ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 10, Issue 6, June 2023

Phenotypic Characterization of River Bacteriophage Against *Pseudomonas aeruginosa* Biofilm Formation

[1] James Patrick C. Picar, [2] Bernadette Anabelle P. Balitaan, [3] Mohanifah Baraocor, [4] Allen Dizon, [5] Julie Dequiña,[6] Venus Camal,[7]Marianne Ruth B. Espina, [8]John Paul Boisa

[1][2][3][4][5][6][7][8] College of Medical Laboratory Science
[1] Research Development and Innovation Center
[1][2][3][4][5][6][7][8] Our Lady of Fatima University
[1] jcpicar@fatima.edu, [2]phberanabelle.mls@gmail.com

Abstract*— Prevalence of multidrug resistant (MDR) pathogens has increased with time due to various anthropologic and environmental factors. Because of this, a challenge that arose in the field of healthcare is to find a corresponding treatment for infections resistant to common antibiotics. Bacteriophage is a virus that kills bacteria and is known as an alternative treatment to multidrug resistant pathogens. This study focuses on the phenotypic characterization of the river water isolate bacteriophage against a well-known pathogen, Pseudomonas aeruginosa. Based on the stability test, the isolated phage is stable at both basic and acidic environment however showed a decrease in titer when exposed to higher alkalinity. The thermal stability test showed stability on cold and imitated body temperatures. The study highlights the phenotypic characteristics and lytic action of the isolated bacteriophage against Pseudomonas aeruginosa biofilm formation.

***Index Terms*— biofilm inhibition, MTT Assay, stability test, microtiter, double overlay plaque**

I. INTRODUCTION

Extreme *Pseudomonas aeruginosa* is a common and ubiquitous environmental gram-negative bacillus that also grow in plant and animal tissues. Its distribution is attributed to its capacity to withstand adverse environmental niches and source their energy from various compounds. However ubiquitous in the environment, it rarely causes infection in immunocompetent individuals. Nearly all clinical cases of its infection have been associated with immunocompromised states such as AIDS and neutropenic patients undergoing sessions of chemotherapy. It causes infections like: a.) bacteremia in severe burn victims; b.) chronic lung infection in cystic fibrosis patients; and c.) acute ulcerative keratitis in people who uses soft contact lenses in long period of time [1].

This pathogen have various virulence factor including biofilm formation [2], which makes it innately resistant to antimicrobials [3]. Biofilms are sessile bacterial communities on surface environments such as in the blood vessels in vivo and catheters in vitro, which may “trap” other organisms and promote gene-exchange resulting to an enhanced survivability [4]. Bacterial biofilm infections are difficult to treat because of cell wall modification that excludes the original drug target site, or if still present, the antibiotics cannot reach to it. Another resistance mechanism the secretion of a sticky carbohydrate armor that is impermeable to antibodies nor antibiotics [5].

The emergence of multidrug resistant bacteria pose a serious problem in modern medicine. Concerns about going back to the era of “preantibiotics” became very real, and the development of alternative treatment have been one of the priorities in research. The use of bacteriophage had been among the innovative strategies to address multidrug resistance [6]. Phages, as antibacterial agents, have properties that make them compelling alternatives to chemical antibiotics. Meanwhile most concerns associated with phage therapy should be manageable through a combination of proper phage selection, effective formulation, and greater clinician understanding of and familiarity with product application [7].

This study focuses on the characterization and efficacy of bacteriophage isolated from sewage waters against biofilm forming, multidrug resistant *Pseudomonas aeruginosa.* This contributes to the body of knowledge regarding the study of bacteriophage and its therapeutic capability; and possibly a contribution on the study of phage therapy that will open new doors for a more complex treatment for drug-resistant infections, and finally for the benefit of those immunosuppressed individuals that is a target of the *Pseudomonas aeruginosa* infection.

