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RESEARCH ARTICLE

Biocontrol of Pseudomonas Aeruginosa Biofilm Using Bacteriophages

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Abstract

Pseudomonas aeruginosa has been mentioned as a remarkable case that contaminated animate and inanimate surfaces. Clinically, it is considered essential in the practical therapy. In current study, the inhibition effect of bacteriophages against biofilm of P. aeruginosa detected by ELISA method had been assessed. 50 samples had been collected from wounds infections in General Baqubah Hospital to isolate Pseudomonas aeruginosa and tested for 10 groups of antibiotics. Befor the treatment, Pseudophage was isolated from sewage water of General Baqubah Hospital through pseudophage enrichment. The biocontrol of biofilm activity of pseudophage was applied by using the ELISA method. For the 50 samples were collected from wounds infections, and 10 isolates only were able to grow on Pseudomonas and Cetrimide agar. P. aeruginosa isolates showed high resistance associated with Cefepime (30 µg), Fusidic acid (10 µg) and Ampicillin (10 µg) 9(90%) while P. aeruginosa isolates showed high resistance associated with Erythromycin 80% then Imipenem and Tetracycline 70%. All 10 P. aeruginosa isolates have the production of biofilm. 4 P. aeruginosa bacteriophages (Pab 1, Pab 2, Pab 3, Pab 4) were isolated from sewage water, the Pab4 had a wide range. P. aeruginosa were sensitive to pseudophage by spotting assay were inhibited the biofilm formation. Active effect of pseudophage to inhibit Pseudomonas aeruginosa' biofilm in vitro.

Introduction

Biofilms are the accumulation of microorganisms which their excretion yields can be attached to animate or inanimate surfaces. Biofilm-associated bacteria are tolerant to antimicrobial agents, can evade the host immune system, and can act as a source for infection [1]. Pseudomonas aeruginosa is the cause of harmful infections that can be hardy to effective treatment with conservative antibiotics.

However, it shows obvious development in biofilms. Pseudomonas aeruginosa had been proposed as a distinguished case that contaminated animate and inanimate surfaces where clinically, it can be considered essential for practical therapy [2]. A number of novel strategies have been proposed to more effectively prevent and control device-associated biofilms. One of these strategies is the utilization of bacteriophages (phages) [3]. Phages have been applied for the treatment of infectious diseases in plants [4], animals [5], and humans [6].

Bacteriophages act differently on bacteria contained within biofilms than do chemical antibiotics or other biocides. At least, there are four mechanisms underlying this difference (1)

- Bacteriophages replicate within their host cells, resulting in localized increases in bacteriophage numbers (amplification). This can lead to an increase in the numbers of infectious progeny bacteriophages into the biofilm. By spreading through the biofilm (1) or during the lytic replication cycle, the infection of a bacterial host cell by a single phage virion will result in the production of dozens or hundreds of progeny phage [7, 8].
- Some of the Bacteriophages have express depolymerizing enzymes that degrade the extracellular polymeric substance EPS matrix of a biofilm. Phages encoding depolymerizes can play a particular role in forming of biofilm cultures [1, 9].

- Bacteriophages can induce depolymerizing enzymes that degrade the EPS of the host genome. (1)
- Persisted cells can be infected by bacteriophages; however, the bacteriophage cannot replicate within them and destroy inactive cells. In contrast, they can remain within these bacteria until they reactivate and then begin the productive infection, which leads to destroying the cells. Thus, phages had been choosing to control biofilms [1, 9].

Due to the limited success of antibiotic therapy in treating biofilms [10], many researchers had studied the potential of using the bacteriophages or deriving the enzymes to treat the biofilms [11, 14].The cause behind this ability is usually due to the expression of depolymerizes capable of dispersing the biofilm through enzymatic digestion of extracellular polymeric substances, the main obstacle to antibiotic treatment or phage therapy [15].

The ability of phages to disrupt this biofilm is a valuable phenotype of phage therapy candidates. The depolymerazes expressed by phages digest these polymeric substances so as to obtain access to cell surface receptors [16, 17], however, it has been noted that the depolymerase activity alone may not be sufficient to disrupt the biofilm and the ability of the phage to amplify in the biofilm is crucial for biofilm treatment [18].

