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Studies on the Application of Bacteriophages and Silver_Nanparticles in The Treatment of *Pseudomonas spp*

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ABSTRACT

This study aimed to study the applications of bacteriophages as a biocontrol agent against certain antibiotic-resistant bacteria causing a chronic wound. Here, the chronic wound caused by *Pseudomonas aeruginosa* were isolated and characterized biochemically using the automated VITEK® 2 system. The antibiotic sensitivity pattern of the isolated chronic wound bacteria was assessed against the selection of antibiotics. These bacteria exhibited resistance against most of the tested antibiotics. To overcome this problem, phage vB_PseM-EB-E44 was isolated, identified and applied to control the growth of *Pseudomonas aeruginosa*. Phage was identified morphologically using TEM and exhibited that VB_PseM-EB-E44 is related to *Myoviridae*, this phage showed high lytic activity, high stability and a narrow host range. *P. aeruginosa* phage vB_PseM-EB-E44 was thermostable at temperatures ranging from 10 to 60 °C, however, and the titer decreased significantly at 70°C, no viable phages were detected at 80°C for 60 min. Phage vB_PseM-EB-E44 stabled at pH range 3 to 9 for 1h, lost its activity at pH 2, 10, 11, 12. The one-step growth curve of the phage showed a burst size of (60) PFU/cell with a latent period of (15) minutes for *P. aeruginosa* phage. *Pseudomonas aeruginosa* is one of the most common causes of healthcare-associated infections, this review collects data of all the *P. aeruginosa* phage sequenced to date, this review further addresses *in vitro* and *in vivo* results obtained by using silver_nanparticles and phages to treat or prevent *P. aeruginosa* infections in addition to the major hurdles related with this therapy. This study indicates that the isolated bacteriophages are promising biocontrol agents that could challenge antibiotic-resistant chronic wound bacteria to announce new successful alternatives to an antibiotic.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, aerobic, non-spore-forming rod that is able of causing an assortment of infections in both immunocompromised and immunocompetent hosts (Kerr and Snelling, 2009). *Pseudomonas aeruginosa* scarcely existed as part of the human microflora in healthy individuals is nonglucose fermenter rod, *P. aeruginosa* is an opportunistic pathogen for humans lead to a broad spectrum of diseases such as burn, urinary, septicemia and respiratory infections (Yang *et al.*, 2011).

P. aeruginosa is an aerobic Gram-negative commonly existed in different environments such as soil, plant, and water (Wagner *et al*., 2008; Pereira *et al*., 2014). That is capable to infect various organisms, including animals, humans, and plants (Lister *et al*., 2009; Breidenstein *et al*., 2011; Pereira *et al*., 2014). *P. aeruginosa* may not be an exemplary *pseudomonad* in natural environments. *P. aeruginosa* requires a simple nutrition supply, and has the ability to even grow in distilled water, it has the ability to also grow well in a medium containing ammonium sulphate (nitrogen source) and acetate (carbon source), the optimum temperature for growth is 37°C, but *P. aeruginosa* also grows at high temperatures 42°C helps distinguish it from many other *Pseudomonas* species (Nazina *et al*., 2005). But this widespread bacterium will grow in the presence of NO_3 but in the absence of O_2 (Robertson *et al*., 1989).

Most strains of *P. aeruginosa* produce one or more pigments, containing pyoverdine (fluorescent yellow-green) and pyocanin (blue-green), and pyorubin (red-brown) (Young *et al.,* 1947; Meyer *et al.,* 2000*)*. Former investigations have suggested that pyocyanin not only contributes to the persistence of *P. aeruginosa* in the lungs of CF patients but also interferes with many mammalians cell functions, containing ciliary beating, cell respiration, epidermal cell growth, calcium homeostasis and prostacyclin release from lung endothelial cells (Caldwell *et al*., 2009). *P. aeruginosa* caused nosocomial infections involving pneumonia, surgical site infections, bloodstream infections, and urinary tract infections, skin infections in the case of burn injuries. Repeated infections and chronic sinopulmonary colonisation from *P. aeruginosa* are seen in patients with cystic fibrosis (CF), infections caused by *P. aeruginosa* are not only widespread (Rello *et al*., 2002; Rello *et al*., 2005). Most of the injuries caused by *P. aeruginosa* are noticeably complex to treat using conventional antibiotic therapies, *P. aeruginosa* possesses an arsenal of virulence factors enabling it to circumvent host defenses and invade host cells (Wagner *et al*., 2008; Ballok *et al*., 2013). These mechanisms of virulence contain dissembled factors such as elastase, pyocyanin, proteases, exoenzyme S, exotoxin A, and exoenzyme S and also cell-associated factors such as pili, lipopolysaccharide (LPS), and flagella (Wagner *et al*., 2008; Strateva *et al*., 2011). All of these resistance mechanisms and virulence factors take part in increased *P. aeruginosa* pathogenicity, resulting in infections that are consequently very complicated to treat (Driscoll *et al*., 2007; Fajardo and Martínez 2008; Breidenstein *et al*., 2011).