II. MATERIALS AND METHODS

A. Sample Collection and Identification

The researchers obtained *Pseudomonas aeruginosa* from Our Lady of Fatima University—Quezon City, College of Medical Laboratory Science. It was then subjected to Vitek Analysis to verify its identity. The researchers collected samples for phage isolates from sewage water treatment plants in Tullahan River, Philippines; following proper and standardized protocol with regards to bacteriophage

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 10, Issue 6, June 2023

isolation.

B. Biofilm formation Assay

To confirm biofilm formation, an overnight culture was prepared in Tryptic Soy Broth whose concentration was corrected to OD 595. Then, 200uL of diluted 1:100 host inoculum was added on a flat-bottom polysterene microtiter plates which were then incubated at 37C for 24 hours over various intervals. After incubation, non-adherent cells were removed by washing the suspension thrice in Phosphate-buffered Saline (PBS). These were then stained with Hucker-modified crystal violet for 30 minutes at room temperature. After, the specimens were washed in distilled water to remove excess stain and then incubated to dry for 30 minutes. The macroscopic characteristics of the biofilm forming agent was photographed. Lastly, 70% ethanol was added to remove crystal violet stain and was then read using the specified optical density [8].

C. Amplification of Bacteriophage

5mL of stock bacterial suspension of *Pseudomonas aeruginosa* was added with 5mL DecaStrength Peptone Broth and 45mL of the collected sewage water sample. It was then maintained at room temperature on a shaker platform for 24 hours [9].

D. Isolation and Plating of Bacteriophage

10 mL of river bacteriophage culture in a sterile conical tube was spun at 35,000 RPM for 10 minutes. Bacteriophage was collected as a supernatant. This was repeated thrice to ensure the separation of sediments from the solution. To further isolate the phage from bacterial debris in the sample, aspirate the centrifuged samples with 10mL syringe barrel with 0.22micron filter [9].

E. Double Overlay Plaque Assay

Pseudomonas aeruginosa was incubated overnight in a nutrient broth (NB). It was then mixed with 5 mL of molten soft agar along with the phage suspension. The plates were labeled as Plate 1, Plate 2, and Plate 3. Plate 1 contained 50uL of phage lysate, Plate 2 had 150uL and plate 3 had 300uL of lysate. This was incubated at 37C for 24 hours. Indication of lysis and death of host bacterium was shown via plaque formation or spots. Stab a plaque-forming colony from the medium and purify the phage lysates [8,9].

F. Determination of Phage Titre

A serial tenfold dilution of the lysate suspension were done. Each tube contained 0.9ml of sterile Phosphate Buffer Saline and 0.1 ml of phage suspension. The following concentrations were 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} prepared. The samples were incubated at 37C for 24 hours. The titre was computed with formulae [9]:

$$\frac{\text{\# of plaques}}{\text{Dilution factor X volume of diluted virus added}} = \text{PFU/ml}$$

G. Biofilm inhibition Assay (MTT Assay)

100uL of *Pseudomonas aeruginosa* culture from TSB was dispensed on a 96-well microtiter plate. A 100uL of phage dilution suspension was then added to the wells and incubated for 16 hours. An addition of 20uL of MTT Reagent was done to differentiate viable from non-viable cells as only live cells can take up the dye. Color development was observed after 24 hours incubation. The solution was aspirated and added with 100uL of DMSO to each well. Absorbance was read at 595nm using a microtiter plate reader [8].

H. Temperature Stability Test

Phage cocktails were incubated at 37°C and 4°C for 24 hours. The cocktail after incubation, was subjected to plaque overlay assay to identify titer of the remaining phages. The procedure was adopted from Jonczyk et.al. with slight modifications [10].

I. pH Stability Test

Phage cocktail were incubated at 2°C for 24 hours while exposed to different pH levels: 3.0 and 4.0 pH for acidic environment and 10.0 for alkaline environment [10]. Cocktail titer was determined by plaque assay method. Adjustment of PBS to match various pH levels was via addition of drops of 1M HCl and 0.5M of NaOH [8].

J. Data analysis

The data collected was analyzed statistically with One-way Analysis of Variance (ANOVA) and Tukey HSD post-hoc analysis. For the other parameter, thermal stability testing, T-Test analysis was utilized.