Phage-associated depolymerase activity can easily be identified in phages of interest through analysis of plaque morphology where depolymerase-expressing phages usually form a plaque surrounded by a large halo indicative of its degrading activity [19]. This phenotype has been observed for phages infecting members of several genera including Pseudomonas [16], Klebsiella [20], Staphylococcus [21] and Escherichia [22], thus representing a useful in vitro marker for phages of interest [23].

Material and Methods

Bacterial Strain Isolation and Identification

This study was carried out from October 2017 till May 2018. 50 samples were collected from wounds infections in General Baqubah Hospital; samples were cultured on Pseudomonas agar then subculture on cetrimide agar. Cultural, Microscopical and Biochemical characterization was applied in order to identification P. aeruginosa [24]. All bacterial strains were cultured at 37°C in nutrient broth or on agar medium.

Antibiotic Susceptibility Testing

The antimicrobial susceptibility assay was performed on Mueller-Hinton agar by the disc-diffusion method (Kirby– Bauer) [25]. Selection of antibiotics and growth inhibition zones were interpreted according to the Clinical Laboratory Standards Institute. The antimicrobial disks: Gentamycin (10 µg), Cefepime (30 µg), Amikacin (30 µg), Imipenem (10 µg), Erythromycin (15 µg), Amoxi/clavulanic acid (30 µg), Cefriaxone (30 µg), Fusidic acid (10 µg), Tetracycline (30 µg), and Ampicillin (10 µg) were of commercial grade (Mast Group, UK).

Detection of Biofilm Production

By microtiter plate and an enzyme-linked immunosorbent assay (ELISA) [26] the plate divided to 4 replicates were prepared for each isolate and Wells for negative control filled with 100µL sterile nutrient broth. The plate was then incubated for 24 h at 37◦C in the incubator.

The liquid contents of the bacterial wells were gently removed, followed by washing with sterile phosphate-buffered saline (pH 7.2) to remove any remaining planktonic cells and air-dried for 15 min to fix adherent organisms. Staining of biofilms with crystal violet was performed for 15 min the stained wells were rinsed off by placing the plate under running tap water and dried for 15 min. the optical densities (OD) of stained adherent bacterial films were read in ELISA reader at 490 nm. The value for each isolate (Ai) was compared with control (Ac) depending on the following:-

Ai>2*Ac (strong biofilm): Ac<Ai<2*Ac (Moderate biofilm): Ai<Ac (no biofilm)

Bacteriophage

Two containers (2 liters) of sewage sludge were obtained from the waste water treatment facility located in Sheishein area at Tikrit- Salah al din Governorate.

To produce larger scale of pseudophage was used broth media as described by Sambrook and Russel [27] with some modifications.

Briefly, the plaques were scraped off using a sterile lance and were transferred to sterile nutrient broth (100ml) containing Pseudomonas aeruginosa and incubated for about 24 h at 37°C. Then was centrifuged at 5000 rpm for 15min and filtered through (0.22µ) Millipore Membrane Filter. Then was collected in sterile bottles finally chloroform was added (1:10) and stored at 4°C until further use [27].

Stocks of 4 P. aeruginosa bacteriophages (Pab 1, Pab 2, Pab 3, Pab 4), prior to each assay, the stock suspension of each bacteriophage was titrated against a selected P. aeruginosa bacterial strain using the soft agar overlay small drop assay, as described below. Equal concentrations of each bacteriophage were used.

Titration by Double Layer Assay

Phage titer was determined by using the double-layer agar method as designated by Adams. Briefly, 100 µl of Pseudomonas aeruginosa overnight culture, 100 µl of diluted Pab phage solution in SM buffer, and 3 ml of molten soft agar (pre-warmed in a water bath at 45 °C) mixed well and directly poured in Petri dish containing a 1.5 % nutrient agar. Then were incubated overnight after which plaques were counted on each plate and multiply by inverted dilution [28].

Sensitivity to Bacteriophage

The ability of phage to infect isolates of strains P. aeruginosa was determined by spotting technique [29] in brief spotting inoculated at the center of each plate cultured by one isolates. Then the plates were incubated at 37°C and examined after 24 h. A clear zone in the bacterial lawn was recorded as host susceptible.