Most of the infections caused by *P. aeruginosa* are noticeably hard to treat using ordinary antibiotic therapies, since this microorganism exhibit high actual resistance to a large range of antibiotics, containing, fluoroquinolones, β-lactams and aminoglycosides (Breidenstein *et al*., 2011). *P. aeruginosa* is extremely isolated from hospital medical equipment, because of the bacterium's capability to survive in biofilms (Inglis, 1993; Donlan, 2001). Infections with multidrug-resistant *P. aeruginosa* require new therapies with present care focused on silver_nanparticles and bacteriophages (phages). Silver compounds have been historically used to control microbial proliferation (Wadhera and Fung, 2005). The antifungal and antibacterial effect of Silver Nanoparticles AgNPs, even against antibiotic-resistant bacteria (Wright *et al*., 1994; Wright *et al*., 1999), has been demonstrated in *in-vitro* conditions. Nowadays, silver compounds are routinely applied in a wide array of industrial and sanitary fields, such as coating of catheters and surgery material, the production of synthetic compounds for odontology, treatment of burn injuries, homeopathic medicine, water purification and textile fabrics (Spencer, 1999; Klasen, 2000; Wadhera and Fung, 2005; Atiyeh *et al*., 2007; Hwang *et al*., 2007), and possess low toxicity

to human cells, high thermal stability and low volatility (Durán *et al*., 2007).

Biodegradable nanoparticulate systems have received considerable attention as potential drug delivery vehicles. Chitosan (CS), a polysaccharide known to be a favorable pharmaceutical material because of its biocompatibility and biodegradability, forms an ideal hydrophilic carrier system (Kreuter, 1991). Moreover, chitosan has been shown to be non-toxic and tissue compatible in a range of tests. Nanoparticles, which can be produced with a wide variety of polymers and nanotechnologies, have also been recently proposed as delivery systems for peptides and proteins through the pulmonary route (Alonso, 2020). In this respect, chitosan is a very attractive polysaccharide due to its reported low toxicity, biodegradability and mucoadhesivity (Cohen and Bernstein, 2020). Bacteriophages are bacterial viruses that were discovered about a century ago (Felix, 1917; Twort, 1961). Phages are viruses that infest bacteria; they cause lysis of bacterial cells (lytic phages) and disconnect the metabolism of bacteria. Especially, any species of phage attacks only certain bacteria as its host; it frequently does not affect total microbial biomass (Wang *et al*., 2000). According to their life cycles, phages may be divided into two classes: temperate phages (lysogenic) and virulent phages (strictly lytic). Temperate phages commonly combine their genome into the host chromosome or sometimes save it as a plasmid that is transmitted, by cell division, to the daughter cells (Guttman *et al*., 2004; Little, 2005).

Lytic phages adsorb to the host cell surface, inject and replicate their DNA, and then stimulate host cell lysis, resulting in the liberation of progeny phages that may be starting another cycle of infection (Guttman *et al*., 2004). In addition to the lysogenic and lytic types of bacteriophage infection, other types may exist. The filamentous bacteriophages typically cause persistent infection of bacterial cells which does not kill the host but results in continued excretion of viral particles (Harper *et al*., 2014). During effective infection, a strictly virulent phage produces typically more than 100 copies of itself (Szafranski *et al*., 2017). If the bacterial cells are larger than the number of phages, next to many generations the population of phages will exceed that of bacterial cells, finally, the total bacterial cells will lyse (Wang *et al*., 2000). Next, with the arrival of the golden era of antibiotics, together with the scientific difference concerning the utilize of phages because of poorly controlled trials, the benefit of phage therapy decreased quickly (Sulakvelidze *et al*., 2001). Antibiotics were inexpensive and frequently effective against bacterial diseases and thus were considered the better solution to fight bacterial diseases. So, following World War II, phage therapy was ignored in the western world but kept being utilized in Poland and the Soviet Union (Sulakvelidze *et al*., 2001; Chanishvili, 2012). One alternative that has recently revived benefit is phage therapy, top proposed in the early 20th century by Felix d'Herelle. Besides, several clinical studies have clarified that the utilization of phages in both animals and humans is active and without side effects (Burrowes *et al*., 2011; Patey *et al*., 2019). Lytic phages are considered as possible alternative candidates to traditional antibiotics for a large range of oral bacterial infections (Lee *et al*., 2019). Phages of the *Pseudomonas* genus were first described in the middle of the 20th century (Kellenberger and Kellenberger, 1957; Holloway *et al*., 1960).