III. RESULTS

3.1 Minimum Inhibitory Concentration (MIC)

Dilution	Trial 1	Trial 2	Trial 3	Mean
1.00E-01	3.000	2.997	2.998	2.998
1.00E-02	2.973	2.973	2.979	2.975
1.00E-03	2.775	2.77	2.772	2.772
1.00E-04	2.554	2.557	2.457	2.522
1.00E-05	2.447	2.449	2.552	2.483
1.00E-06	1.99	1.992	1.998	1.993
1.00E-07	1.435	1.432	1.434	1.434
1.00E-08	1.254	1.355	1.379	1.329
1.00E-09	1.035	1.039	1.045	1.04
1.00E-10	1.022	1.019	1.021	1.02
Control:	0.982	0.98	0.989	0.984

Table 1 Biofilm Formation Assay – Mean Absorbance

Table 1 presents the biofilm formation of the host bacterium in the following dilutions. The $1x10^{-1}$ dilution shows the greatest mean absorbance while $1x10^{-10}$ is the least absorbance value.

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 10, Issue 6, June 2023

	Trial 1	Trial 2	Trial 3	Mean
1 x 10^{-1}	1.342	1.342	1.349	1.344
1 x 10^{-2}	1.002	1.006	1.003	1.004
1 x 10^{-3}	1.012	1.011	1.014	1.012
1 x 10^{-4}	0.29	0.33	0.35	0.323
1 x 10^{-5}	0.326	0.323	0.329	0.326
1 x 10^{-6}	0.502	0.501	0.507	0.503
1 x 10^{-7}	0.532	0.537	0.533	0.534
1 x 10^{-8}	0.546	0.549	0.544	0.546
1 x 10^{-9}	0.675	0.677	0.679	0.677
1 x 10^{-10}	0.994	0.991	0.997	0.994
Control:	3.23	3.231	3.229	3.230

Table 2 Minimum Inhibitory Concentration (Treated with Bacteriophage) – Mean absorbance

Table 2 presents three trials for the minimum inhibitory concentration. It can be noted that dilutions 1x10^{-4} and 1x10^{-5} the least absorbance, indication the MIC.

DILUTION	Untreated	Treated
1.00E-01	2.998	1.344
1.00E-02	2.975	1.004
1.00E-03	2.772	1.012
1.00E-04	2.522	0.323
1.00E-05	2.483	0.326
1.00E-06	1.993	0.503
1.00E-07	1.434	0.534
1.00E-08	1.329	0.546
1.00E-09	1.04	0.677
1.00E-10	1.02	0.994
control	0.984	3.23

Table 3 Comparison of Mean between Untreated and Treated

Table 3 is shown to compare side by side the following means between the untreated and treated concentrations stated. 1x10^{-1} untreated concentration is higher than treated and can indicate the loss of bacteria via the lysate.

t-Stat	T Critical	Interpretation	Decision
3.126511909	6.313751515	Significant	Reject Ho

Table 4 t-Test Assuming Two Sample of Unequal Variances

Table 4indicates the t-Test analysis of the untreated vs. treated means to identify if there is a significant difference or none. The t-Stat value is 3.127 and the T critical is 6.314 which indicates significant difference.

3.2 pH and Thermal Stability Test

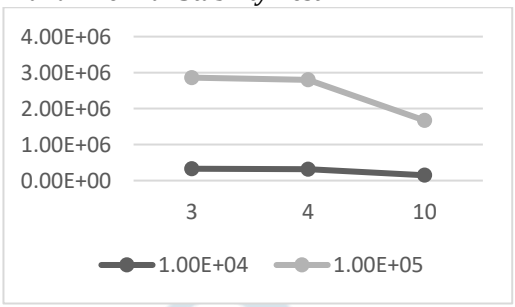

Figure 2 pH stability testing

Figure 2 shows the plaques counted as PFU/mL as opposed to pH exposure of the phage. The lysate 1 x 10 ^{4} shows stability on pH 3.0 to pH 10.0. Although it has a stability in pH 10.0, the colonies decreases as it is mostly stable in an acidic environment. While the lysate 1 x 10^{5} shows stability only on pH 3.0 and pH 4.0.

