Mesopo. Environ. j., Special Issue C :64-82, 2017
ISSN 2410-2598
proceeding of 1st National conference of science and Art
University of Babylon

Mesopotemia Environmental journal
journal homepage:www.bumej.com

The Molecular and Biological Study of the Isolated Bacteriophages Infecting *Pseudomonas aeruginosa* From Sewage water

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To cite this article:

Alsaffar. M.F, Jarallah. E.M, The Molecular and Biological Study of the Isolated Bacteriophages Infecting *Pseudomonas aeruginosa* From Sewage water *Mesop. environ. j*., 2017, Special Issue C.;64-82.

Abstract

Pseudomonas aeruginosa is one of the Multi-Drug-Resistant organisms mostfrequently isolated worldwide. And because of a shortage of new antibiotics, bacteriophages are considered an alternative for its treatment. Previously, *P. aeruginosa* phages were isolatedbest candidates were chosen based on their ability to form clear plaques and their host range. This work aimed to characterize one of those phages and evaluate the antibacterial activity of isolated bacteriophages in laboratory animal . This studywas carried out during the period from June 2015 to January/ 2016. It included used of *P.aeruginosa* phage samples collected from sewage water.Fourtype of *P.aeruginosa*phage wereidentified according to genetic study, However, analysis of the *P. aeruginosa* phages genome revealed that it was show closed related to NCBI-Blast Pseudomonas phage (KU297675.1) and others The local Pseudomonas phage isolates (F2 FF, E1 EF, and F1 FF) were show as unique isolates and different than NCBI-Blast Pseudomonas phage. The results showed the vast diversity of *P. aeruginosa*phages may be useful in treatment of infection with antibiotic resistant bacteria.We studied the biological activity of *P.aeruginosa* phage isolate in the burned Rabbits model.The result showedresponsible for a significant reduction in the bacterial population. The burned mouse model showed an animal survival between 80% and 100%, significantly different from the control animals (0%).All the rabbits completely recovered after 8 days of treatment and showed the absence of signs after 18 days after phage application in treatment.So, considered as a good candidate for phage-therapy.

Key words: *P.aeruginosa* bacteriophages,phage therapy, phage genomics.

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1- Introduction:

Infections caused by Multiple Drug Resistant bacteria (MDR) wasconsiders as a major public health problem by the World Health Organization (WHO). One of the organisms contributing to this problemis *Pseudomonas aeruginosa* MDR, which is an opportunistic gram-negative pathogen [1]. *Pseudomonas* is one of the most diverse genera known, with members in diverse environmental niches, such as soil, water, animals, and plants. *Pseudomonas aeruginosa* differs from other species of this genus in being pathogenic to animals [2]. This Gram-negative rodshaped bacterium is an opportunistic pathogen, causing various types of infection (e.g., skin, eyes, ears, respiratory tract, urinary tract, gut-derived sepsis). Immunocompromised patients and patients on immunosuppressive treatments, such as patients suffering from cystic fibrosis, burn wounds, AIDS, and cancer, are the most frequently infected by this organism. *Pseudomonas aeruginosa* strains are naturally resistant to several antibacterial drugs [3].

In addition, some clinical isolates have a hypermutator phenotype, facilitating the rapid evolution of resistance to drugs to which they were initially sensitive. *P. aeruginosa* strains can also acquire resistance from mobile genetic elements, such as plasmids [4]. Most *P. aeruginosa* strains are still susceptible to colistin, a polymyxin antibiotic, but increasing numbers of reports of pan-resistant strains are emerging [5,6] . A number of Intensive Care Units ICUs have reported incidents of *P. aeruginosa* infections resistant to every available antibiotic, leaving a patient with no viable treatments. The problem is compounded when the search for new antibiotics is expensive, arduous and not incentivized. It is often not profitable for pharmaceutical companies to develop new antimicrobials, especially when resistant strains are found within less than two years after their introduction [7]. Establishing the use of viruses (Bacteriophages) as antimicrobial treatments, Bacteriophages (or phages) are the viruses that infect bacteria [8]. There are at least 12 distinct groups of phages; and, each phage species is specific to its bacterial host. The exact morphology and genetic material (DNA or RNA) varies according to the phage species. The typical structure of a phage is a hollow head filled with phage DNA or RNA and a tunnel-like tail for injecting the genetic material into the bacteria [9].

Several bacteriophages against *P. aeruginosa* MDR were isolated and characterized to select the best candidates for phage therapy. Host ranges, genome size, morphology, its activity against biofilms and planktonic cells in vitro, and in vivo using rabbits models and, lastly, genomic sequencing and annotation were performed for phage [10]. Genome sequencing and annotation is important for the assessing of presence of potential toxins and genes associated with the lysogenic cycle, which is one concern when the therapeutic phage application is discussed [11]. In this study, the genome analysis contradicted the previous collected evidence that suggested *P. aeruginosa* phages as a good candidate for phage therapy, showing that researchers should consider genomics as an early step in the characterization of phages intended for therapeutic application.

Objectives of this study:

The present study aimed to evaluate the antibacterial activity of isolated bacteriophages from sewage water. By the following objectives:

1. Detection of bacteriophages endolysin gene by PCR and Sequencing.
2. Studying the antibacterial activity of isolated bacteriophage in laboratory animal.

3- Materials and Methods

3-1- Samples Collection:

3-1-1-Bacterial Isolates: In this study, 40 isolates of *P.aeruginosa* were obtained from AL-hilla Teaching Hospital and mirgan Teaching Hospital. *P.aeruginosa* isolates were tested for their sensitivity to 18 commonly used antibiotics by disk diffusion method [12]. Results were recorded by measuring the inhibition zone (in millimeters) and interpreted according to Clinical and Laboratory Standards Institute documents CLSI, [13].

3-1-2-Phage Isolation and Propagation:

Samples of sewage water were collected from different environmental sources in a cleancontainers about 500 ml from this samples. In brief, sewage samples were collected, centrifuged (10,000 rpm, 10 min, 4^{o}C) and supernatants were filter sterilized (0.45 µm Millipore filter). 50 ml filtered sewage sample and 50 ml sterile nutrient broth was mixed with 5.0 ml overnight culture of *P.aeruginosa*and incubated at 37^{o}C overnight. The bacteria were removed by centrifugation; supernatant was filtering sterilized and checked for the presence of phages [14].