Pseudomonas aeruginosa is one of the most problematic opportunistic pathogens involved in hospital-acquired infections (Aloush *et al*., 2006; Driscoll *et al*., 2007). The possibility to handle efficiently multidrug-resistant (MDR) bacteria with phages has recently spurred regenerate interest in this field (Gordillo and Barr, 2019). Phages exhibit several features over traditional antibiotics, the leading one being their specificity, since they usually target a single bacterial species, while dismissal the host-microbiota unaffected. Secondly, phage replication is dependent on the existence of the host-bacterium and is therefore self-limiting. The main interest for the therapeutic

utilization of phages is the potential to transfer virulence or antibiotic resistance genes, requiring in-depth analysis of the phages' genomes (Torres-Barcelo, 2018). More than 90% of phages have large, double-stranded DNA genomes located in heads with icosahedral symmetry, with tails of diversity lengths. All fall within the order *Caudovirales* and belong to three major morphological groups. These are the families *Podoviridae* (with short, noncontractile tails), *Myoviridae* (with long, rigid, contractile tails), and the *Siphoviridae* (with long, flexible, noncontractile tails). The genome and morphology type of the remaining phage families are highly changeable, and they can own RNA or DNA genomes. One notable group with single-stranded DNA genomes (*Inoviridae*) appears as long filaments (Maniloff, 2006). For therapeutic utilizes, obligately lytic phages are so desired. Due to they result in the fast killing of their target host cell, bacteriophage numbers rise rapidly and transduction is relatively unusual. DNA sequencing of bacteriophage genomes is now utilized to confirm both absence of unfavourable elements and identity, like functional bacterial toxins or lysogenic components. Like toxin genes are known to be particularly related with some bacteriophages, for example, the *Shiga* toxins of *Escherichia coli*, which would be of real interest if existent in a therapeutic bacteriophage formulation. Early tryings to utilize phages for therapy of bacterial infections were compromised by the rareness of understanding of the nature of the agents involved. Work in this area dates back to 1919, but as delayed as 1941, reliable reports were yet challenging the theory that bacteriophages were viral in nature (Krueger and Scribner, 1941). In this study, bacteriophage isolation and identification were achieved as novel candidates appropriate for bacteriophage therapy silver_nanparticles against *P. aeruginosa* as alternatives to conventional antibiotics.

MATERIALS AND METHODS

Growth Media and Buffers Recipes:

1. Nutrient Agar (NA) Medium (Eaton *et al*., 1995):

Nutrient agar continues to be an extensively utilized general-purpose medium for growing non-fastidious microorganisms. If required, enrichments can be added to this medium. It consists of 3 g/l beef extract; 5 g/l Peptone, 5 g/l NaCl and 20 g/l agar. The ingredient of the medium was suspended in one liter of distilled water. pH adjusted to 7.0 before sterilization.

2. Muller-Hinton Agar (Atlas, 1993):

This medium is utilized in antimicrobial susceptibility testing by the disk diffusion method. This formula conforms to clinical and laboratory standard Instited (2009) and this medium is low in trimethoprim, sulphonamide and tetracycline inhibitor, and supply satisfactory growth of most non-fastidious pathogens. This medium

3. Brain Heart Infusion Media (Seth, 1970)

A solid medium that contains the highly nutritious infusions recommended for the cultivation of fastidious organisms.it is consists of Brain infusion solids 12.5 g/L, Beef heart infusion solids 5g/L, peptone 10 g/L, sodium chloride 5g/L, glucose 2g/L, disodium phosphate 2.5g/l, agar 10g/L, the ingredient of the medium was suspended in one liter of distilled water. pH adjusted to 7.4 before sterilization.

4. SM Phage Buffer (Giudice and Mutolo, 1967):

SM buffer consist of NaCl (Sigma) 5.8 g/L, $MgSO_4$ (Sigma) 1.2 g/L, Tris-HCl 1 M (pH=7.5) (Fluka) 50mM, Gelatin (Sigma) 0.1 g/L, these Ingredients dissolved in1 liter of deionized water before sterilization.

Bacterial Strains and Growth Conditions:

This study was performed on antibiotic-resistant chronic wound isolates that were isolated formerly from patients with wound plaques in Qalubiya governorate, Egypt. All strains were stored at -80°C in Brain-Heart-Infusion broth complemented with 20% (v/v) glycerol. Freshly overnight grown cultures were prepared by inoculating a single colony into 10 ml of Basal salt medium with yeast extract and incubating for 16 h at 37°C with shaking at 200 rpm.

Morphological And Biochemical Identification of the *P. aeruginosa*:

Biochemical and Morphological identification (Table S1, Supplementary data) of the chronic wound bacteria were done according to Bergey's Manual of Systematic Bacteriology. This isolate was approved by VITEK® 2 COMPACT automated instrument for ID/ AST testing (Pincus, 2006)

Gram's Stain and Microscopic Examination (Davies *et al*., 1983):

Air-dried and heat-fixed smear were stained for 1 minute with crystal violet solution then the slide was washed in a gentle and indirect stream of tap water, the slide was flooded with the Iodine for 1 min, The slide was washed in a gentle and indirect stream of tap water, The slide was washed with a decolorizing agent until decolorizing agent running from the slide and become clear, The slide was flooded with safranin stain for 1/2 min, The slide was washed with tap water until no color appears in the effluent and then blot dry with absorbent paper and the slide was examined under oil immersion lens (100X) using a bright field microscope.