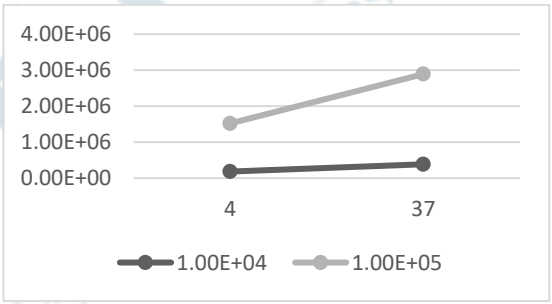

Figure 3 Thermal stability testing

Figure 3 illustrates the thermal stability testing of bacteriophage exposed to two different temperatures. It will determine the ideal storage and further identification of river bacteriophage. The table shows that the thermal stability of bacteriophage against *Pseudomonas aeruginosa* in a dilution 1 x 10^{4} and 1 x 10^{5} is from 37C and 4C.

The table below shows the minimum inhibitory concentration of the pH stability testing under the dilution of 1 x 10^{4}. As seen, the minimum inhibitory concentration of pH 3.0 with the dilution of 1 x 10^{4} shows the mean of 3.29 x 10^{5}. In the pH 4.0 the mean is 3.15 x 10^{5} and in pH 10.0 the mean is 1.48 x 10^{5}.

P pH	Trial 1	Trial 2	Trial 3	Mean
3	3.20x10^{5}	3.34x10^{5}	3.32x10^{5}	3.29x10^{5}
4	3.12x10^{5}	3.15x10^{5}	3.17x10^{5}	3.15x10^{5}
10	1.45x10^{5}	1.48x10^{5}	1.52x10^{5}	1.48x10^{5}

Table 5 Minimum inhibitory concentration (pH) 1 x 10^{4}

The table below shows the MIC of the pH stability testing in dilution 1 x 10^{5}. As seen, the MIC of pH 3.0 with the dilution of 1 x10 ^{5} shows the mean of 2.86 x 10^{6}. On the other

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)

Vol 10, Issue 6, June 2023

hand, the mean of pH 4.0 is 3.14 x 10^6 and in pH 10.0 the mean is 1.79 x 10^6.

pH	Trial 1	Trial 2	Trial 3	Mean
3	2.86x10^6	2.84x10^6	2.88x10^6	2.86x10^6
4	3.12x10^6	3.15x10^6	3.16x10^6	3.14x10^6
10	1.77x10^6	1.79x10^6	1.80x10^6	1.79x10^6

Table 5.1 Minimum inhibitory concentration (pH) 1x10^5

The table below shows the thermal stability testing of river bacteriophage in temperatures 4C and 37C, using the dilution 1 x 10^4. The minimum inhibitory concentration of 4C with the mean of 1.83 x 10^5 and 37C with the mean of 3.85 x 10^5.

Thermal	Trial 1	Trial 2	Trial 3	Mean
4C	1.80x10^5	1.82x10^5	1.86x10^5	1.83x10^5
37C	3.80x10^5	3.85x10^5	3.89x10^5	3.85x10^5

Table 5.2 Minimum inhibitory concentration (thermal) 1x10^4

The table below shows the thermal stability testing of river bacteriophage in various temperatures (4C and 37C) using the dilution 1 x 10^5. The MIC of 4C with the mean of 1.52 x 10^6 and 37C with the mean of 2.89 x 10^6.

Thermal	Trial 1	Trial 2	Trial 3	Mean
4C	1.48x10^6	1.52x10^6	1.56x10^6	1.52x10^6
37C	2.90x10^6	2.87x10^6	2.91x10^6	2.89x10^6

Table 5.3 Minimum inhibitory concentration (thermal) 1x10^5

Table 6.0 shows the statistical treatment used to analyse the pH stability of two concentrations. The treatment used is One Way-Analysis of Variance. This is done to decide whether the researcher should accept or reject the stated null hypotheses. F computed value is 35.214 and the F critical is 5.143. P value given is 0.000484 and the degree of freedom is 8. The table is interpreted as having significant difference and reject null hypothesis.