3-2-Phage DNA Extraction:

The phage DNA was extracted from phage lysate using the Viral Nucleic Acid Extraction (VNE) Kit (Vavorgen) and according to the manufacturer's instructions.

3-2-1-Amplification by Polymerase Chain Reaction:

Single plex and multiplex were used in this study, the PCR reaction mixture consisted of 20-50 ng template DNA, 250 mM of each dNTP, 5µl buffer, 1 U Taq DNA polymerase (Bioneer), 20 pmol of each primer and 30mM $MgCl_2$ in 20 µl of total reaction volume.

Two pairs of oligo- nucleotide primers were used in this study. These primers used for detection of bacteriophage endolysin gene. These primers were provided by (Bioneer company, Korea), Primer 1 (F) 5'GTAAAACGACGGCCAGT3'(R) 5'CAGGAAACAGCTATGACCATG3'. The PCR conditions used were denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, with a final extension at 72°C for 5 min [15] .And Primer 2 (F) 5'GTAGAGGTTATCATATGAAAGTATTA3' (R) 5'TGCTACCTCGAGTTTTCT'3 The PCR conditions used were denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min[16].

DNA sequencing method was performed for genotyping and phylogenetic analysis study of *P. aeruginosa* Phageisolates. The sequencing of the PCR product, 97 bp,500 bprespectively, where the PCR product was purified from agarose by using (EZ EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic, Canada).

3-3- Activity of Bacteriophages in the Burned Rabbits Model:

Male rabbit of (*Oryctylagusconniculus*), weight (1000-1500g) were brought from the laboratory of the College of Medicine /University of Baghdad. All animals were housed for one weeks in steel cages and kept under a light-dark cycle. Groups of rabbits were separated (4 rabbits/cage) according to the protocol reported by [17].

Hair were clipped from the backs circle with a diameter of 10 cm of anesthetized rabbits (rabbits were anesthetized by using 1 ml of Lodocaine Hydrochloride by the injection under the skin of the area to be burned), the area denuded with a commercially available hair remover and after (6-7) minutes, sterile surgical blade was used and heated to redness, it used to burn the skin of the rabbits and keep attention to not access to the soft tissue under the skin. Immediately after the burn, all the rabbits were injected intraperitoneally (i.p.) with 0.5 ml of sterile physiological saline for fluid replacement to prevent it from shock [18,19]

Bacterial inoculum was prepared by inoculating 10^6 cfu/ml of *P. aeruginosa*in nutrient broth, incubating at 37^oC overnight followed by repeated centrifugation (10,000 rpm for 10 min) and washing, finally it was resuspended in normal saline. Sterile dry swab was submersed in the suspension of bacterial broth (10^6 cfu/ml that used in this study) and swabbed the area that was burned of all rabbits groups except the healthy control group rabbits. The rabbits were let until we notice the sign of inflammation redness, swelling and pus [19].

3-3-1-Topical Application of Phage Carrier:

A carrier is the basic material was mixed with the phage titer (10^8 pfu/ml that was used in the treatment) as ointment, cream, oil, and even distilled water to prepared phage suspension then it applied directly to the surface of the burning rabbits or submerged the gauze piece with phage suspension or used impregnated dressing [20]. Vaseline was used as a carrier in this study.

Rabbits were used in this study divided into four groups (five rabbits for group):

Group 1: Healthy (control –ve) rabbits.

Group 2: Burned (control +ve) rabbits.

Group 3: Burned *P.aeruginosa* infected rabbits.

Group 4: Burned *P.aeruginosa* infected rabbits treated with phage.

3-3-2-Histopathological Study:

The preparation ofhistopathological sections of Rabbits skins depended on standard methods of [21].

3-4- Statistical Analysis:

Data analyses were performed with Graph Pad Prism version 5.04 Software (Graph Pad Software Inc., San Diego, CA, USA). All of the descriptive variables were expressed as the mean ± standard error (SE).The group analyses were performed using one-way ANOVA and Turkey's post-hoc analyses by determination of LSD on probability level 0.05 [22].

Mesopotamia Environmental Journal **ISSN 2410-2598**
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4- Results:

4-1- Molecular Genetic Study:

To provide a fingerprint of the phages isolated from the sewage samples, DNA sequencing method was performed for genotyping and phylogenetic analysis study of *P. aeruginosa* Phage**.** Isolates was performed with the DNA samples isolated from *Pseudomonas* phage, Two primers were used. The isolated phage showing that the phage could be distinguished by PCR analysis and sequencing. Primer 1 and 2provided bands with phage. Figure (4-1, 2, 3) showed the electrophoresis pattern of DNA extract.

The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Pseudomonas phage isolates (E2 EF) were show closed related to NCBI-Blast Pseudomonas phage (KU297675.1) and others The local Pseudomonas phage isolates (F2 FF, E1 EF, and F1 FF) were show as unique isolates and different than NCBI-Blast Pseudomonas phage. Figure (4-4): Multiple sequence alignment analysis of the partial Pseudomonas phage endolysin gene sequence in local Pseudomonas phage isolates with for NCBI-Genbank. And Figure (4-5) showed Phylogenetic tree analysis based on the Pseudomonas phage endolysin gene partial sequence that used for Pseudomonas phage detection.