Catalase Test (Varghese & Joy, 2014):

Transfer a small amount of bacterial colony to the surface of a clean, dry glass slide utilizing a loop or sterile wooden stick. Place a drop of 3% H_2O_2 onto the slide and mix. A positive result is the rapid evolution of oxygen (within 5- 10 s) as evidenced by bubbling. A negative result is no bubbles or only a few scattered bubbles

Coagulase Test (Chessbrough, 2000):

Procedure: Slide Coagulase Test: A drop of rabbit plasma was placed on a clean glass slide, then A colony of the organism was emulsified in the rabbit plasma using a sterile inoculating loop and the slide rocked back and forth gently for one minute. The results may be Positive: white clumps in plasma, Negative: no clumps

Motility test (Shields and Cathcart, 2016):

Motility of different pure bacterial isolates was observed using MIO media (HIMEDIA). A loopful of bacterial culture from a 24 hrs old liquid culture was immersed in a slant containing MIO media to observe motility and non-indole and non-ornithine production.

Urease test (Brink, 2010):

The test organism is cultured in a urease medium slant (Oxoid), which contains urea and the indicator phenol red. Pure bacterial cultures containing the urease enzyme are able to hydrolyze urea to ammonia and though there was red or pink color; the result was positive.

Confirmatory Identification of The Selected Bacteria by Vitek® 2 Compact System:

Selected isolates were cultured on nutrient agar and incubated for 24 hr. at 37 °C to ensure purity and to get single colonies, after isolation of bacterial colonies on culture media, isolates were identified by Vitek® 2 compact auto analyzer system in Microbiology Lab., Mabret Elasafra hospital, Alexandria governorate, Egypt (Abbas & Radhi, 2016).

Antibiotic Sensitivity Test:

Antibiotics sensitivity testing was performed on Mueller-Hinton agar by the disc diffusion method (Biemer, 1973) for the following antibiotics (Oxoid, UK); Aztreonam (ATM, 30 µg), Amoxicillin (AX, 25 µg), Tobramycin (TOB, 10 µg), Streptomycin (S, 10 µg), Imipenem (IPM , 10 µg), Ceftazidime (CAZ, 30 µg), Cefotaxime (CTX, 30 µg), Cefaclor (CEC, 30 µg), Cephalexin (CL, 30 µg), Ceftriaxone (CRO, 30 µg), Cefoxitin (FOX, 30 µg), Ciprofloxacin (CIP, 5 µg), Norfloxacin (NOR, 10 µg), Ofloxacin (OFX, 5 µg) and Trimethoprim-sulfamethoxazole (SXT, 25 µg). The results were interpreted conferring to the guidelines of the Laboratory and Clinical Standards Institute (CLSI) (Wayne, 2010).

Preparation Of Silver and Chitosan Nanoparticles:

Silver nanoparticles have been prepared by the chemical reduction method as reported by Turkevich (Turkevich *et al*., 1951; Lee and Meisel, 1982). A solution of $AgNO_3$ has been used as Ag^{1+} ions precursor. The PVP has been used as stabilizing agent and borohydrate as a mild reducing agent. The color of the solution slowly turned into a

grayish-yellow, indicating the reduction of the Ag^{1+} ions to Ag nanoparticles.

Chitosan nanoparticles were prepared according to the ionotropic gelation process (Hasanin *et al*., 2018). Blank nanoparticles were obtained upon the addition of a tripolyphosphate (TPP) aqueous solution to a chitosan solution

Susceptibility Tests Assess the Antimicrobial Activity of Nanoparticles:

Nanosilver and nano chitosan were brought from specialized company in distilled water to different concentrations (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml). The test was performed by the disc diffusion method on muller hinton agar media. This experiment used a sterilized paper disc (5mm) impregnated with different concentrations of nanoparticles. Bacteria overnight cultured was spread-plated on muller hinton agar plate then sterilized disc was put over them and the plates were incubated at 37 °C for 24 hrs, after this time the inhibition zone was measured to determine their antimicrobial activity (George *et al*., 2010).

Isolation of Bacteriophages:

The clinical isolates *P. aeruginosa* was utilized as hosts for the propagation and isolation of bacteriophages. Samples from 15 healthy individuals and 85 burn injuries in affected patients and drainage samples from dermatology clinics have been collected from Benha city, Qalubiya governorate, Egypt for bacteriophage screening. This sample was centrifuged at 10,000 rpm for 10 min, supernatant fluids were collected, and membrane filtered using 0.22 µm membrane filters. Enrichment of phages and isolation were performed as described formerly (Van Twest and Kropinski, 2009). Briefly, 5 ml of a 0.22 µm-filtered sample was mixed with 20 ml double-strength Tryptic Soybean Broth (TSB) medium and 100 µl of a mid-log culture of *P. aeruginosa* strain vB-pesM-EB-E44 and incubated for 48 h at 37°C with shaking at 200 rpm. Later, bacteria were harvested by centrifugation at 10,000 rpm for 10 min, supernatant fluids have been recovered, and membrane-filtered using 0.22 µm. Phages were screened by spotting five microliters of the enriched samples onto double-layered plates containing a lawn of the indicator bacteria strain and incubated for 48 h at 37°C (Huang *et al*., 2018). Plates were inspected for the presence of clear lysis zones, the clear zone was cut and propagated in a fresh culture. This lysate was serially diluted, spotted onto double-layered plates, and incubated as described above.

