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Department of Medical Biotechnology

Isolation and Characterization of *Proteus mirabilis* Phages

A Research
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<[إِنَّ هَذَا الْقُرْآنَ يَهْدِي لِلَّتِي
هِيَ أَقْوَمُ وَيُبَشِّرُ الْمُؤْمِنِينَ
الَّذِينَ يَعْمَلُونَ الصَّالِحَاتِ
أَنَّ لَهُمْ أَجْرًا كَبِيرًا (9)]>

صدق الله العلي العظيم

[الإسراء: 9]

Dedication

I dedicate this study to my parent's, my strong pillar, and my sources of inspiration, wisdom, knowledge and understanding. He has been the sources of my strength throughout this the course of the study. Thank you.

Ali - Maysam

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My deepest gratitude goes to God who has provided all that was needed to complete this study for which it was undertaken. There was never lack or want. Throughout this entire study, He took care of everything that would have stopped me in my tracks and strengthened me even through my most difficult times.

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Finally, I thank my parents and for their prayers, words of motivation and words of comfort that come in just in time. God bless you all. You all will not lose your reward in Ali name. Amen.

Ali - Maysam

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Summary

This study was conducted during the period from October 2017 to April 2018. This work aimed to isolate and characteristics the phage that infected of proteous .This research was done on several steps. In the first step, bacterial were obtained from Al-Diwaniyah Teaching Hospital laboratory. While bacteriophages sample were collected from sewage sample, municipal water and heavy sewage in quantities ranging from 500 ml to 1000 ml. Results showed the study of bacteria isolated from those hospitals showed a positive result, for bacteriophage sample were diagnosed microbiological according to biochemical and phenotypic. Phage assay should that their two types of anti-Proteus mirabilis lytic bacteriophages primarily named Phage 1 and Phage 2 were isolated from sewage water. The burst size of Phage 1 and Phage 2 were 1±1.5 pfu\cell and 1±2 pfu\cell respectively. Complete lysis time of Phage 1 particles was 4 hours while the complete lysis time of Phage 2 particles was 5 hours. The Phage 1 and Phage 2 particles were stabled at a wide range of pH (6-8) and temperatures (30-50°C), the optimal temperature of two phages were 37°C.

Introduction

Proteus is one of the bacteria that found symbiotic with human as normal flora of the gastrointestinal tract. It can be found free living in water and soil. When this bacterium enters into the urinary tract, wounds and lungs, they may cause pathologic complication. *Proteus mirabilis* is usually, main cause of urinary tract infections and stones formation.**(1)**

Was found alkaline, so cause that resulting from the bacteria have ability to produces urease in increased quantities, whereas transform the urea into ammonia by using urease enzyme .that make alkalinity of the urine may be a cause pathologic complication .If the infection is not early treated of infection. Whereas increase the alkalinity lead to formation of crystals, which are going to eventually become kidney stones. Kidney stones when reach it renal system leading to obstruction and even to renal failure. *Proteus mirabilis* that causes many infectious diseases as structural abnormalities at the level of the urinary tract, urethral instrumentation, and other risk factors include – unprotected anal intercourse and uncircumcised penises .**(2)**

Proteus mirabilis belong to the family of the Enterobacteriaceae, it is a small gram-negative bacilli and a facultative anaerobe.**(3)** *Proteus mirabilis* characterized by swarming motility (coordinated translocation of the bacterial population across solid surfaces). its ability to ferment maltose, and its inability to ferment lactose , has the ability to make fishy odor, due to the production of hydrogen sulfide gas and ability to the formation of struvite stones to elongate itself and secrete a polysaccharide, when it comes in contact with solid surfaces. **(4)** Phage is a virus that can be infect and replicated within bacteria. **(5)**