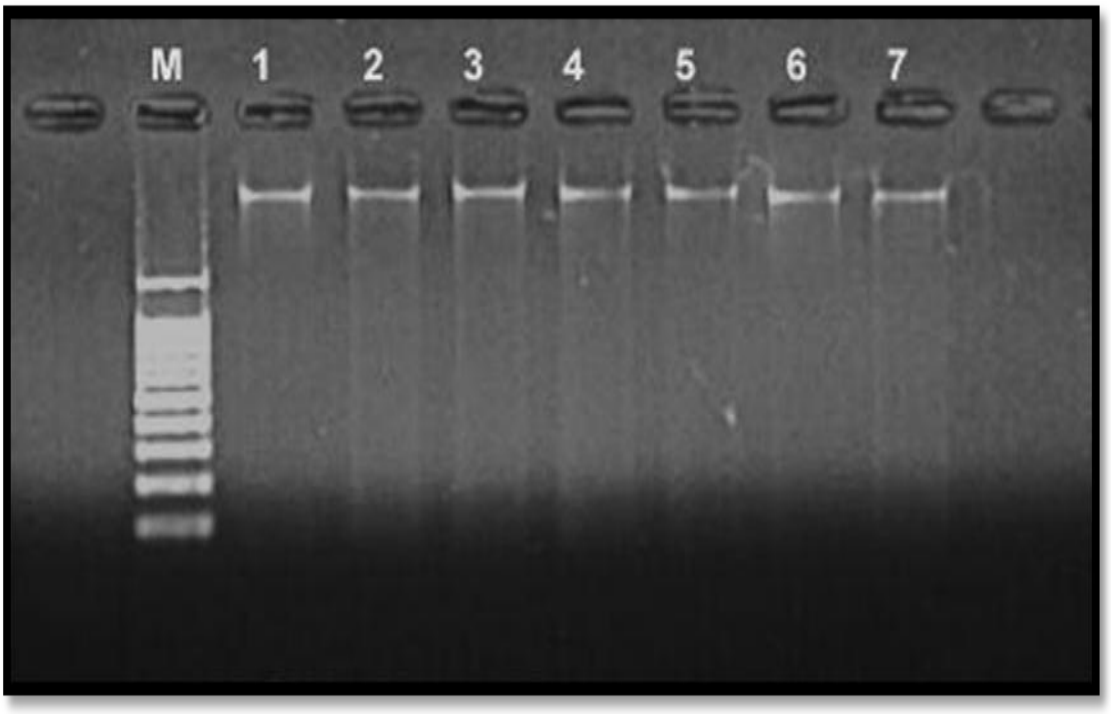

Figure (4.1): Agarose gel electrophoresis of DNA extracted from phage lysate, (1.5% agarose, 75V, 20 mA for 1- hr). Lane (1-7) DNA of extracted phage lysate. (10 µl in each well) .(M: ladder).

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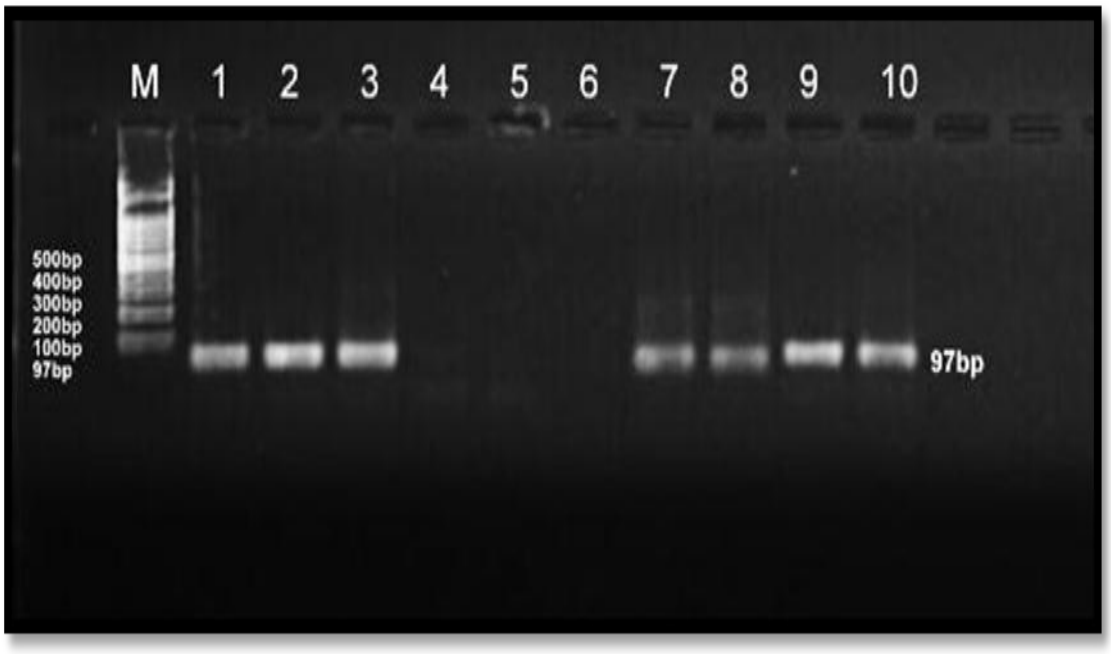

Figure (4-2): Agarose gel electrophoresis (1.5% agarose, 75V, 20 mA for 1 hr). for endolysin gene, this amplification product was one band 97 bp Lane M DNA ladder, lan (1,2,3,7,8,9,10) of PCR product show positive result , (4,5,6) of primer show negative result. (10 µl in each well).