Transmission Electron Microscopy of Bacteriophage:

Ten microliters of highly purified phage suspension were fixed onto 300 by 300 mesh copper grids (Electron Microscopy Sciences) supported by carbon-coated Formvar film (Ackermann, 2012). After 5 minutes, fixed phages were negatively stained with 2% (w/v) aqueous uranly acetate, pH7.2 for 1 min, andair-dried at room temperature for 1 h. A transmission electron microscope (A JEOL JEM-2100) was used for attaining the phage particle images at the Electron Microscope Facility, Al-Azhar University, Egypt.

One-Step Growth Curve:

Phage's growth phases and burst size were determined as described formerly (Huang *et al*., 2018). A known number of an exponential-phase culture (ca. 4×10^7 CFU/ml) of each bacterial host was infected with each specific phage individually at an MOI of 1, phages were allowed to adsorb for 5 min at room temperature. The mixture was then centrifuged at 5,000 rpm for 5 min and the supernatant was decanted to remove free-unbound phages. The bacteria-phage pellet was then washed twice and resuspended in 10 ml of TSB and maintained at 37°C with continuous shaking. At appropriate times phage titers were enumerated using plaque assay (Kropinski *et al*., 2009).

Determination of the Bacteriophages Host Range:

The host range for the isolated phage vB-PesM-EB-E44 was determined against a collection of twenty bacterial isolates as formerly described with some modifications (Clokie and Kropinski, 2009; Danis-Wlodarczyk *et al*., 2015). Ten microliters of each phage suspension (about 10^8 PFU/ml)

were spotted, in duplicate, onto the TSA bacterial lawn plates and incubated at 37 °C for 16–18 h.

Thermal and pH Stability:

The thermal and pH-stability of the isolated phages were tested as described before (Huang *et al*., 2018). For the assessment of thermal stability, 900 µl of preheated 0.22 µ m filter-sterilized SM buffer (5.8 g $NaCl_2$ 2.0 g $MgSO_4$ •$7H_2O_2$ 50 ml 1 M Tris-HCl pH 7.4, in 1-liter dH_2 O) were added to 100 µl of each of phage lysates (8 log10 PFU/ml). Tubes were incubated at 10°C, 37°C, 50°C, 60°C, 70°C, and 80°C for 1 h. Aliquots were collected after 60 min of incubation to determine phage titers. For pH-stability assessment, phage lysates (8 log10 PFU/ml) were added to tubes containing sterile SM buffer with pH values ranging from 2-12 adjusted with NaOH and HCl. The tubes were incubated at 37°C for 60 min. Subsequently, the phage solutions were serially diluted and the recovered phage titers were determined using bacterial hosts employing the double-layer agar method (Kropinski *et al*, 2009). Each temperature and pH treatment were performed in triplicates and the average of triplicate counts was calculated. Phage thermal/pH stability (%) = (Recovered phage titers following the treatment / Initial phage titer before treatment) ×100%

RESULTS

Bacteria and Antibiotic Sensitivity Testing:

In the current study, *P. aeruginosa* were isolated formerly from infected patients with the chronic wound (Fig. 1). The isolates were identified biochemically using conventional methods (Table S1, Supplementary Data) and were confirmed by Vitek 2 system. An antibiotic sensitivity test was performed for isolated bacteria against a selection of fifteen antibiotics (Fig. S1, Supplementary data). Qualitative data from the antibiograms (Table 1) revealed that *P. aeruginosa-S-EB3* were resistant to at least twelve antibiotics with a resistance percentage of 87% against the tested antibiotics, respectively. *P. aeruginosa-S-EB3* resisted Tobramycin, Amoxoicillin, Norfloxacin, Cefaclor, Cephalexin, Ceftriaxone, Cefoxitin, Streptomycin, Vancomycin, Ceftazidime, Ofloxacin, and Trimethoprim/sulfamethoxazoe but was susceptible to Aztreonam.

Table1. Antibiotic sensitivity pattern of the isolated chronic wound bacteria against a selection of fifteen antibiotics

Antibiotic Category	Antibiotic used	*P. aeruginosa-S-EB3*
Monobactams	Aztreonam (ATM, 30 µg)	S
Penicillins	Amoxicillin (AX, 25 µg)	R
Aminoglycosides	Tobramycin (TOB, 10) Streptomycin (S, 10)	R R
Carbapenems	Imipenem (IPM, 10)	I
1st Generation Cephalosporins	Cephalexin (CL, 30)	R
2nd Generation Cephalosporins	Cefaclor (CEC, 30) Cefoxitin (FOX, 30)	R R
3rd Generation Cephalosporins	Ceftriaxone (CRO, 30) Cefotaxime (CAZ, 30 µg) Ceftazidime (CTX, 30 µg)	R R R
Quinolones	Ciprofloxacin (CIP, 5 µg) Norfloxacin (NOR, 10 µg Ofloxacin OFX, 5)	I R R
Sulfonamides	Trimethoprim-sulfamethoxazole (SXT,25)	R

* Denotes for Resistant (**R**), Intermediate (**I**), and Susceptible (**S**).