Bacteriophages is a type of virus that infects bacteria, because bacteriophages destroy their host cells. Thousands of varieties of phage exist, each of which may infect only one type or a few types of bacteria or archaea that bacteria have their own sets of viruses, so they are very specific. A virus, for example, that infects proteous bacteria would not infect a different species of bacteria. Where over 90% of all viruses found that infect bacteria fall into three families or 'types' of viruses, but for the thousands of types of bacteria that exist, there are even more sub-types of viruses within these three families.**(6)** Phages are classified in a number of virus families; some examples include Inoviridae, Microviridae, Rudiviridae, and Tectiviridae. Like all viruses, phages are simple particales that consist of a core of genetic material.**(7)** All bacteriophages are composed of proteins that enclosed the DNA or RNA geneome called protein capsid and may have relatively simple or complex structures, , Phages increase within the bacteria after infection in the cytoplasm. Bacteriophages are one of thousands of the most common viruses, which are found everywhere **.(8)** Phages are found where the bacteria are presence .**(9)**

In addition, spread widely in locations populated by bacterial hosts, such as soil or the intestines of animals .**(10)**

Phage therapy was discovered to be antibacterial agents; phages were used during the 1920s and 1930s for treating bacterial infections. Phages, short for bacteriophages, are bacteria-specific viruses that have been used as a treatment against pathogens such as *Shigella dysenteriae* as early as 1919 .**(11)** However, they were abandoned for general use in the west for several reasons as the advent of pharmaceutical antibiotics in the mid-20th century. Along with a better understanding of disease and sanitation, revolutionized healthcare and drastically improved both quality of life and life expectancy in the industrialized world medical trials

were carried out, but a basic lack of understanding of phages made these invalid .**(12)** The study's results demonstrated, that the safety of therapeutic application of bacteriophages but did not show efficacy .**(13)** The authors explained that the use of certain chemicals that are part of standard wound care (e.g. lactoferrin or silver) may have interfered with bacteriophage viability. **(14)**

Lytic phages are similar to antibiotics in that they have remarkable antibacterial activity. However, therapeutic phages have some advantages over antibiotics, and phages have been reported to be more effective than antibiotics in treating certain infections in humans and experimentally infected animals **.(15)**

Phage therapy can be very effective in certain conditions and has some unique advantages over antibiotics. Bacteria also develop resistance to phages, but it is incomparably easier to develop new phage than new antibiotic. A few weeks versus years are needed to obtain new phage for new strain of resistant bacteria. As bacteria evolve resistance, the relevant phages naturally evolve alongside. When super bacterium appears, the super phage already attacks it. We just need to derive it from the same environment**. (16)** Phages have special advantage for localized use, because they penetrate deeper as long as the infection is present, rather than decrease rapidly in concentration below the surface like antibiotics. The phages stop reproducing once as the specific bacteria they target are destroyed. Phages do not develop secondary resistance, which is quite often in antibiotics. With the increasing incidence of antibiotic resistant bacteria and a deficit in the development of new classes of antibiotics to counteract them, there is a need to apply phages in a range of infections. **(17)**

For that, this work aimed to isolate and Characteristics the phage that infected of proteous.

2- Materials and Methods

2-1. Materials

2-1- 1. Equipment

Laboratory equipment and tools

Table (3-1): The equipment and laboratory tools used in the study and the manufacturers

No.	Equipments	Manufacturing
1	Autoclave	Delama, USA
2	Burner	Amal, Turkey
3	Centrifuge –universal 16 A	Hittch Germany
4	Digital camera	Sony, Japan
5	Fine and adjustable micropipettes	Gilson, France
6	Freezer	Ishtar (Iraq)
7	Hot plate	Gallenkamp (England)
8	Incubator	Memmert(Germany)
9	Laminar air flow	Prettl , Germany
10	Light microscope	Olympus(Japan)
12	Magnetic stirrer with hot plat	Vision , Korea
13	Micro pipettes	Eppendorff\Germany
14	Oven	Olympus, Japan
15	PH-meter	Thermo Electron , USA
16	Refrigerator	Arcelik, Turkey
17	Sensitive balance	Sartorius (U.S.A)
18	Vortex	Griffin, Germany
19	water bath	Tafesa , Germany