F Value	F crit	P value	dF	Interpretation
35.21	5.143	0.000484	8	Significant

Table 6.0 Statistical Analysis of pH Stability Testing using One Way-ANOVA

One Way ANOVA was helpful enough to state if the data has significant difference or none, however it cannot identify, if significant, where the difference lies. With this, Table 5.0 shows the Post Hoc Analysis data of pH stability testing. Post Hoc used was Tukey's Procedure to identify which parameter gave its significance. The treatment pair pH 3.0 vs 4.0 showed a significant interpretation due to its Tukey HSD p- value of 0.005, being lower than 0.05. pH 3.0 vs 10.0 p-value is 0.899 and has no significant difference. Lastly, pH 4.0 vs 10.0 shows a p-value of 0.899, same with the previous treatment pair and is also interpreted as having no significance. The p-value is compared to the alpha value of 0.05 to identify its significance or lack thereof.

Treatment Pair	Tukey HSD Statistic	Tukey HSD p-value	Interpretation
pH 3.0 vs 4.0	7.8745	0.0050963	Significant
pH 3.0 vs 10.0	0.6152	0.8999	Not significant
pH 4.0 vs 10.0	0.582	0.8999	Not significant

Table 7.0 Post Hoc Analysis: Tukey Procedure

The table below shows a data analysis through T-test for thermal stability. T computed has a value of -2.774 and T critical value is 6.313. This is interpreted as having no significant difference from each other and the decision to accept the null hypothesis.

T computed	T Critical	Interpretation
-2.774384014	6.31375151	Not Significant

Table 8.0 T-Test Two-Sample Assuming Unequal Variances

IV. DISCUSSION

Cystic fibrosis, an infection caused by *Pseudomonas aeruginosa* through its biofilm formation characterized as spherical microcolonies in the lumen of the patients' airways. *Pseudomonas aeruginosa* could prolong patient's morbidity and, even cause mortality for those with cystic fibrosis [11]. Bacteriophage therapy may be an alternative treatment for bacterial infections. The effectiveness of this study started form 1980's from animal model that was conducted on western countries [12]. Bacteriophages are a viral intracellular parasite that recognizes bacteria as their host [13]. For this reason, researchers collected water sample from a river to isolate bacteriophage that could possibly kill *Pseudomonas aeruginosa.* In this study, the researchers isolated a bacteriophage from a sewage sample collected in Tullahan River. The lysate was obtained through the use of 0.22um syringe filter, removing the debris that produced a concentrated phage lysate.

In the river bacteriophage, *P. aeruginosa* can produce a minimum inhibitory concentration of 0.323 PFU/mL at 1 x 10^4 dilution. The phage lysis activity in general is present at much higher dilutions. However, 1 x 10^1 dilution shows maximum effect with 1.344 PFU/mL concentrations [10, 14]. Variation in the results of plaque assay may be due to the presence of several phages having different stability and characteristics. The result may be affected by the temperature, pH and period of incubation used in the

procedure since phages have different thermal and pH stability [10, 14, 15]

In early studies that helped to demonstrate the potential of bacteriophages for biofilm control, Hanlon et al. found that *Pseudomonas aeruginosa* bacteriophages could destroy bacteria in a mature (20-day-old) biofilm [5, 16]. The significant difference between the bacteriophage isolated and the biofilm formed by *Pseudomonas aeruginosa* is seen at Table 4, where t-stat shows 3.126511909 and the T Critical shows 6.313751515. Therefore, rejecting the null hypothesis indicating significant difference between treated and untreated samples.