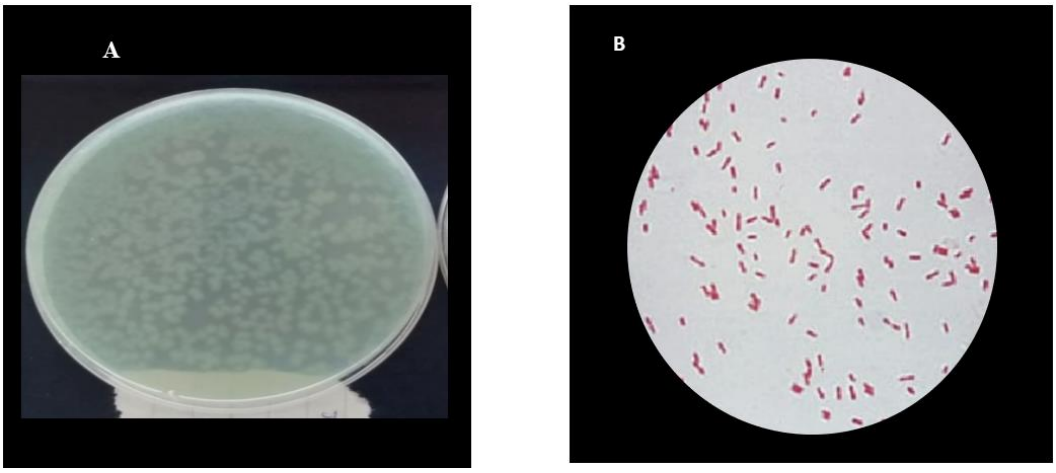

Fig. 1. (A): Single colony representative of ***P. aeruginosa***, (**B**) **Microscopic** examination representative of *P. aeruginosa* using 100X oil-immersed lens of B-150 OPTIKA microscope.

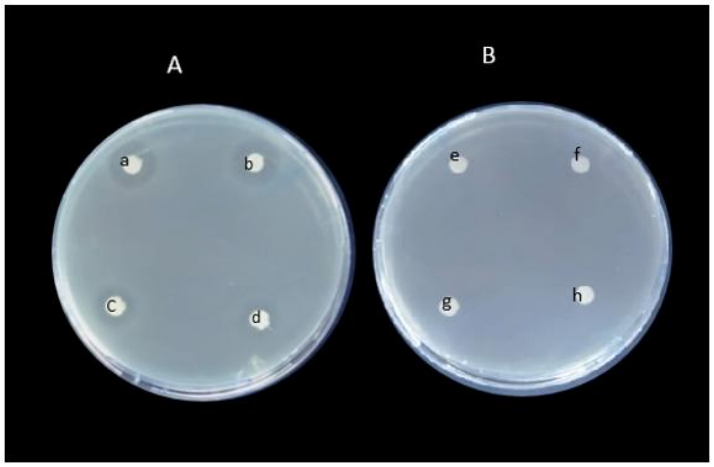

Fig. 2. (**A**): Nano sliver susceptibility test showing zones of clearance surrounding paper Discs (a=1mg/ml, b=0.5mg/ml, c=0.25mg/ml, d= 0.125mg/ml) against multidrug resistance *P. aeruginosa-S-EB3*, (**B**): Nano chistosan susceptibility test isn't effective on the bacteria with different concentration in (e=1mg/ml, f=0.5mg/ml, g=0.25mg/ml, h= 0.125mg/ml) against multidrug resistance *P. aeruginosa-S-EB3*.

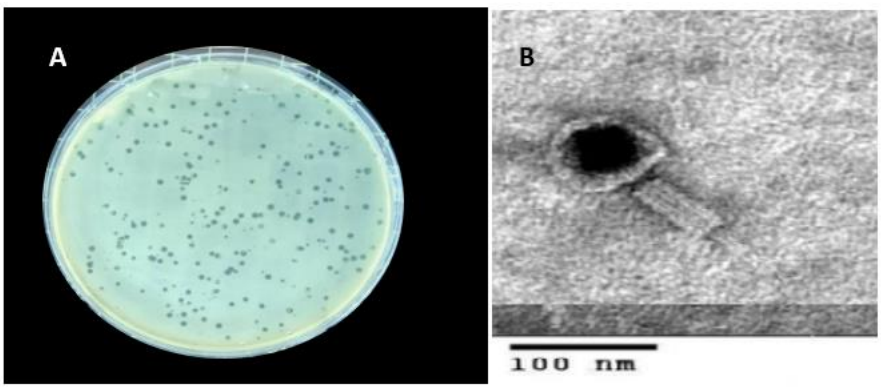

Fig.3. Plaque phenotype and TEM morphology of the isolated phage. (**A**) Image of bacterial plaque formed by the isolated phage in top-agar lawns of *P. aeruginosa*-S-EB3, plaque appearance was detected and imaged after culturing 48 h on their hosts. (**B**) TEM micrographs of phages vB_PseM-EB-E44 were negatively stained with 0.2% uranyl acetate as described in Materials and Methods. Scale bar= 100 nm.