2-1- 2. Plastic and Glass Wares:

Table (2-2): Plastic and Glass Wares

No.	***Plastic and Glass Ware***	***Manufacturing***
1	Automatic pipette	Birhit, Finland
2	Disposable Petri-dishes	EAPIF (Germany)
3	Gloves	Hungary
4	Pasteur's pipettes	Volac (England)
5	Plain tubes	AFMH, England
6	Test tubes (10 ml)	AFMA ,Jordan
7	Millipore Filter paper	AFMH, England
8	Millipore unit (0.44 , 0.27)	EAPIF (Germany)
9	Whattman filter papers	MEHE, China

2-1- 3. Biological Materials:

Table (2-3): Biological Materials

	Biological Materials	**Manufacturing**
1	MacConkey agar	Oxoid (England)
3	Nutrient agar	Oxoid (England
4	Nutrient broth	Oxoid (England)
5	Chlorophorm	BDH Chemical, England
6	Peptone water	Oxoid (England)

2-2. Methods

2-2-1. Samples Collection:

2-2-2-1. Bacteria was obtained from Diwaniyah Teaching Hospital. Samples were subjected to laboratory culturing, Nutrient agar container of Peptone water broth for 18 hours at 37 ° C. After that, labeling was applied to each plate, which contain of bacteria. Growth and propagation of the culturing was observed on all petri dish. Tests were included Biochemical and serological tests .**(18)**

2-2-2-2. Phage samples: were collected from the sewage water and from different environmental sources within the closed laboratory bottle with covered at the size of about 500 ml of these samples. Then were centrifuged (10,000 rpm, 10 minutes, and 4 ° C) and passed through super-sterilization filters (0.45µm Milliporee1 filter). filtered sewage were mixed at 1.5 ml to 3.5 ml sterile nutrient broth which contain bacteria in the test tube.**(19)**

Mixture from phage and bacteria were incubated for 18-24 hours in the incubator at 37°C. Chloroform organic solvent was taken at 0.5 ml and added to the bacteria, so that all the bacteria were eliminated except phage in the test tube for 5 minutes. **(20)**

2-2-2. Sterilization Methods

- Equipment's, Biological Materials were Sterilization during the experiment was performed using an Autoclave at 121 ° C and one p.s.i for 15 minutes.
- Liquids were sterilized which used in the experiment by mili pore paper using filters with diameters ranging from 0.27 microns to 0.4 microns to ensure the elimination of contaminant.**(21)**

2-2-3. Preparation Nutrient agar

Nutrient agar powder were weighted at 28 g of nutrient in 1 liter of distilled water, then putting on the hotplate to dissolve completely. Suspension were sterilized by using Autoclave as required. Suspension were Cooled to 55°C. Cotton plug and flame were taken the mouth of the flask over a Bunsen burner, and then suspension was poured into sterile empty of Petri dishes (10-15 mL into each Petri dish). And the Petri dishes were kept horizontally until the medium completely solidifies.**(22)**

2-2-4. Plaque Assay and Spot Test:

Phages were verified presence in the test tube by Plaque Assay was performed as previously known. **(23)** for the isolation phage and determination through a double layer agar technique, each of diluted tube were mixed with 0.5 ml of phages in 4.5 ml soft agar that contain host bacteria as *Proteous* into other tube , then mixture that

contain the phages and bacteria were poured to plate .**(23)** Plates were incubated for 24 hour at 37^{o}C.