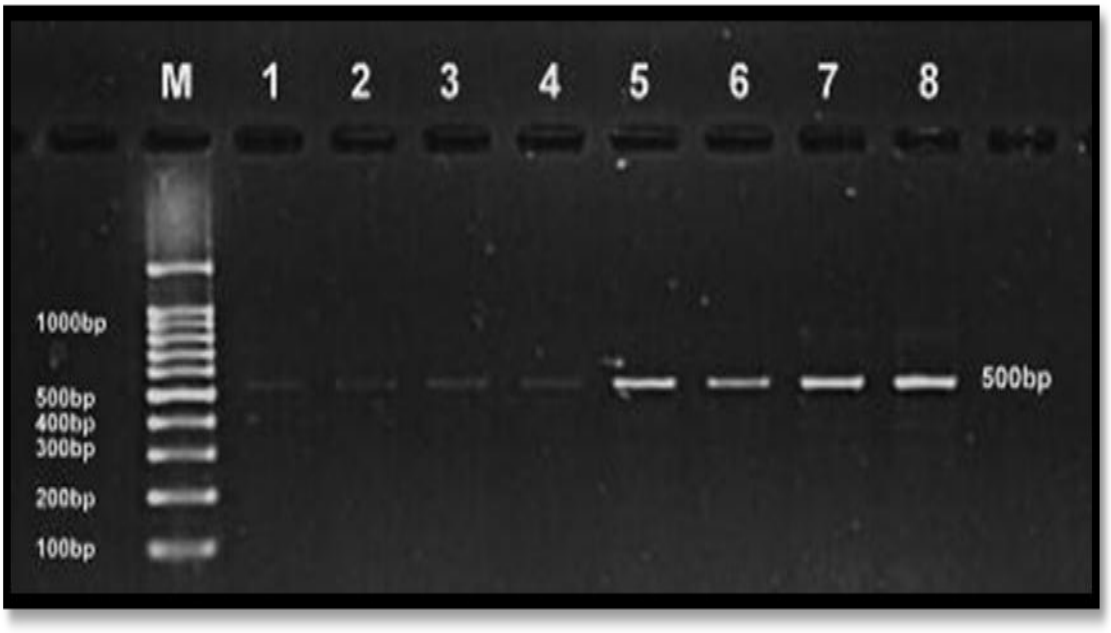

Figure (4-3): Agarose gel electrophoresis of PCR product for endolysin gene, this amplification product was one band 500 bp, lane M DNA ladder, lane 1-8 PCR product, (1.5% agarose , 75v, 20 mA for 1hr.(10µl in each well).

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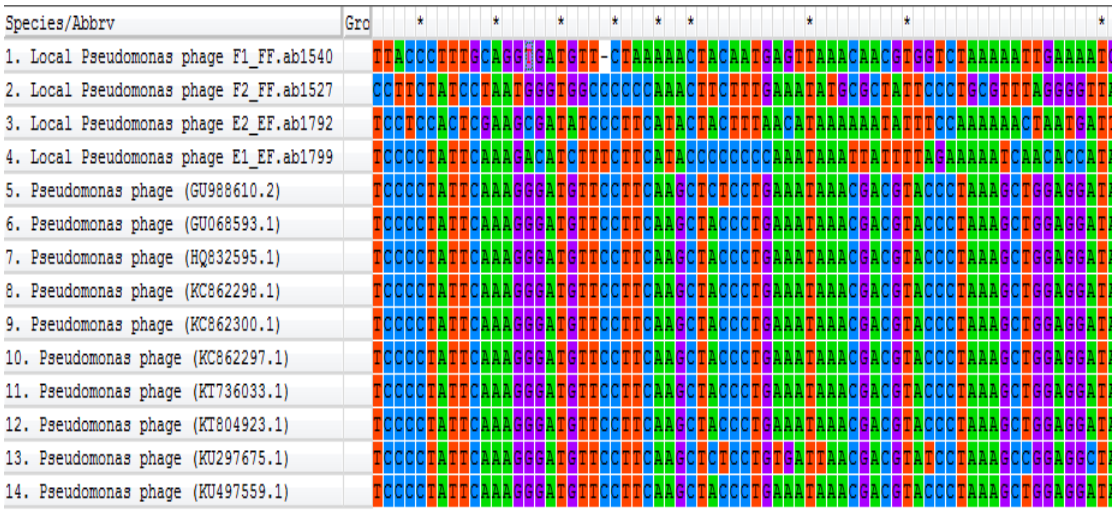

Figure (4-4): Multiple sequence alignment analysis of the partial Pseudomonas phage endolysin gene sequence in local Pseudomonas phage isolates with for NCBI-Genbank Pseudomonas phage based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity (*) and differences in endolysin gene nucleotide sequences.

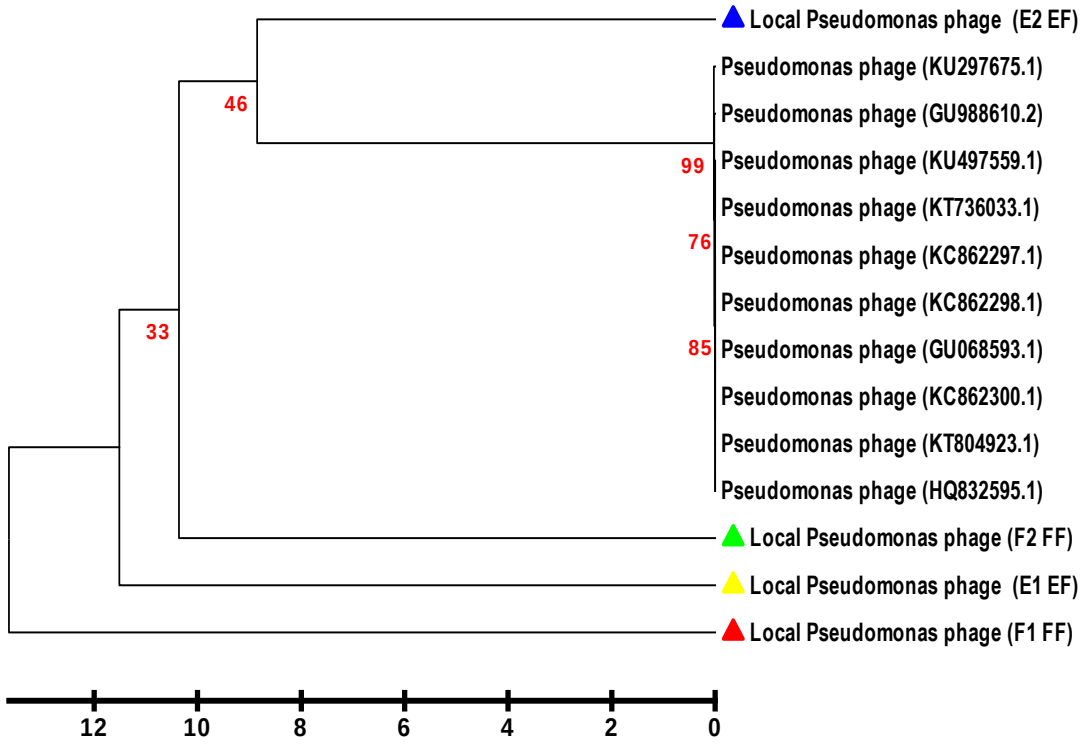

Figure (4-5): Phylogenetic tree analysis based on the Pseudomonas phage endolysin gene partial sequence that used for Pseudomonas phagedetection.