The minimum inhibitory concentration termed as lowest concentration that will inhibit the growth of microorganisms. It is used in research studies to confirm the unusual resistance and release a definite answer. For the minimum inhibitory concentration, the colonies of *Pseudomonas aeruginosa* were transferred to Tryptic Soy Broth. The broth is incubated at 37C until it becomes turbid equal or greater than 0.5 McFarland standard. It can be done photometrically at 595 OD. The biofilm formation of the host bacterium showed the highest concentration of $1x10^{1}$ having the mean of 2.998 and with $1x10^{10}$ as the least concentration with a mean of 0.92.

In pH stability testing of the isolated bacteriophage it is shown that in a concentration of 1×10^{4} was highly stable to pH 3.0, pH 4.0 and pH 10.0. According to Jin et.al (2012), Even though it has a stability in pH 10.0, the colonies decreases as the isolated bacteriophage losses its affectivity and it is mostly stable in an acidic environment. In concentration of 1×10^{5} the bacteriophage was stable in pH 3.0 and pH 4.0 [16, 17]. This shows that the bacteriophage isolated were mostly stable at pH 3.0 to pH 10.0. Generally the bacteriophage was stable at pH 5.0 to pH 6.0 but according to Kerby et.al the optimum physical stability of pH for this bacteriophage was pH 6.0 to pH 8.0 for long storage [10, 18].

Further study shows that the isolated bacteriophage was stable at 37C with a concentartion of 1×10^{4} and 1×10^{5}. It becomes altered when exposed to temperatures lower than 37C. According to Tey et.al (2009) higher temperature can prolong the lengtt of latency stage of bacteriophage. Thermal stability testing analyzes heat resistant capability of bacteriophage at pH 3.0 and pH 4.0 [4, 17]. It will also determine the ideal storage and further identification of river bacteriophage. Phage lysate aliquot with the concentration of 1x104 and 1x105 was incubated at different temperatures [19]. 1ml of phage was distributed to centrifuge tubes labeled with their incubation temperature and incubation period for 24 hours. The results showed that the phage against *Pseudomonas aeruginosa* is highly stable at 37C with the concentration of 1x104 and 1x105. Although there is a decrease in titre upon storage in 4C, there is still stability of the bacteriophage. After incubation, phage lysates were subjected to plaque formation assay and all assays was done in 3 trials. [15, 17, 19]

There is a significant difference between the three pH concentration parameters exposed on the bacteriophage. Stability on alkaline environments showed a decrease in titre, nevertheless still present. Acidic environments work mostly on the isolated bacteriophage as it showed great significance upon statistical treatment [10, 19, 20].

In thermal stability, no significant difference was present between the two parameters, therefore conluding that both temperatures works best on the isolated lysate against *Pseudomonas aeruginosa.*

V. CONCLUSION

The study highlights the phenotypic characteristics of the isolated bacteriophage (lysate) against *Pseudomonas aeruginosa* biofilm formation. It also indicates the lysate's possible potential in eliminating highly biofilm forming agents, such as *P.aeruginosa.*

Based on the statistical analyses done, the isolated lysates were stable at pH 3 and 4. At pH 10, stability was also noted, however with decreasing number of plaque forming units.

Another phenotypic character observed was thermal stability. Upon exposure to 4C and 37C for 24 hours, the bacteriophage showed stability in both the stated.

In conclusion, the isolated bacteriophage is effective against the host bacterium, *Pseudomonas aeruginosa.*

REFERENCES

[1] Lyczak JB., Cannon CL., Pier GB. Establishment of Pseudomonas aeruginosa infection: Lessons From a Versatile Opportunist. *Microbes and Infection*, *2*(9), 1051–1060. December 2000. Volume 2(9), 1051-1060.https://doi.org/10.1016/S1286-4579(00)01259-4

[2] Mah TC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. April 2001. Vol. 9. p34–39

[3] Costerton, J. W., P. S. Steward, and E. P. Greenberg. Bacterial biofilms: a common cause of persistent infections. 1999. Science 284. p1318–1322.

[4] Donlan RM. "Biofilm Formation: A Clinically Relevant Microbiological Process." Clinical Infectious Diseases 33, no. 8. October 15, 2001. p1387–1392. https://doi.org/10.1086/322972.