2-2-5. Phage Propagation and Purification:

Phages were isolated by one Petri Dishes and sequentially until the dishes are obtained homogenously container on Plaque .**(24)** During this period. The Plaque were taken by sticky loop once of all Petri Dishes at time in a well-sequenced mode. Under ideal conditions where as providing a sterile atmosphere when removal it, for ensure as not to contaminate them with fungus scattered in the air, bactericidal and bacteria scattered anywhere and inject them into the 5.0 ml nutrient broth, which contain bacteria proteous . Mixture, which contain specific phage and these bacteria, were left 24 hour of the host strain and then incubated at 37^{o}C. for complete decomposition. Until ensure the replicated of phage .**(25)**

2-2-6. Lysis Time:

from petri dishes plaque forming unit were taken at 10^{-7} pfu/ml at 10-ml to added to soft agar colony forming at 10^{-7} cfu/ml ,then left for grown in test tube at five to six minutes. Whereas suspension were, separate into flasks until to stop adsorption. For calculate time, untreated free phage and infected cells were mixed of infective centers at various time points to determine changes in titer. To give a graph (3-6) which showing growth in bacteria and phage .**(26)**

3- Results

3-1. Phage assay

Obtain of results should that there are two proteous phages; type 1 and 2. Characteristics of these phages were determined by the plaques appearance, diameter, turbidity and the presence of a halo in the culture media as in table (4-1). Infection of *Proteous meribilus* by phage exhibited the clear plaques on the surface of nutrient agar. From plaque assay, all the *Proteous meribilus* isolates were susceptible to phages lytic infection, plaques on agar plates revealed a big hollow zone with slightly wrinkled margin as in figure ((4-1), (4-2)) that showed plaques of *Proteous* phages.

Table (4-1): Characteristics of *Proteous meribilus* phages

Name of Phage	Phage diameter |mm|	Number of Plagues	Turbidity	Halo
Phage 1	2 mm	7	C	—
Phage 2	1-2 mm	25	C	—