Inhibition Biofilm by Bacteriophage

The microtitre plate biofilm formation assay as described by O'Toole was used to assess the ability of 4 locally isolates of bacteriophages to eradicate P. aeruginosa biofilms in vitro [30]. Treatments included each of the 4 phages in nutrient broth at concentrations of 107 and 108 PFU/mL, as well as equivalent volumes of phage stocks of the 4 phages, with nutrient broth as positive control and nutrient broth with bacteria as a negative control.

180 µL of each treatment was plated in duplicate and biofilms were assessed at 24 h after treatment. The biofilm plates were gently washed twice with sterile PBS, and then stained with 190 µL/well of 0.5% crystal violet for 30min. The stained plates were rinsed off by placing the plate under running tap water and dried for 15 min, and left to dry. And the plate was incubated at room temperature for 30min. Absorbance at 490 nm was measured for each well using the ELISA reader.

Results and Discussion

Isolation Bacteria

For the 50 samples which collected from wounds infections in General Baqubah Hospital, 10 isolates were identified as P. aeruginosa.

Sensitivity to Antibiotics

The result of antibiotics sensitivity test of 10 P. aeruginosa isolates showed high resistance associated with Cefepime (30 µg), Fusidic acid(10 µg) and Ampicillin(10 µg) 9(90%) followed by Amoxi- Clavulanic acid 80%then Gentamycin 60% and Amikacin and ceftriaxone 50%. While P. aeruginosa isolates showed high resistance associated with Erythromycin 80% then Imipenem and Tetracycline 70%. From this results indicated that the 10 isolates were multidrug resistance as in Figure (1).

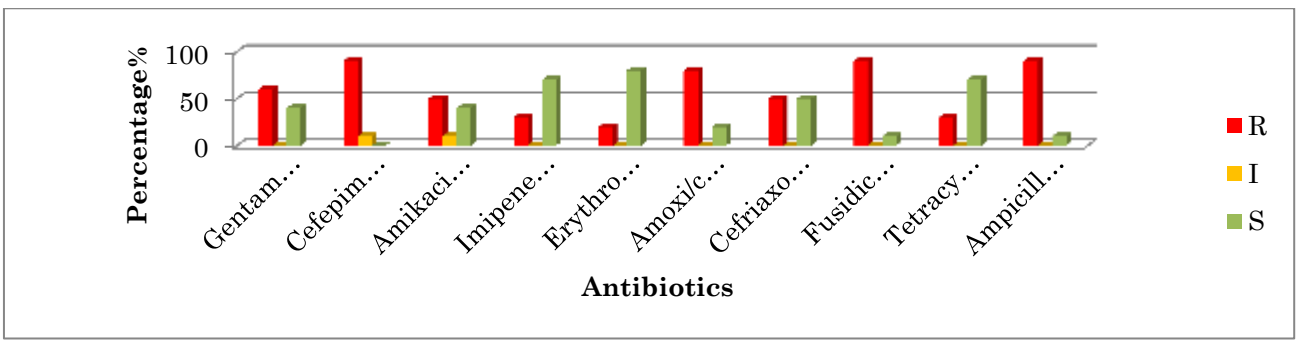

Figure 1: Sensitivity of P. aeruginosa isolates to antibiotics

Biofilm Formation

All 10 P. aeruginosa isolates have the production of biofilm when detection in micro titer plate by ELISA. Biofilm-associated bacteria are tolerant to antimicrobial agents and populations diversify (1), it is necessary to have monitoring programs to continually evaluate prevalent strains of problematic pathogens.

Isolation of Pseudophage

4 P. aeruginosa bacteriophages (Pab 1, Pab 2, Pab 3, Pab 4) were isolated from sewage water by enrichment method. The titer of Pab 1, Pab 2, Pab 3, Pab 4 was 5*109 PFU/ml, 2*108 PFU/ml, 1*109 PFU/ml and 6*107 PFU/ml respectively. Then each stock was stored in the refrigerator after chloroform added (1:10) until use. Many previous studies isolated pseudophages [2, 31] at the isolation step, in these study only virulent phages (lystic) were selected as candidates for phage therapy while temperate phages (lysogenic) were excluded as these will easily convert hosts into (phage-resistant) lysogens, thus making them incapable of causing immediate lysis [32]. Plaques with turbidity were avoided, typical of temperate phages will assist in the selection of virulent phages produced clear plaques [33].