The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Pseudomonas phage isolates (E2 EF) were show closed related to NCBI-Blast Pseudomonas phage (KU297675.1) and others The local Pseudomonas phage isolates (F2 FF, E1 EF, and F1 FF) were show as unique isolates and different than NCBI-Blast Pseudomonas phage.

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4-2-Histopathological Study and Phage Therapy:

To examine the efficacy of phage therapy in *P.aeruginosa* infection, thermally injured rabbit model used in the present study. All rabbit were housed for four weeks in steel cages. Animals challenged superficial with 10^6cfu/ml of *P. aeruginosa* and incubation occurred at room temperature for 72 hours (3 days).

The results of this study showed that the phage had a remarkable role in the treatment of *P. aeruginosa* infection in the burned-rabbit models. The effect of phage appeared on a healing time 8 days after phage application in treatment and reduce the mortality of rabbit to zero in comparison to untreated *P. aeruginosa* infected group which was 100% after 4 days, all the rabbits completely recovered and showed the absence of signs after 18 day of using the phage in treatment as in figure (4-6).

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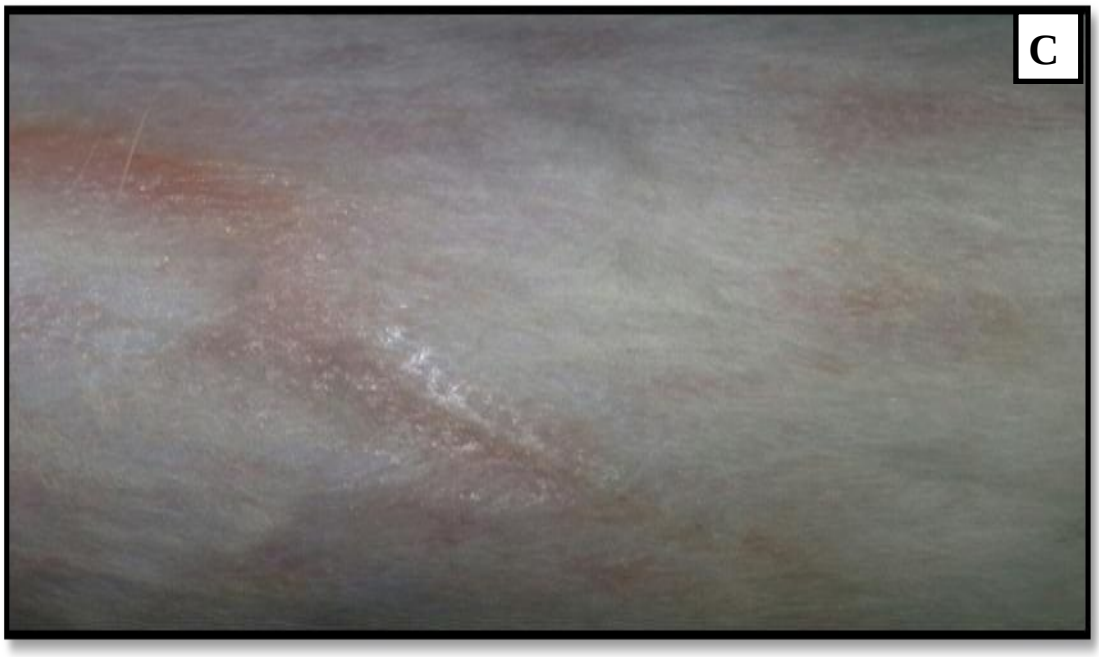
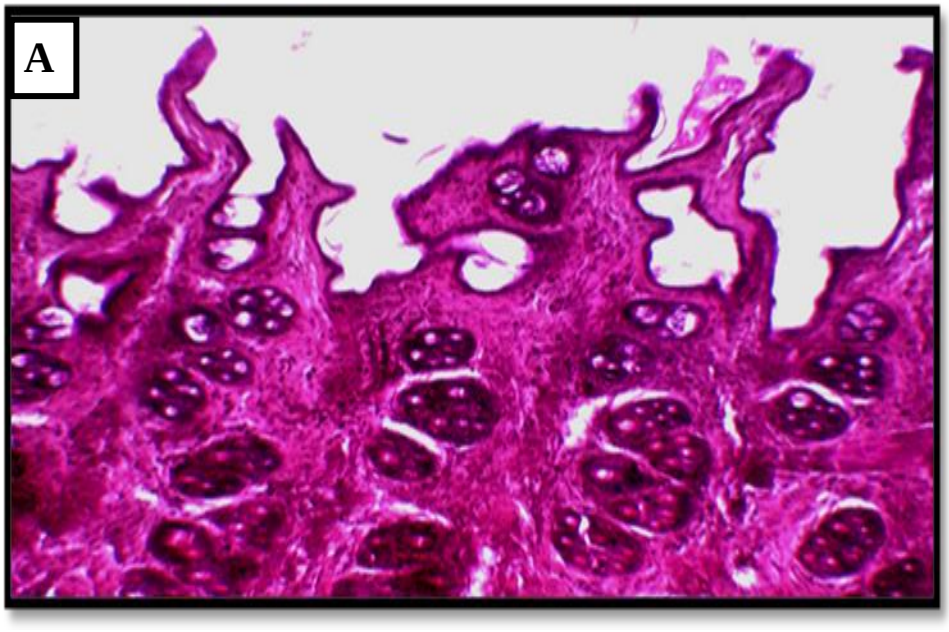


Figure (4-6) (A):Inflammation of skin rabbit after infection by *P. aeruginosa*.

(B, C) Complete healing of burned rat skin: 18 day after phage application in treatment.

The observed results showed that after histopathological examination of the rabbit skin lesions were demonstrated loss of skin reti-riges, thickening of epidermis, discontinuation of epidermal layer, incomplete repair process, acute and chronic inflammatory cell infiltration and all the skin appendages (hair follicles, sebaceous gland…. etc.) were damaged and congested blood vessel as in figure (4-9) in comparison to normal healthy control in figure (4-7) and burned control in figure (4-8).



