compared to the control. there are significant differences at P-values < 0.05.

Effect of QSI on Protease:

results of this study indicate that both compounds have an inhibitory effect on the production of proteases, as it was found that there was a significant reduction when the isolates were treated with SA&CA,(Mean,±SD) was (0.4396±0.2280), (0.0528±0.0595)and (0.0830±0.0504) for Control, SA and CA respectively (figure 2).

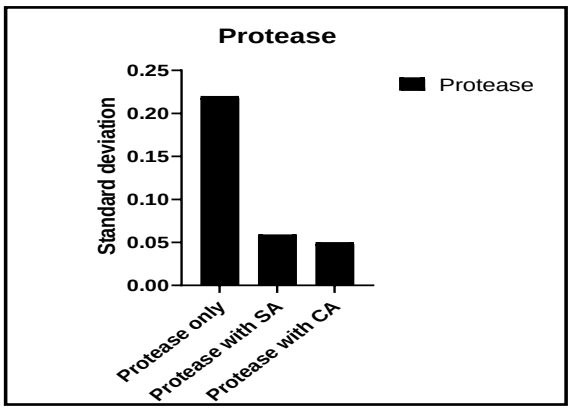

Figure2; Standard deviation values of Protease in the presence of inhibitors compared to the control treatment. there are significant differences at P-values < 0.05.

Statistical analysis

The statistical program GenStat-Tenth Edition, version 10.3.0.0 was used to analyze the data in accordance with the simple experiment system, which applied the Complete Randomized Design (CRD) with three replications. The significant differences of the mean and standard deviation were tested using the LSD (least significant difference test) at a probability level of 0.0516.

Result

Diagnoses of *P. aeruginosa*: After microscopic and biochemical identification, a total of 50 isolates were Pseudomonas aeruginosa. The Sub-MIC of CA and SA were 0.25 µg/ml and 250 µg/ml respectively.

Effect of QSIs on Elastase:

Elastase was measured at a wavelength of 495 nm using a spectrophotometer. The current study's findings show that, albeit in different amounts, 31 (62%) of the P. aeruginosa isolates were able to produce elastase. Elastase had a maximum absorbance of 0.7 and a minimum absorbance of 0.03. to test the ability of CA observed a significant (p- values >0.05) reduction in the enzyme. The results in the current study showed a decrease in elastase with CA was 27.6% and with SA 59.6%, compared to the untreated. The results of the current study showed that there were significant differences in the mean of the elastase coefficients (figure 1), where by the treatment of the isolates with SA gave the highest inhibition rate, with a mean 0.1138, compared to CA, which reached the lowest inhibition, where the mean was 0.2043 compared to the untreated 0,2825. The ±SD value was ± 0.151 for SA and ± 0.217 for CA, while for control was ± 0.256.

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than that of SA, where the treatment of cells with CA reduced the gene expression of rhlR gene from 1 to 0.2-fold, and for lasI gene, the decrease was from 1 to 0.3-fold. As for the treatment of cells with SA at a concentration 250 µg (sub-MIC), it also caused inhibition of the rhlR and lasI QS genes. It led to a reduction of rhlR from 1 to 0.5-fold, but the total reduction of this inhibitor was less than CA.

Effect of QSI on Gene Expression

The current investigation found that the tested isolates that generated Lasl and RhlR also produced protease and elastase, while SA and CA inhibited these two genes. Concentration sub-MIC of CA (0.25 µl/ml) and SA (250 µg/ml) led to a decrease in the gene expression of RhlR and lasI genes compared to the control (without treatment). The effect of CA on those genes was greater

Table1; Effect of CA and SA on Virulence factors, QS lasl and RhlR genes in *P. aeruginosa* isolates.

Elastase production	Protease production	*Lasl* Ct	*RhlR* Ct	NO.
0.05	0.7	20.41	19.38	Ps control
0	0.1	22.15	21.2	P1 with CA
0.03	0.009	20.84	21.87	P1 with SA
0.09	0.12	21.44	23.36	P2 with CA
0.01	0.03	21.11	21.21	P2 with SA

Discussion

Natural substances significantly reduce the expression of some genes; SA suppresses the expression of QS genes. Compared to the SA, which successfully inhibited the regulatory proteins (RhlR) and the AHL synthases, the chemical CA was more efficient in inhibiting those genes. (Lasl). This mean that CA displayed strongest interaction with the QS genes LasI binds to the LasR transcriptional activator and triggers the synthesis of many virulence enzymes, including LasA protease, LasB elastase [21]. RhlI and RhlR are the two components of the Rhl system, while lasI and lasR are the two components of the Rhl system [22]. CA is considered the most active in this study in suppressing genes and virulence factors than SA, and this is due to its large molecular weight that qualified it to possess this high activity. It also works to stop a number of metabolic processes inside the cell. And acts as an anti- AHL las, and Rhl which, Modulation or inhibition of QS by blocking the binding of AHL to its receptors [23].