Host Range

The 10 biofilm isolates described above (designated biofilm variants) that were tested for their susceptibility to 4 environmental phages isolated in this study by spotting method. The results are shown in figure (2). 6/10 isolates (60%) were lysed by Pab4, 4/10 isolates (40%) were lysed by Pab3, 2/10 isolates (20%) were lysed by Pab1 and Pab2 with full activity spots (clear zone) seen on the small drop plaque assay. So the Pab4 had wide range while Pab1 and Pab2 have narrow range. One of the reasons associated with phage therapy is the emergence of phage-resistant variants of pathogenic bacteria with increased fitness.

The host range of a phage reflects its ability to (lytically) infect strains within a given test panel where narrow host range phages infect a small number of strains and broad host range phages infect a wide range of strains. Phages may exhibit narrow or broad host range depending on (i) the presence of anti-phage mechanisms in the test strains and; (ii) the presence of generalized (highly conserved) or specialized (variable, non-conserved) host-encoded phage receptors.

While broad host range phages are generally more acceptable due to the increased likelihood that clinical isolates that emerge will be infected, narrow host range phages may be useful in certain scenarios. In contrast to antibiotics, which are broad spectrum antimicrobial agents, the use of narrow host range phages presents a new opportunity (23).

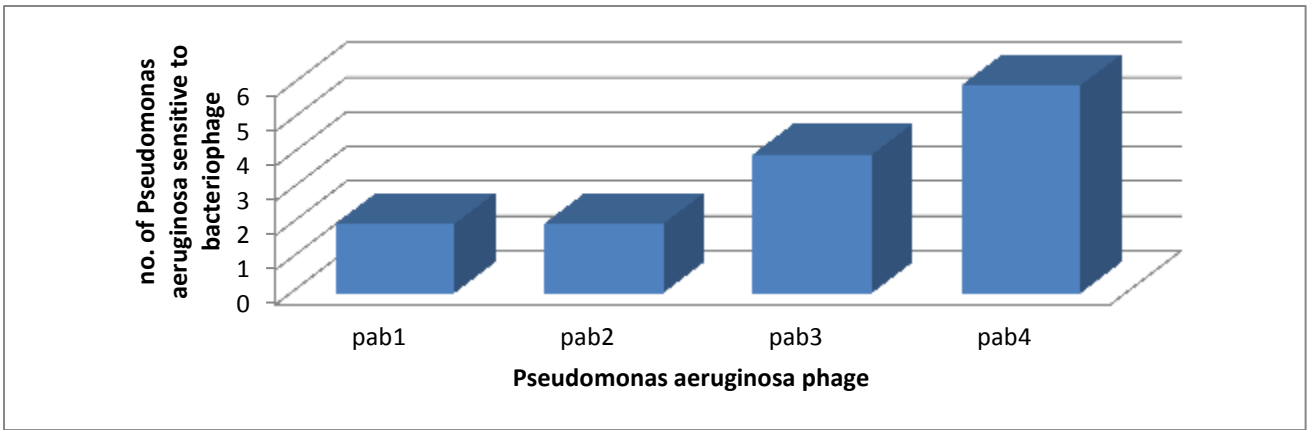

Figure 2: Host range of phages clinical isolates

While targeted narrow host range phage therapies may have the potential for specific applications, it is likely that broad host range phages will continue to be the preferred option as they possess a more powerful destructive potential against a wider range of pathogenic isolates. Furthermore, even those phages that are classified as exhibiting a broad host range would still be considered to possess a narrow activity spectrum relative to antibiotics. Antibiotics may be effective against multiple genera of bacteria, while phages are rarely genus-specific, but most species-or strain-specific [34, 35].

Phage-host interactions require both a host-encoded receptor(s) and a phage-encoded receptor binding protein (RBP). The receptor presented on the cell surface may be a carbohydrate, protein [36] all Pseudomonas phage-host systems characterized to date attach to saccharidic receptors. Therefore, it is clear that there is a diverse array of interactions at play among these phage-host combinations [23].