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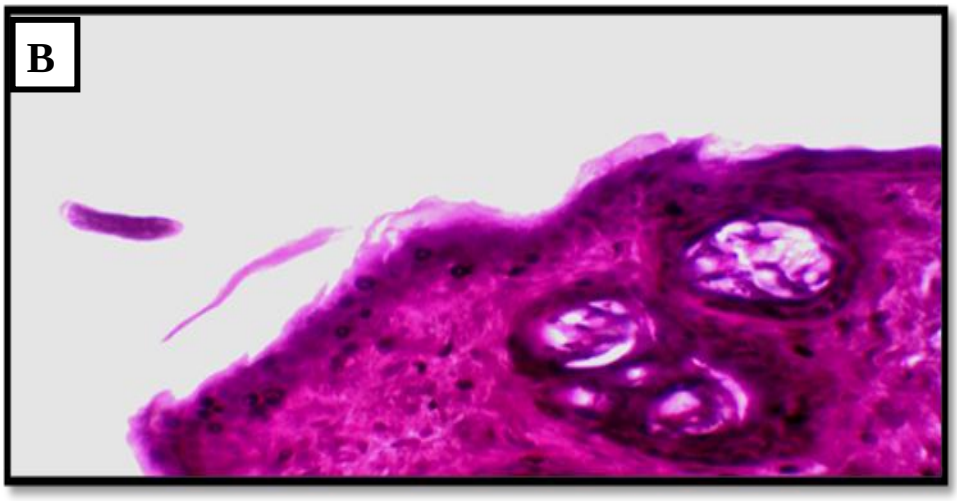

Figure (4-7A10X,B40X): Skin sections of control rabbit: Epidermis and dermis are normal in morphology and structure, keratinized squamous epithelium , normal skin appendages , scattered congested blood vessel. (stain :haematoxylin).

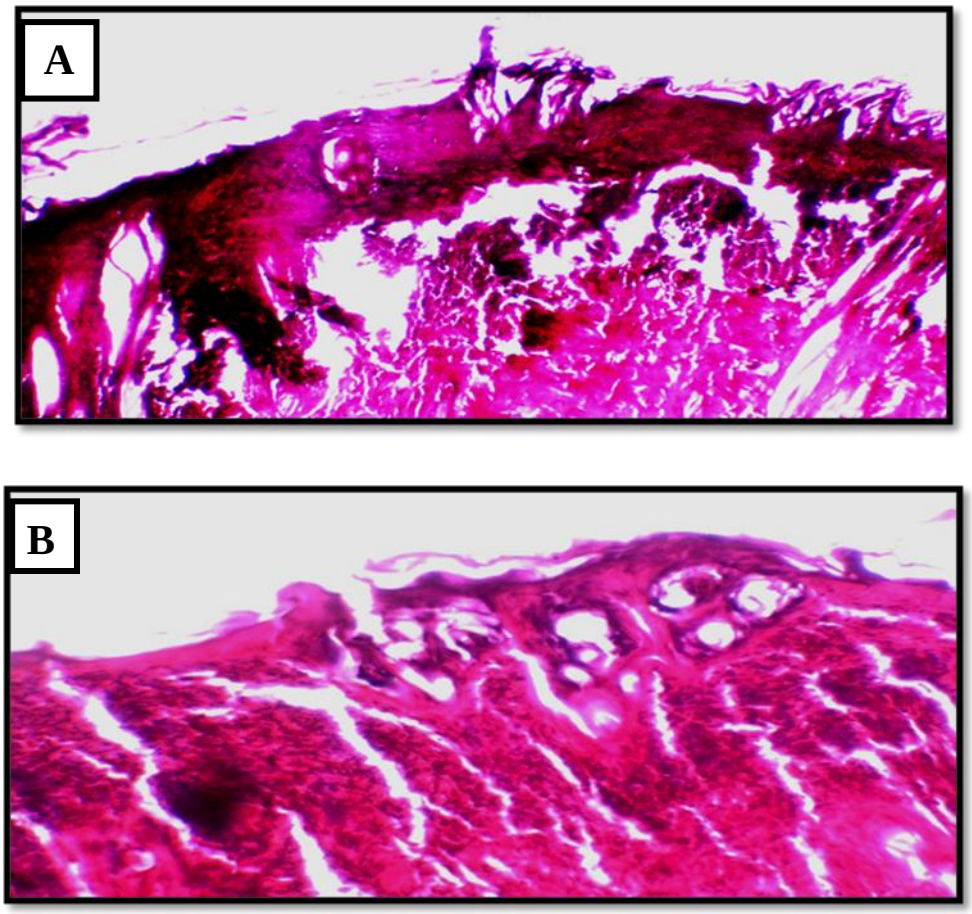

Figure (4-8A10X, B40X): Skin sections of burned rabbit skin: Loss of skin reti-riges and upper corneal layer, slight epidermal thickening with hyper keratodermia. Dermal heavy acute inflammatory cells, congested blood vessel .(stain :haematoxylin).