Protease is a virulence factor that functions as a proteolytic enzyme during an acute infection. Protease production is controlled by the Rhl and Las systems. The current study found that the tested isolates that generated Lasl and RhlR produced proteases, and that inhibiting these two genes with SA and CA decreased protease production by 87% and 81%, respectively, compared to the control. Several previous investigations have shown that defective QS genes in isolates result in low amounts of protease [12], which agrees with our findings (table 1). Increased protease activities were associated with tissue damage. Therefore, a significant decrease in protease production with SA suggests that SA may reduce infection virulence and the inflammatory response during acute infections [18].

Tissue injury was correlated with increased protease activity. In light of this, a substantial reduction in protease synthesis with SA implies that SA may lessen the virulence of infections and the inflammatory response in acute infections [18].SA decreased P.

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aeruginosa's ability to invade corneal epithelial cells and to cause the death of epithelial cells. Moreover, growing in SA markedly decreased P. aeruginosa's synthesis of proteases. The results were consistent with [19], which showed that natural compounds are highly effective in reducing virulence factors, including the production of proteases, where treatment of the isolates with CA and SA reduced the production of proteases. While [20] revealed that the isolates were treated with SA, proteases were decreased by 50%, and salicylic acid was found to adversely regulate virulence factors in P. aeruginosa. It exploits the Las QS system, which comprises of LasI interacting with the LasR transcriptional activator to activate the protease virulence gene, leading in protease synthesis.

Furthermore, Elastase The pathogenicity of P. aeruginosa is largely responsible for the severe tissue damage it causes. shown that the isolates under investigation that expressed Lasl and RhlR were elastase-producing, and that when SA and CA inhibited these two genes, the amount of elastase produced dropped by 27.6% and 59.9%, respectively. QS regulates the formation of elastase, which is produced by LasI and lasR. These results are consistent with the findings of [17], which indicated in his research that SA has a significant effect on the inhibition of elastase in P. aeruginosa. It is also in line with observation [12], which demonstrated that these organic substances decreased the synthesis of elastase without influencing the development of the bacteria. This study shown that naturally occurring substances originating from plants significantly hinder P. aeruginosa's ability to suppress its QS systems. This can lower these components' production without influencing the development of the microorganisms that produce them. The steadily declining effectiveness of antibiotics necessitates alternative approaches. Furthermore, natural substances are more targeted than antibiotics

Conclusion

Natural compounds CA & SA significantly reduced the phenotypic virulence factors; protease, elastase and reducing the expression of the RhlR and lasl genes. Thus, results could give hope in the fight against resistant P. aeruginosa isolates by inhibiting QS genes regulating the gene expression of virulence factors.

References:

1. Özen AI, Ussery DW. Defining the Pseudomonas genus: where do we draw the line with Azotobacter?. Microbial ecology. 2012 Feb;63:239-48.
2. Diggle SP, Whiteley M. Microbe Profile: Pseudomonas aeruginosa: opportunistic pathogen and lab rat. Microbiology. 2020 Jan;166(1):30.
3. Faure E, Kwong K, Nguyen D. Pseudomonas aeruginosa in chronic lung infections: how to adapt within the host?. Frontiers in immunology. 2018 Oct 22;9:2416.
4. Kessler E, Safrin M. Elastinolytic and proteolytic enzymes. Pseudomonas Methods and Protocols. 2014:135-69.
5. Grabski H, Tiratsuyan S. Mechanistic insights of the attenuation of quorum-sensing-dependent virulence factors of Pseudomonas aeruginosa: Molecular modeling of the interaction of taxifolin with transcriptional regulator LasR. bioRxiv. 2018 Dec 19:500157.
6. Badawy MS, Riad OK, Taher FA, Zaki SA. Chitosan and chitosan-zinc oxide nanocomposite inhibit expression of LasI and RhlI genes and quorum sensing dependent virulence factors of Pseudomonas aeruginosa. International journal of biological macromolecules. 2020 Apr 15;149:1109-17.
7. Le Berre R, Nguyen S, Nowak E, Kipnis E, Pierre M, Ader F, Courcol R, Guery BP, Faure K. Quorum-sensing activity and related virulence factor expression in clinically pathogenic isolates of Pseudomonas aeruginosa. Clinical microbiology and infection. 2008 Apr 1;14(4):337-43.
8. Boakye YD, Osafo N, Danquah CA, Adu F, Agyare C. Antimicrobial agents: Antibacterial agents, anti-biofilm agents, antibacterial natural compounds, and antibacterial chemicals. Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods. 2019 Feb 13;13:75.
9. Sendamangalam V, Choi OK, Kim D, Seo Y. The anti-biofouling effect of polyphenols against Streptococcus mutans. Biofouling. 2011 Jan 1;27(1):13-9.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(6), 59-65 | ISSN:2251-6727