[5] Harper DR, Parracho HM, Walker J, Sharp R, Hughes G, Werthen M, et. al. Bacteriophages and Biofilms. Antibiotics. June 2014. Vol. 3 (ISSN 2079-6382). p270-280.

[6] Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage Therapy. 2001. *Antimicobial Agents and Chemotherapy* Vol. *45*(3). p649–659. https://doi.org/10.1128/AAC.45.3.649-659.2001

[7] Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. Future Microbiol. 8 (2013) p769-783.

[8] Yele AB, Thawal ND, Sahu PK, Chopade BA. Novel lytic bacteriophage AB7-IBB1 of Acinetobacter baumannii: isolation, characterization and its effect on biofilm. *Archives of Virology*. 2012. *157*(8), p1441–1450. https://doi.org/10.1007/s00705-012-1320-0

[9] Clokie, M. R. J., & Kropinski, A. M. (Eds.). *Bacteriophages*. 2009. Totowa, NJ: Humana Press. Vol 501. https://doi.org/10.1007/978-1-60327-164-6

[10] Jończyk, E., Kłak, M., Międzybrodzki, R., & Górski, A. The influence of external factors on bacteriophages—review. *Folia Microbiologica*. 2011. *56*(3), 191–200. https://doi.org/10.1007/s12223-011-0039-8

[11] Head, N. E., & Yu, H. (2004). Cross-Sectional Analysis of Clinical and Environmental Isolates of Pseudomonas aeruginosa: Biofilm Formation, Virulence, and Genome Diversity. *Infection and Immunity*, *72*(1), 133–144. https://doi.org/10.1128/IAI.72.1.133-144.2004

[12] Shigenobu M, Mohammed R, Jumpei U, Shingo S, Takako U, et. al. Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. J Infect Chemother (2005) 11:211–219. DOI 10.1007/s10156-005-0408-9

[13] Boyd, E. F., Davis, B. M., & Hochhut, B. (2001). Bacteriophage–bacteriophage interactions in the evolution of pathogenic bacteria. *Trends in Microbiology*, *9*(3), 137–144. https://doi.org/10.1016/S0966-842X(01)01960-6

[14] Caldentey, J., and Bamford, D.H. (1992) The lytic enzyme of the Pseudomonas phage phi 6. Purification and bio-chemical characterization. Biochim Biophys Acta 1159:44–50.

[15] Chang, H.-C., Chen, C.-R., Lin, J.-W., Shen, G.-H., Chang, K.-M., Tseng, Y.-H., & Weng, S.-F. (2005). Isolation and Characterization of Novel Giant Stenotrophomonas maltophilia Phage SMA5. *Applied and Environmental Microbiology*, *71*(3), 1387–1393. https://doi.org/10.1128/AEM.71.3.1387-1393.2005

[16] Jin WJ, Sib SG, Hyoun Joong K, Sae Kil Y, Cheng C, Ji Young C, Byeong CL, et. al. Bacteriophage application to control the contaminated water with Shigella. Scientific Reports. 2016. Vol 6:22636. DOI: 10.1038/srep22636 2

[17] Bassetti, M., Vena, A., Croxatto, A., Righi, E., & Guery, B.. How to manage Pseudomonas aeruginosa infections. Drugs in Context. 2018.Vol 7, 1–18. https://doi.org/10.7573/dic.212527

[18] Adedeji G.B., Fagade O.E., and Oyelade A.A.. Prevalance of Pseudomonas aeruginosa in Clinical Samples and its sensitivity to Citrus Extract. 2007. African Journal of Biomedical Research, vol. 10: 183-187.

[19] Webb, J.S., Lau, M., and Kjelleberg, S. (2004). Bacteriophage and Phenotypic Variation in Pseudomonas aeruginosa Biofilm Development. Journal of Bacteriology 186, 8066–8073

[20] Gupta V & Saxena HM, Phage based diagnosis of bacterial infections. J Clin Pathol Microbes, 1 (2016) 1.