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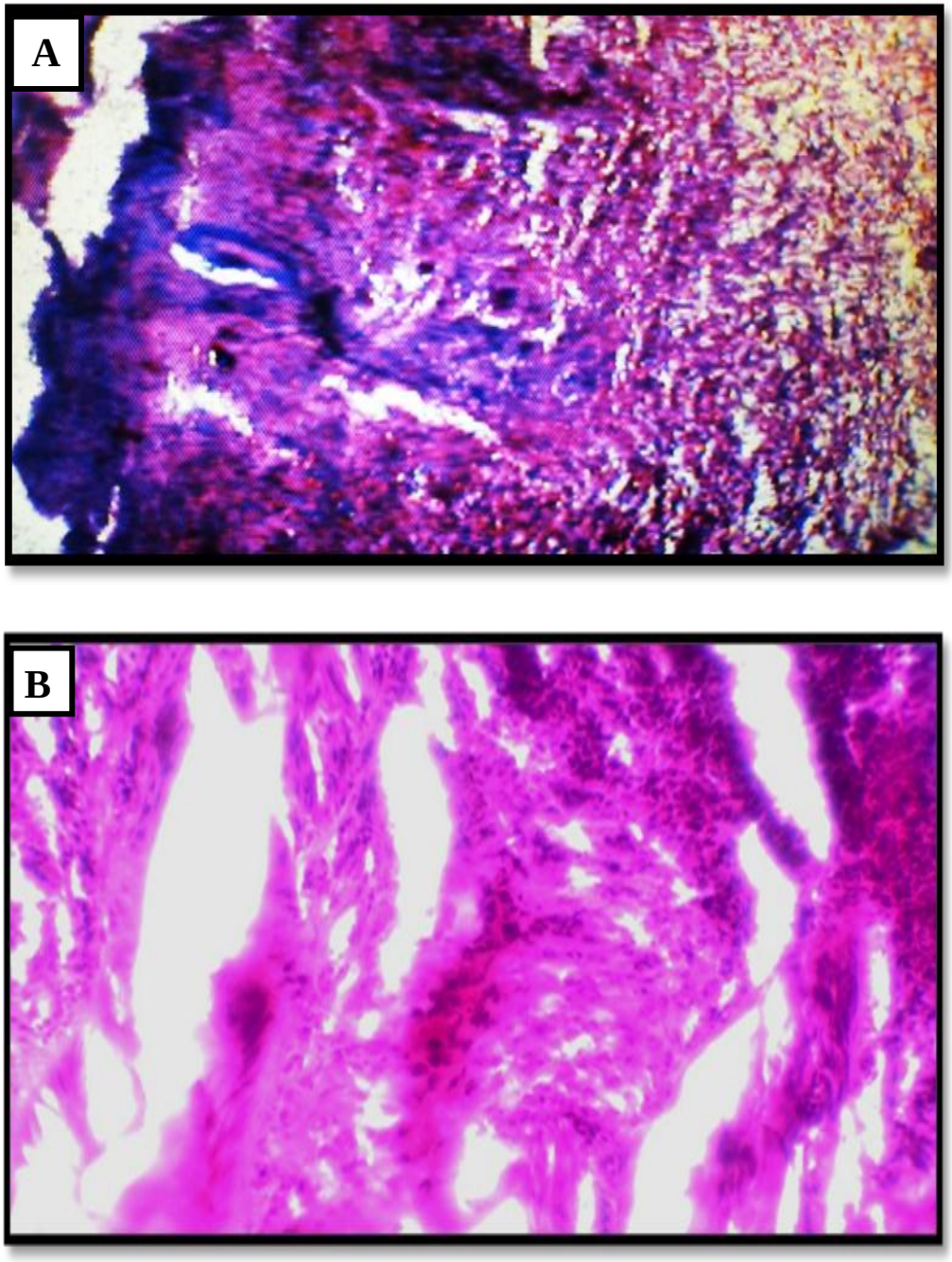

Figure (4-9:A10X.B40X):Skin sections of burned rabbit skin: four days after infection by *P.aeruginosa* (10^6 CFU per ml). sloughed epithelium(ulcer), acute and chronic inflammatory cells, congested blood vessel. (stain :haematoxylin).

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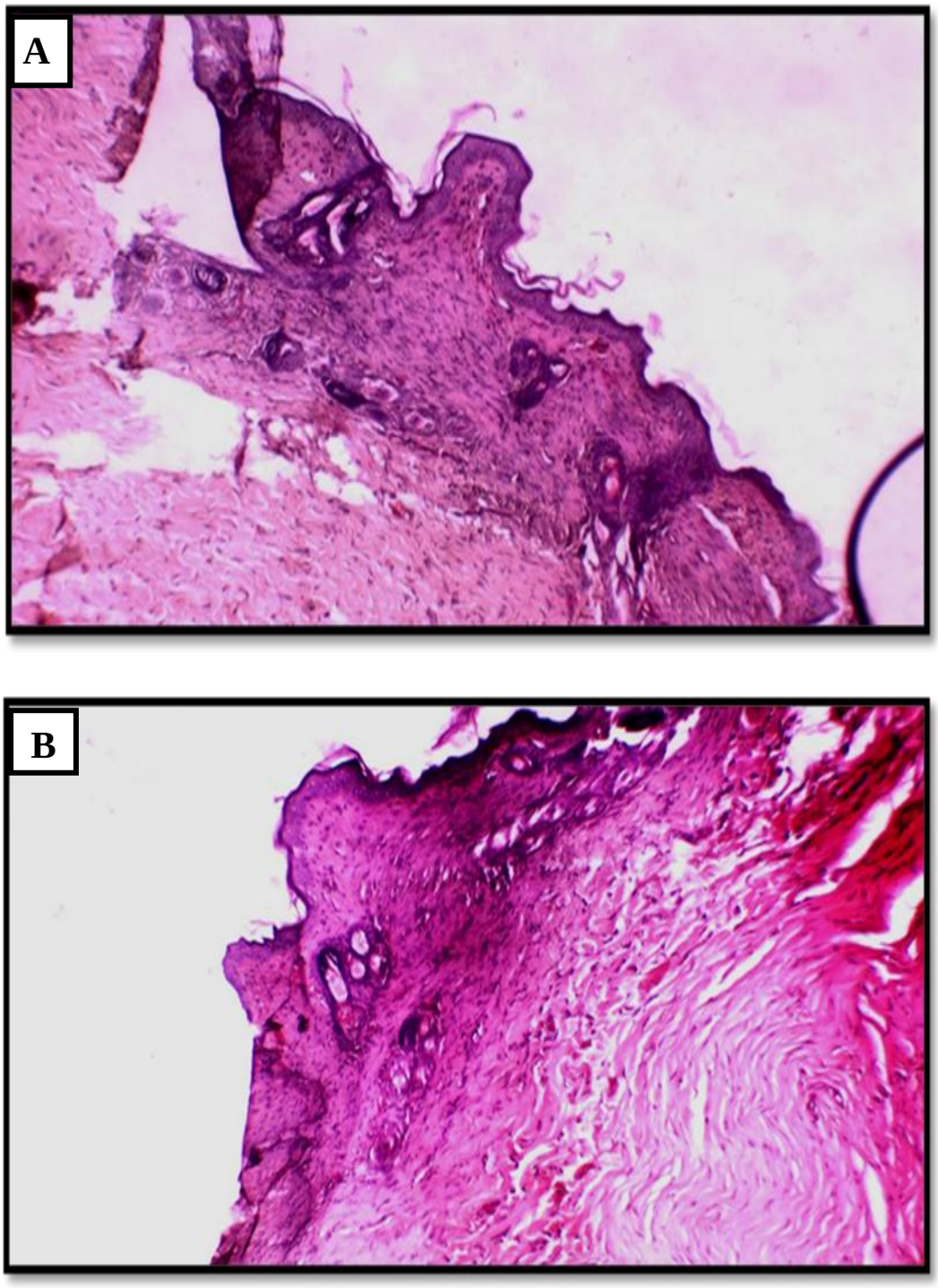