Morphology of Bacteriophage:

Phage plaque morphology (Fig3. A) targeted chronic wound causing bacteria, *P. aeruginosa* was successfully obtained after a screening of 100 samples of wounds. Successive double soft-layer agar assays led to pure phage isolation, titrated and processed at 4°C. (Fig3. A) shows clear plaques produced by VB_PseM-EB-E44 on *P. aeruginosa -S-EB3* as host strains respectively. Phage VB_PseM-EB-E44 produces small circular clear plaques with a diameter of 2 mm. The concentration of phage was $4x10^7$ PFU/ml. Transmission Electron microscopy (Fig3. B) allowed us to infer that VB_PseM-EB-E44 belong taxonomically to order *Caudovirales*. Dimensions of the isolated phage were measured and summarized in (Table 2). The particle of VB_PseM-EB-E44 had a contractile tail with 192.3 nm in length and head with a diameter of about 61.5 nm a typical member of *Myoviridae* family.

Table2. Dimensions of the isolated phage

Phage	Bacterial host	Plaques diameter (mm)	Head diameter (nm)	Tail length (nm)	Proposed family
vB_PseM-EB-E44	*P. aeruginosa-S-EB3*	2	61.5± 2	192.3± 1	*Myoviridae*

Growth-Kinetics and Host Range:

One-step growth kinetics of the isolated phage (Fig. 4) exhibited typical growth kinetics of most bacteriophages. Phage vB_PseM-EB-E44 gave a burst size of 60 PFU/cell with latent periods of 15 minutes. The host range of the isolated phages was estimated and results were summarized in (Table 3). Phage established a narrow spectrum of lytic activity.

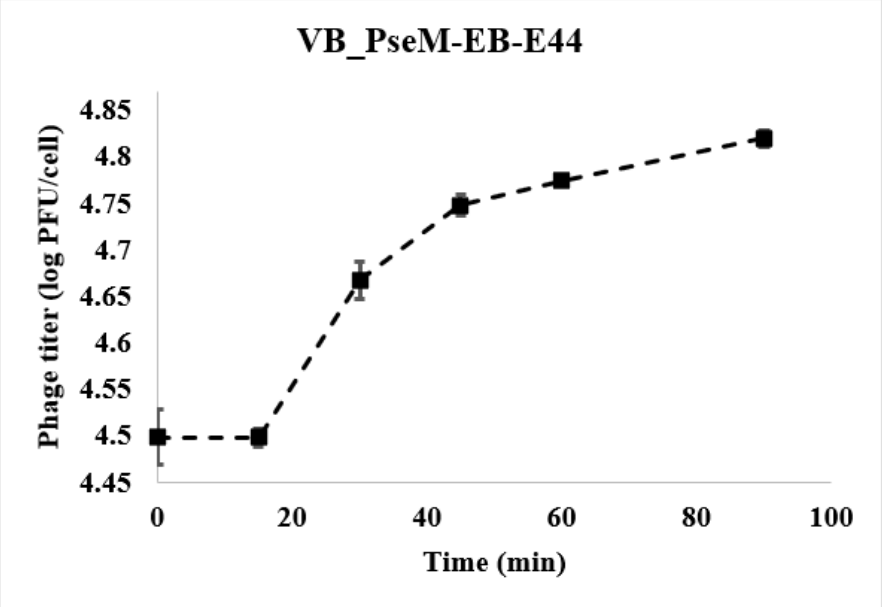

Fig.4. One-step growth curves of phage vB_PseM-EB-E44 on their corresponding hosts. Data show the mean of two replicates and error bars show the deviation in the values.

Table 3. Host ranges of the isolated phage

Species	Strain ID number	Lysis by bacteriophage
		Phage vB_PseM-EB-E44
Pseudomonas aeruginosa	*P.aeruginosa-S-EB1*	-
	P.aeruginosa-S-EB2	-
	P.aeruginosa-S-EB3	+
	P.aeruginosa-S-EB4	-
	P.aeruginosa-S-EB5	-
	P.aeruginosa-S-EB6	-
	P.aeruginosa-S-EB7	-
	P.aeruginosa-S-EB8	-
	P.aeruginosa-S-EB9	-
Klebsiella Pneumoniae	Kp01	-
S. aureus	Sa01	-
	SA101	-
	SA1E	-
	EG-AE1	-
E. coli	Ec01	-
Acinetobacter baumannii	Ab01	-
	Ab02	-
Salmonella enteritidis	EG. SmE1	-
	EG. SmE2	-
Proteus	Pm1	-
Number (N=20)	2	-

"–," no clearing; "+," completely clear

Thermal and pH Stability:

Thermal and pH stability patterns of phage vB_PseM-EB-E44 were tested based on residual phage titers after incubation under different pH values and temperatures (Fig 5. A, B). The *P. aeruginosa* phage (vB_PseM-EB-E44) was thermostable at temperatures ranging from 10 to 60 °C. But, after heating at 70°C for 60 min, phage titers decreased by 60% and no viable phages were detected after heating at 80°C for 60 min (Fig. 5A). Phage vB_PseM-EB-E44 resisted a pH range between pH 4 and 9 for 1h. lost its activity at pH 2, 10, 11, 12. (Fig. 5B).