10. Topa SH, Subramoni S, Palombo EA, Kingshott P, Rice SA, Blackall LL. Cinnamaldehyde disrupts biofilm formation and swarming motility of Pseudomonas aeruginosa. Microbiology. 2018 Sep;164(9):1087-97.
11. Ragno R, Papa R, Patsilinakos A, Vrenna G, Garzoli S, Tuccio V, Fiscarelli E, Selan L, Artini M. Essential oils against bacterial isolates from cystic fibrosis patients by means of antimicrobial and unsupervised machine learning approaches. Scientific reports. 2020 Feb 14;10(1):2653.
12. Ahmed SA, Rudden M, Smyth TJ, Dooley JS, Marchant R, Banat IM. Natural quorum sensing inhibitors effectively downregulate gene expression of Pseudomonas aeruginosa virulence factors. Applied microbiology and biotechnology. 2019 Apr 2;103:3521-35.
13. Wang H, Tu F, Gui Z, Lu X, Chu W. Antibiotic resistance profiles and quorum sensing-dependent virulence factors in clinical isolates of Pseudomonas aeruginosa. Indian journal of microbiology. 2013 Jun;53:163-7.
14. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008 Jun;3(6):1101-8.
15. Todorović MM, Zvrko EZ. Immunoregulatory cytokines and chronic tonsillitis. Bosnian journal of basic medical sciences. 2013 Nov;13(4):230.
16. Bandara BM, Sankaridurg PR, Willcox MD. Non-steroidal anti inflammatory agents decrease bacterial colonisation of contact lenses and prevent adhesion to human corneal epithelial cells. Current eye research. 2004 Jan 1;29(4-5):245-51.
17. Caballero A, Thibodeaux B, Marquart M, Traidej M, O'Callaghan R. Pseudomonas keratitis: protease IV gene conservation, distribution, and production relative to virulence and other Pseudomonas proteases. Investigative ophthalmology & visual science. 2004 Feb 1;45(2):522-30.
18. Guendouze A, Plener L, Bzdrenga J, Jacquet P, Rémy B, Elias M, Lavigne JP, Daudé D, Chabrière E. Effect of quorum quenching lactonase in clinical isolates of Pseudomonas aeruginosa and comparison with quorum sensing inhibitors. Frontiers in microbiology. 2017 Feb 14;8:227.
19. Li WR, Ma YK, Shi QS, Xie XB, Sun TL, Peng H, Huang XM. Diallyl disulfide from garlic oil inhibits Pseudomonas aeruginosa virulence factors by inactivating key quorum sensing genes. Applied microbiology and biotechnology. 2018 Sep;102:7555-64.
20. Mukherjee S, Moustafa D, Smith CD, Goldberg JB, Bassler BL. The RhlR quorum-sensing receptor controls Pseudomonas aeruginosa pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. PLoS pathogens. 2017 Jul 17;13(7):e1006504.
21. El-Mowafy SA, Abd El Galil KH, El-Messery SM, Shaaban MI. Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in Pseudomonas aeruginosa. Microbial pathogenesis. 2014 Sep 1;74:25-32.
22. Kim HS, Lee SH, Byun Y, Park HD. 6-Gingerol reduces Pseudomonas aeruginosa biofilm formation and virulence via quorum sensing inhibition. Scientific reports. 2015 Mar 2;5(1):8656.
23. Bjarnsholt T, Jensen PØ, Jakobsen TH, Phipps R, Nielsen AK, Rybtke MT, Tolker-Nielsen T, Givskov M, Høiby N, Ciofu O, Scandinavian Cystic Fibrosis Study Consortium. Quorum sensing and virulence of Pseudomonas aeruginosa during lung infection of cystic fibrosis patients. PloS one. 2010 Apr 12;5(4):e10115.