Inhibition Effect of Pseudophage on Biofilm

The 10 biofilm isolates described above (designated biofilm variants) that were tested for inhibition biofilm formation by 4 pseudophages isolated in this study by using a microtiter plate. The results are shown in Table (1). All P. aeruginosa were sensitive to pseudophage by spotting assay were inhibited the biofilm formation. Pab4 displayed suitable anti-biofilm activity in vitro.

Table 1: inhibition biofilm formation by pseudophage

Phage id Bacterial id	Pab1	Pab2	Pab3	Pab4
1	N	N	I	I
2	N	N	N	N
3	I	N	I	N
4	N	I	I	I
5	N	N	N	I
6	N	N	I	I
7	N	N	N	N
8	I	I	N	I
9	N	N	N	N
10	N	N	N	I

I (inhibition biofilm); N (no inhibition biofilm)

The first experiments involving the use of phages in fighting biofilms were published in 1995 [37] Many experiments using various bacteriophages and various bacterial biofilms have been conducted to date (11, 12, 13, 14), suggesting that phages are capable of reducing the bacterial population in this particular form of bacterial cultivation. In theory, biofilm should become infected faster than planktonic cells, as the vicinity of the cells in the biofilm structure may increase the phage replication rate [38].

But, the structure and composition of the biofilm, as well as the physiology of biofilm cells, may impose some limitations in this regard. Various imaging techniques, including con-focal microscopy with fluorescent in situ hybridization (FISH) and atomic force microscopy have revealed the heterogeneity of biofilm structures with a diverse distribution of cells, matrix, and water-filled channels and pores.

It is worth mentioning that many biofilms have open structures with water-filled channels that facilitate phage access inside the biofilm [39, 40, 41] demonstrated the ability of Lactococcus phage c2 to penetrate the biofilm through water channels and cell clusters in addition the radial movement of T4 phage molecule across the biofilm, similar to the process of forming clear patches in the bacterial lawn, suggesting that biofilms may be destroyed by single phage doses. The studies showed that both single- and dual-species biofilms may be effectively con¬trolled by phages; Doolittle et al. showed that progeny phage will propagate radially through a biofilm. At least in theory, a single phage dose should be capable of treating a biofilm infection as progeny phage infect adjacent cells and degrade the biofilm matrix [41].

Further research on simultaneous used in combination phages with other antimicrobial agents also seems justified [42]. Curtin and Donlan [43] demonstrated that a phage that is active against Staphylococcus epidermidis could be incorporated into a hydrogel coating on a catheter and significantly reduce biofilm formation by this organism in an in vitro model system.

The application of Listeria phage with a quaternary ammonium compound displayed a synergistic effect [44]. An 85% reduction in Pseudomonas fluorescens biofilm mass was observed after treatment with phage FS1 [45]. Fu 2010 showed that the pretreatment of catheters with pseudophages cocktail reduced the 48-h mean biofilm cell density by 99. 9 %, but fewer biofilm isolates were

resistant to these phages [9]. Phage AZ1 significantly reduced both planktonic cells of Pseudomonas aeruginosa and 48 h old biofilm biomass about 3-fold as compared to control [46].