Figure (4-10:A10X, B40X): Skin sections of burned rabbit skin: 18 day after used phage in therapy. Skin sections shows: Loss of skin reti-riges, thinning of epidermis and dermal edema. Scattered mild dermal chronic inflammatory cells (near normal). (stain :haematoxylin).

Discussion:

Molecular Genetic Study:

Genome sequencing of Pseudomonas phage:

*P.aeruginosa*causes a broad spectrum of infections which are associated with urinary, respiratory and gastrointestinal tract, burn, wound, eyes and ears [23] The main goal of this study was to explain the global genomic diversity of virulent bacteriophage infecting the opportunistic pathogen *Pseudomonas aeruginosa*. Figure (4-1, 2, 3) showed the electrophoresis pattern of DNA extract. DNA fingerprinting using PCR amplification used as first indication [24], but classification in subfamilies and genera and elucidation of underlying evolutionary mechanisms can only be revealed by genome sequencing [25]. Genomic sequencing and annotation were performed for*P. aeruginosa* phage,
Figure (4-4) showed multiple sequence alignment analysis of the partial Pseudomonas phage endolysin gene sequence in local Pseudomonas phage isolates for NCBI-Genbank. And figure (4-5) showed Phylogenetic tree analysis based on the Pseudomonas phage endolysin gene partial sequence that used for Pseudomonas phage detection. Results showed the local Pseudomonas phage isolates (E2 EF) were show closed related to NCBI-Blast Pseudomonas phage (KU297675.1) and others The local Pseudomonas phage isolates (F2 FF, E1 EF, and F1 FF) were show as unique isolates and different than NCBI-Blast Pseudomonas phage.

Phage encoded cell wall hydrolytic enzymes lyse the bacterial cell from within. These enzymes are also very useful for pathogen detection and control. Large libraries of lytic enzyme-encoding genes identified by either genome sequencing, prophage identification or functional (meta-) genomics approaches [26] (Nelson *et al*., 2012). Knowledge of phage-encoded gene functions, and their influence on the bacterial host gene expression and general understanding of the molecular diversity of phage life cycle are rather limited. Recent advances in genome sequencing, comparative genomics combined with functional genomic studies will play a major role in filling this knowledge gap and increase our understanding of phage biology for better utilization of these organisms for bacterial detection and therapeutics [27].

This results highlights the importance of phage genome sequencing for its use as an alternative therapy, prior to further experiments. *P. aeruginosa* phage, which seemed to be an ideal candidate for controlling *P. aeruginosa* MDR infections [28,29].

5-1-3-Histopathological Study and Phage Therapy:

P. aeruginosa was chosen for the animal infection model since the phage caused the greatest reduction and effective in biofilm control. Burned skin seems to be especially susceptible to infection by *P. aeruginosa* [30] Phage therapy with phage experimentally improved survival and health in the Burned rabbit Model.

P. aeruginosa phages were isolated from sewage samples were evaluated in this study for the treatment of burn wound infection. The three main characteristics for use bacteriophage therapy from antibiotic therapy:(1) bacteriophages multiply at the infection site; (2) they target only specific bacteria, with no effect oncommensal flora; and (3) they can adapt to resistant bacteria [31].

Results of this study showed that the phage have great role in the treatment of *P.aeruginosa* infection in the burned-rabbit models. The effect of phage appeared on a healing time 4days after phage application in treatment and all the rabbits were full recovered and was showed the absence of symptoms (signs) after 18 days of using the phage in

treatment. This results were agreed with results being reported by [32-35]. There are reports available in literature where phages have been used to treat variety of bacterial infections in animal model systems [36,37] demonstrated the ability of phages to prevent rejection of skin grafts in guinea pigs. On this basis it was suggested that bacteriophages can play a role in the treatment of burn wound infection by *P.aeruginosa by* using cocktail of different *P.aeruginosa* specific phages.

Phages are nontoxic and can be applied to surface infections without adverse effects as described in Rhoads *et al.* [38]. On the other hand, the application of phages orally or intravenously is still highly discussed, because little is known about the reaction of the immune system to the phages, the clearance of the phages in the body and the actual effects on the infection [39]

A number of previous reports [8,40] indicate that a bacteriophage count of 10^2-10^3plaque forming unit (PFU) is sufficient to initiate a therapeutic replication with a 10^6-10^9 CFU per ml proliferation threshold of bacteria *in vivo*. Trials to date have used between 10^5 and 10^9 PFU of individual bacteriophages[41,42,43].

That phages were able to prevent the bacteria spreading into the blood but a high concentration of bacteria remained confined at the inoculation site, causing a strong immunological response and cutaneous mastocytosis. In the treatments at 24 and 48 h after bacterial challenge, the infection advanced systematically, so the phages possibly could kill bacteria in the blood [39].

Previous studies have shown the same results; a phage therapy study subjected immunocompromised mice to *Staphylococcus aureus* and no negative side effects were observed; instead, the animals did experience a beneficial immune-function replacement effect [44].

Any negative side effects of the phage therapy in vivo has been so far associated with insufficient purification protocols [1] As well as cell lysis, potentially releasing endotoxins and/or other superantigens that simulate immune responses [11] In a previous study, Golkar*et al*. showed the effectiveness of a lytic phage to control *P. aeruginosa* in mice model of wound infection[45].

The results showed that a single dose of the *P. aeruginosa* phage could significantly decrease the mortality of thermally injured, *P. aeruginosa* infected rabbits and that the route of administration was particularly important to the efficacy of the treatment.

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