C**:** clear ; **-:** no visible halo

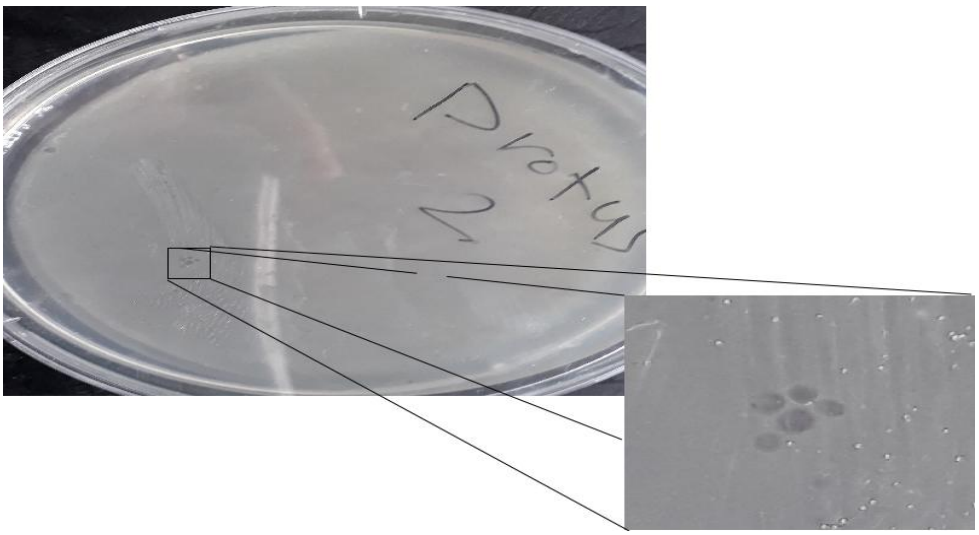

Figure (4-1) shown in it plague

DCM= 15 Mega pixel

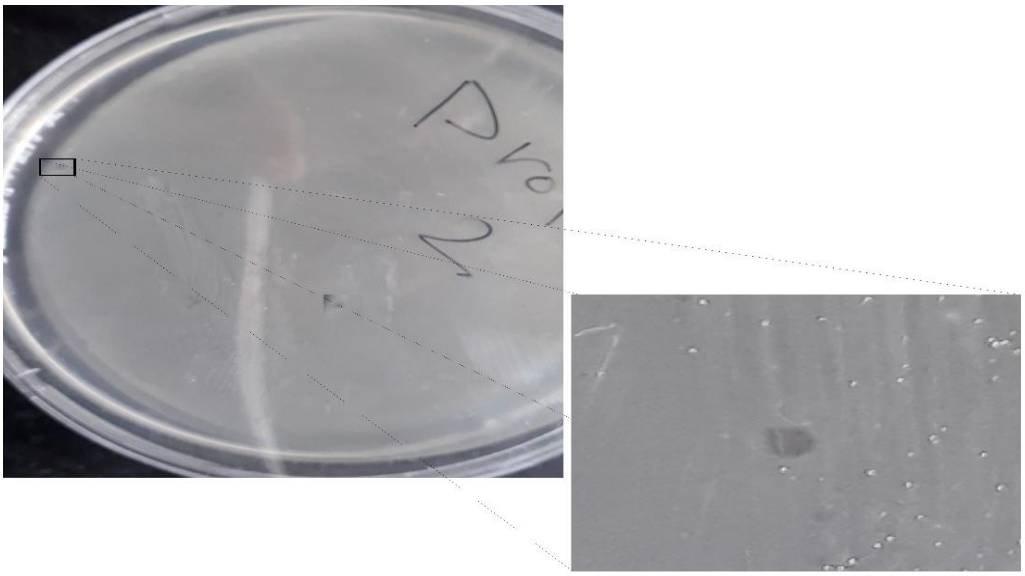

Figure (4-2) show it plague

DCM = 15 Mega pixel

3-2. Lysis Time:

The present study showed that the isolated phage particles recorded highly significant ability ($P > 0.05$) to cause lysis of the bacterial cells and these effects reached to the higher rate after one hours of mixing of phage particles with bacterial cells. Also showed that there is a dramatic decrease in number of bacterial cells that correlated with time. Complete lysis time of Phage 1 particles was 4 hours while the complete lysis time of Phage 2 particles was 5 hours as in figure (3-6).

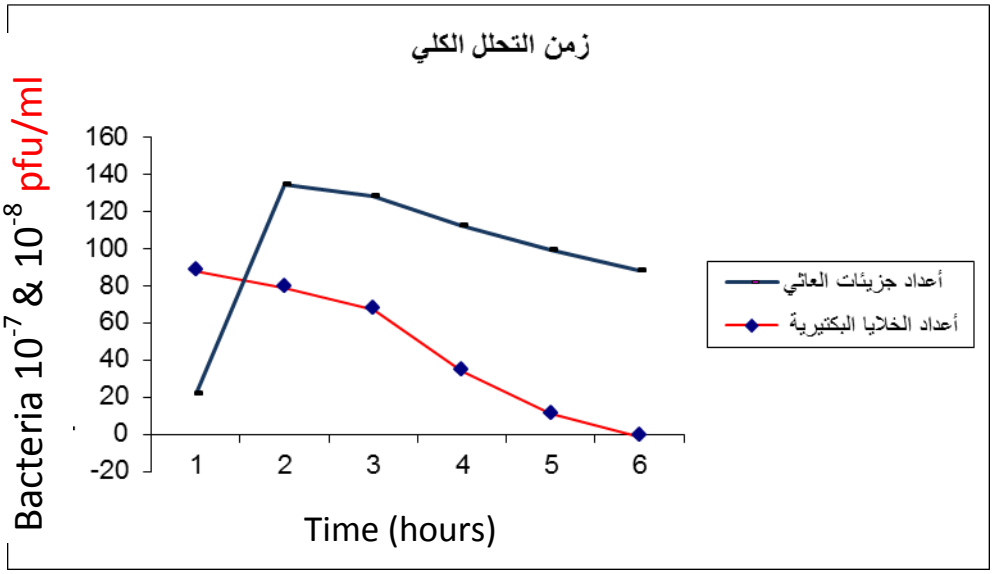

Figure (3-6): Lysis time of **Proteous mirabilis**

Discussion

This proteous, which cause of urinary tract infections, especially in some types of bacteria that show new resistance mechanisms and spread. **(27)** It can be found free living in water and soil. It can infected animal and human and can be transmitted from person to another. **(28)** where it is important factors that help spread infectious disease be had bad infection, eating contaminated food, poor health conditions, genetic and immunity system disorder etc. During this bacteria exposure for many antimicrobial resistance appears in naturally form resistance for this antimicrobial over time through genetic modification, so could be find many of human that use of antibiotics in which unknown uses. This bad uses is excessive and bad in human's makes high resistance for any antibiotics. **(29)**