References

1. David R, Helena M, Parracho A, James W, Richard Sh, Gavin H, Maria W, Susan L, Sandra M (2014) Bacteriophages and Biofilms, Antibiotics, 3: 270-284.
2. David R, Enright M (2011) Bacteriophages for the treatment of Pseudomonas aeruginosa infections. Journal of Applied Microbiology, 111: 1-7.
3. Donlan RM (2009) Preventing biofilms of clinically relevant organisms using bacteriophage. Trends Microbiol., 17 (66-72): 4-26.
4. Momol (2007) Bacteriophages for plant disease control. Annu. Rev. Phytopathol., 45: 245-262.
5. Barrow P, Lovell M, Berchieri A (1998) Use of lytic bacteriophage for control of experimental Escherichia coli septicemia and meningitis in chickens and calves. Clin. Diagn. Lab. Immunol., 5: 294-298.
6. O'Flaherty S, Ross R, Meaney W, Fitzgerald G, Elbreki M, Coffey A (2005) Potential of polyvalent anti-Staphylococcus bacteriophage K for control of antibiotic resistant staphylococci from hospitals. Appl. Environ. Microbiol., 71: 1836-1842.
7. Hughes K, Sutherland I, Jones M (1998) Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbiology, 144: 3039-3047.
8. Hanlon G, Denyer S, Olliff C, Ibrahim L (2001) Reduction of exopolysaccharide viscosity as an aid to bacteriophage penetration through Pseudomonas aeruginosa biofilms. Appl. Environ. Microbiol., 67: 2746-2753.
9. Weiling F, Terri F, Oren M, John JC, Susan ML, Rodney MD (2010) Bacteriophage Cocktail for the Prevention of Biofilm Formation by Pseudomonas aeruginosa on Catheters in an In Vitro Model System, Antimicrobial Agents And Chemotherapy, 54(1): 397-404.
10. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. Int. J. Antimicrob. Agents, 35: 322-332.
11. Montañez-Izquierdo VY, Salas-Vázquez DI, Rodríguez-Jerez JJ (2012) Use of epifluorescence microscopy to assess the effectiveness of phage P100 in controlling Listeria monocytogenes biofilms on stainless steel surfaces. Food Control, 23: 470-477.
12. Khalifa L, Brosh Y, Gelman D, Coppenhagen-Glazer S, Beyth S, Poradosu-Cohen R, Que YA, Beyth N, Hazan R (2015) Targeting Enterococcus faecalis biofilms with phage therapy. Applied and Environmental Microbiolog., 81(8): 2696-2705.
13. Fenton M, Keary R, McAuliffe O, Ross RP, O'Mahony J, Coffey A (2013) Bacteriophage-derived peptidase CHAPK eliminates and prevents Staphylococcal biofilms. International Journal of Microbiology; ArticleID625341, 8.
14. Gutierrez D, Ruas-Madiedo P, Martinez B, Rodriguez A, Garcia P (2014) Effective Removal of Staphylococcal Biofilms by the Endolysin LysH5. PLOS ONE, 9(9):1-8.
15. Flemming H-C, Wingender J (2010) The biofilm matrix. Nat. Rev. Microbiol., 8: 623.
16. Cornelissen A, Ceyssens P-J, Krylov VN, Noben J-P, Volckaert G, Lavigne R (2012) Identification of EPS-degrading activity within the tail spikes of the novel Pseudomonas putida phage AF. Virology, 434: 251-256.
17. Majkowska-Skrobek G, Łaꞏtka A, Berisio R, Maciejewska B, Squeglia F, Romano M, Lavigne R, Struve C, Drulis-Kawa Z (2016) Capsule-targeting depolymerase, derived from Klebsiella KP36 phage, as a tool for the development of anti-virulent strategy. Viruses, 8: 324.
18. Pires DP, Melo LD, Boas DV, Sillankorva S, Azeredo J (2017) Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. Curr. Opin. Microbiol., 39: 48-56.

19. Kostakioti M, Hadjifrangiskou M, Hultgren SJ (2013) Bacterial biofilms: Development, dispersal, and therapeutic strategies in the dawn of the post antibiotic era. Cold Spring Harb. Perspect. Med., 3: a010-306.

20. Hsu C-R, Lin T-L, Pan Y-J, Hsieh P-F, Wang J-T (2013) Isolation of a bacteriophage specific for a new capsular type of Klebsiella pneumoniae and characterization of its polysaccharide depolymerase. PLoS ONE, 8: e70092.

21. Gutiérrez D, Martínez B, Rodríguez A, García P (2012) Genomic characterization of two Staphylococcus epidermidis bacteriophages with anti-biofilm potential. BMC Genomics, 13: 228.

22. Guo Z, Huang J, Yan G, Lei L, Wang S, Yu L, Zhou L, Gao A, Feng X, Han W (2017) Identification and Characterization of Dpo42, a Novel Depolymerase Derived from the Escherichia coli Phage vB_EcoM_ECOO78. Front. Microbiol., 8: 14-60.

23. Eoghan C, Douwe V, Jennifer M (2018) In Vitro Characteristics of Phages to Guide 'Real Life' Phage Therapy Suitability, Viruses, 10: 163: 1-20.

24. Mahon CR, Lehman DC, Manuselis G (2007) Textbook of Diagnostic Microbiology. 3rd edition. Elsevier. 508.

25. Clinical and Laboratory Standards Institute (CLSI) (2012) Performance standards for antimicrobial susceptibility testing, Seventeenth informational supplement, 27 (1).

26. Pires DP, Melo LD, Boas DV, Sillankorva S, Azeredo J (2017) Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. Curr. Opin. Microbiol, 39: 48-56.

27. Sambrook J, Russell D (2001) Molecular Cloning: A Laboratory Manual New York: Cold Spring Harbor Laboratory Press, Cold Spring Har.

28. Adams, MH (1959) Methods of study of bacterial viruses, 447-448. In Bacteriophages. Inter science Publishers, London, United Kingdom.

29. Douglas J (1975) "Bacteriophages". Chapman and Hall publishers, 4th ed., London. Pp.20-46.

30. O'Toole GA (2011) Micro titer Dish Biofilm Formation Assay. J. Vis. Exp., (47): e24-37, doi: 10.3791/2437

31. Jameel ZJ (2016) Isolation and Characterization of PAP a Lytic Bacteriophage Specific for Pseudomonas aeruginosa. Diyala Journal for Pure Science, 12 (4): 112-125.

32. Lin DM, Koskella B, Lin HC (2017) Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World J. Gastrointest. Pharmacol. Ther., 8(3):162-173.

33. Khan Mirzaei M, Nilsson AS (2015) Isolation of Phages for Phage Therapy: A Comparison of Spot Tests and Efficiency of Plating Analyses for Determination of Host Range and Efficacy. PLoS ONE 10(3): e0118-557.

34. Chatterjee S, Rothenberg E (2012) Interaction of bacteriophage l with its E. coli receptor, LamB. Viruses, 4: 3162-3178.

35. Xia G, Maier L, Sanchez-Carballo P, Li M, Otto M, Holst O, Peschel A (2010) Glycosylation of wall teichoic acid in Staphylococcus aureus by TarM. J. Biol. Chem., 285: 13405-13415.

36. Bertozzi Silva J, Storms Z, Sauvageau D (2016) Host receptors for bacteriophage adsorption. FEMS Microbiol. Lett., 363: fnw002.

37. Doolittle MM, Cooney J, Caldwell D (1995) Lytic infection of Escherichia coli biofilms by bacteriophage-T4. Can. J. Microbiol., 41: 12-18.

38. Hanlon GW (2007) Bacteriophages: an appraisal of their role in the treatment of bacterial infections. Int. J. Antimicrob. Agents 30: 118-128.

39. Sutherland IW, Hughes K, Skillman LC, Tait K (2004) The interaction of phage and biofilms. FEMS Microbiol. Lett., 232: 1-6.

40. Donlan RM (2009) Preventing biofilms of clinically relevant organisms using bacteriophage. Trends Microbiol., 17: 66-72.

41. Doolittle MM, Cooney J, Caldwell D (1996) Tracing the interaction of bacteriophage with bacterial biofilms using fluorescent and chromogenic probes. J. Ind. Microbiol., 16: 331-341.

42. Sillankorva SP Neubauer, J Azeredo (2010) Phage control of dual species biofilms of Pseudomonas fluorescens and Staphylococcus lentus. Biofouling, 26: 567-575.

43. Curtin JJ, RM Donlan (2006) Using bacteriophages to reduce formation of catheter associated biofilms by Staphylococcus epidermidis. Antimicrob. Agents Chemother, 50: 1268-1275.

44. Pires DP, Vilas Boas D, Sillankorva S, Azeredo J (2015). Phage therapy: a step forward in the treatment of Pseudomonas aeruginosa infections. J. Virol., 89: 7449-7456.

45. Sillankorva S, R Oliveira, MJ Vieira, IW Sutherland, J Azeredo (2004) Bacteriophage phiS1 infection of Pseudomonas fluorescens planktonic cells versus biofilms. Biofouling, 20: 133-138.

46. Saadia A, Fazal J, Muhammad I, Muhamma A, Tahir H, Chythanya R (2017) Isolation and characterization of a bacteriophage and its utilization against multi-drug resistant Pseudomonas aeruginosa-2995 Life Sciences, 190(1): 21-28. (abstract)