For example, *Proteus mirabilis* for account large percentage of infected by these bacteria in hospital-acquired urinary tract infections, septicemia, pneumonia and soft tissue infections, where consider medically important type. Therefore, phage studies and development of effective treatment are one of the top priorities in public health in the world, which FDA are seeking it. The aimed of this study include the use of bacteriophage to control this bacterial population. All of the bacteriophage isolates which collected from sewage water able to infect proteous. Through isolation and diagnostic according to biochemical and phenotypic test. procedures we have obtained two isolated of phages designated (phage1 and phage 2) which have been categorized into virus classification criteria which include (plague diameter mm, number of plaques, turbidity , halo) and their two from isolated phages (phage1 and phage 2) are highly virulent phages which plague were round , clear , no halo and medium sized (1.0-2.0 mm in diameter) plaques .phage1 and phage 2 were capable to lysis *Proteus mirabilis* . After left phage1 and phage 2 to infected this bacteria

for complete lysis time was recording note of two reading in 4 hours and 6 hours ,where it found significant decrease in the

number of bacteria over time compared to the increment in the number of phages, which it leading isolated phages of this studies were infected bacterial cells and were able to lysis it . The phages isolated in this study are playing an important role not only in treatment such cases was proposed in this studies, to be candidate for medicinal and pharmaceutical applications due to its strong lysis capability and its broad host range. Phage therapy has many advantages compared with other chemical or antimicrobial drugs. In terms of efficiency and safety in treatment, where the antimicrobial drugs were left passive effect when it uses for treatment bacteria cases it kills the beneficial bacteria in the body such as normal flora. Phages that helping to killed or reduced of effective of proteous without side effect because this phage is specific for this bacteria. To reduce the side effect of this bacteria, the phages secreted of enzyme, supported with enzymes (e.g., EPS de-polymerase) on the surface of the capsid that degrade the extracellular polymeric substances and disseminate bacterial biofilms, permitting the phage to reach bacteria embedded within the extra polymer substance matrix .**(30)** In particular conditions this will be considerable advantage considering the well-authenticated.

Finally, Phage therapy may be successfully but may be left side effects unknowingly so many studies require to know the most important potential side effects of this treatment. This study discussed how to isolate and characteristics the phage that infected of proteous.

References

1. Li X, Zhao H, Lockatell CV, Drachenberg CB, Johnson DE, Mobley HL. 2002. Visualization of Proteus mirabilis within the matrix of urease-induced bladder stones during experimental urinary tract infection. *Infection and immunity* 70: 389-94
2. Baquero F, Hsueh P-R, Paterson DL, Rossi F, Bochicchio GV, Gallagher G, Lantz K, Villasenor JB, McCarroll K, Abramson MA. 2009. In vitro susceptibilities of aerobic and facultatively anaerobic gram-negative bacilli isolated from patients with intra-abdominal infections worldwide: 2005 results from Study for Monitoring Antimicrobial Resistance Trends (SMART). *Surgical infections* 10: 99-104
3. Sherman J, Niven Jr C, Smiley K. 1943. Streptococcus salivarius and other non-hemolytic streptococci of the human throat. *Journal of bacteriology* 45: 249
4. Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318-22
5. Hanlon GW. 2007. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International journal of antimicrobial agents* 30: 118-28
6. Bohannan BJ, Lenski RE. 2000. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. *Ecology letters* 3: 362-77
7. Parker HG, Kim LV, Sutter NB, Carlson S, Lorentzen TD, Malek TB, Johnson GS, DeFrance HB, Ostrander EA, Kruglyak L. 2004. Genetic structure of the purebred domestic dog. *science* 304: 1160-4
8. Ackermann H-W, Prangishvili D. 2012. Prokaryote viruses studied by electron microscopy. *Archives of virology* 157: 1843-9

9. Kellenberger E, Ryter A, Séchaud J. 1958. Electron Microscope Study of DNA-Containing Plasms: II. Vegetative and Mature Phage DNA as Compared with Normal Bacterial Nucleoids in Different Physiological States. *The Journal of Cell Biology* 4: 671-8
10. Haft RJ, Mittler JE, Traxler B. 2009. Competition favours reduced cost of plasmids to host bacteria. *The ISME journal* 3: 761
11. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, Abedon ST. 2010. Phage therapy in clinical practice: treatment of human infections. *Current pharmaceutical biotechnology* 11: 69-86
12. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. 2011. Phage treatment of human infections. *Bacteriophage* 1: 66-85
13. Zobell CE. 1943. The effect of solid surfaces upon bacterial activity. *Journal of bacteriology* 46: 39
14. Rhoads D, Wolcott R, Kuskowski M, Wolcott B, Ward L, Sulakvelidze A. 2009. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *Journal of wound care* 18: 237-43
15. Fischetti VA. 2008. Bacteriophage lysins as effective antibacterials. *Current opinion in microbiology* 11: 393-400
16. Rahman A. 2014. *A STATISTICAL ANALYSIS OF NEED AND POSSIBILITY OF ADMINISTRATION OF BACTERIOPHAGE THERAPY*. Presented at 12th International Conference
17. Lehman SM. 2007. Development of a bacteriophage-based biopesticide for fire blight.
18. Al-Abboodi AKA, Al-Rubiaee GH, Zaki NH. 2018. Effects of green algae, Chlorella Vulgaris, extracts and its bioactive substances on the biofilm-producing microorganism. *International Journal of Research in Pharmaceutical Sciences* 9: 165-73

19. Boehm AB, Fuhrman JA, Mrše RD, Grant SB. 2003. Tiered approach for identification of a human fecal pollution source at a recreational beach: case study at Avalon Bay, Catalina Island, California. *Environmental Science & Technology* 37: 673-80
20. Tsuei A-C, Carey-Smith GV, Hudson JA, Billington C, Heinemann JA. 2007. Prevalence and numbers of coliphages and Campylobacter jejuni bacteriophages in New Zealand foods. *International journal of food microbiology* 116: 121-5
21. Heineman RH, Bull JJ. 2007. Testing optimality with experimental evolution: lysis time in a bacteriophage. *Evolution* 61: 1695-709
22. Hayashi N, Guan W, Tsutsui S, Tomari T, Hanada Y. 2006. Sterilization of medical equipment using radicals produced by oxygen/water vapor RF plasma. *Japanese journal of applied physics* 45: 8358
23. Keer NS. 2010. *Physiological influence of Organic, Chemical and Integrated Nutrient Management on Growth Productivity and Quality of Isabgol (Plantago ovata Forsk)*. jnkvv, jabalpur
24. Sosa V, Schlapp G, Zunino P. 2006. Proteus mirabilis isolates of different origins do not show correlation with virulence attributes and can colonize the urinary tract of mice. *Microbiology* 152: 2149-57
25. Butel JS, Morse SA. 2007. *Medical microbiology*: McGraw-Hill Publishing
26. Delbrück M. 1940. The growth of bacteriophage and lysis of the host. *The journal of general physiology* 23: 643-60
27. Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews* 15: 167-93
28. Bradley DJ. 1993. Human tropical diseases in a changing environment. *Environmental change and human health*: 146-62

29. Davies J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264: 375-82
30. Coimbra EC. 2012. Desenvolvimento de estratégias vacinais contra o Câncer de colo de útero baseadas em "Vírus-Like Particles" e imunização genética.