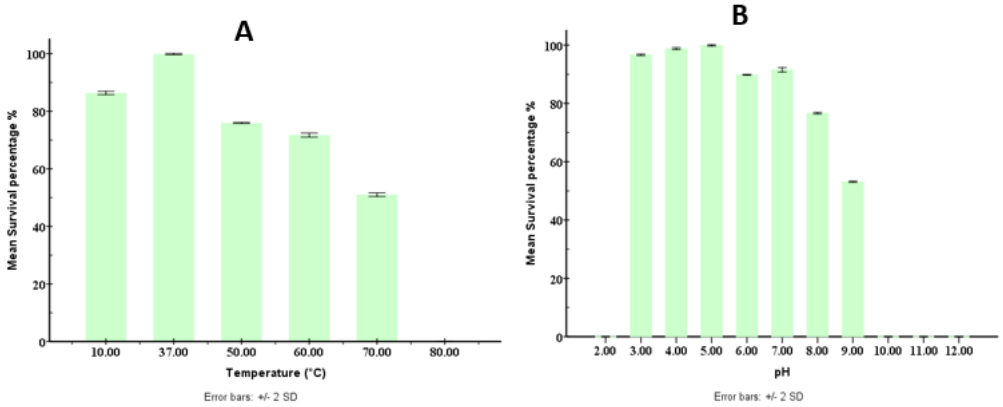

Fig.5. Thermal and pH tolerance test of phage vB_PseM-EB-E44. (**A**) Thermal tolerance, and (**B**) pH stability of vB_PseM-EB-E44 phage, respectively. Temperature experiments were performed for 1 h at pH7. pH tolerance was performed for 60 min at 37°C. Data showed the percentages of the remaining phages after each treatment as normalized from the control. Data shown are the mean of two replicates and error bars show the deviation in the values.

DISCUSSION

P. aeruginosa is related to a potent increase in human infections (Kerr and Snelling, 2009). This opportunistic human pathogen founds chronic or severe infections in immunodeficient patients and hospitalized (Trautmann *et al*., 2005). A group study noted that *P. aeruginosa* had the greatest burden of healthcare-acquired infections in European intensive care units (Lambert *et al*., 2011). *P. aeruginosa* is spread in healthcare cases due it is a usual companion of patients under medical care and also it has the ability existence on biotic and abiotic surfaces like medical equipment, resisting disinfection methods during transmissible from patient-to-patient (Russotto *et al*., 2015). The isolated bacterial candidates were characterized microscopically and identified biochemically using conventional methods and were confirmed by Vitek 2 system according to previous studies (Ligozzi *et al*., 2002; Nonhoff *et al*., 2005). The utilizing traditional methods and were confirmed by Vitek 2 system according to former studies (Ligozzi *et al*., 2002; Nonhoff *et al*., 2005). Recently, most bacteria have had the potential to develop resistance against different classes of antibiotics. Antibiotic resistance is one of the top concerns that threaten global health (CDC, 2019). Egypt is one of the countries that have less severe restrictions on antibiotic remedies (Esmat *et al*., 2017; Esmael *et al*., 2020).

That enhances the chance for bacteria to resist antibiotics. In the current study, antibiotic sensitivity testing of *P. aeruginosa* against a selection of fifteen antibiotics showed that the isolates resisted most of the tested antibiotics. Resistance mechanisms against antibiotics by *P. aeruginosa* were reported (Miller *et al*., 2014). Antibiotic resistance can be developed through mutations in chromosomal genes or by mobile genetic elements (horizontally acquired resistance) (Foster, 2017). In that view, a resistance that is acquired through mutation, mechanism of horizontally acquired resistance, or overexpression of the drug efflux were discussed previously (Jensen and Lyon, 2009; Miller *et al*., 2014). Hence, the current study used nanoparticles and bacteriophages as an alternative strategy to control the spread of these organisms. Silver compounds have been historically used to control microbial proliferation (Wadhera and Fung, 2005). The antifungal and antibacterial effect of silver nanoparticles AgNPs, even against antibiotic-resistant bacteria (Wright *et al*., 1994; Wright *et al*., 1999). Thus, nanosilver is now considered one of the most viable alternatives to antibiotics due it shows to possess high potential to solve the problem of multidrug resistance, which is often noted in several bacterial strains (Rai *et al*., 2012; Salomoni et al., 2015). Chitosan has been shown to be non-toxic and tissue compatible in a range of tests. Nanoparticles, which can be produced with a wide variety of polymers and nanotechnologies, have also been recently proposed as delivery systems for peptides and proteins through the pulmonary route (Alonso, 2020).

In this respect, chitosan is a very attractive polysaccharide due to its reported low toxicity, biodegradability and mucoadhesivity (Cohen and Bernstein, 2020). Phages are small viruses that possess the capability to settle bacteria however they do not affect the cell strains from other organisms (Wittebole *et al*., 2014). Bacteriophages are the most plentiful organisms in the biosphere (Yu *et al*., 2017). Phages are anywhere where their bacterial host is existent (Weinbauer, 2004). Bacteriophages play a significant role in the organization of microbial ecology, virulence and diversity (Penadés *et al*., 2015). Bacteriophages have been sought as one of the novel therapeutic approaches to control antibiotic-resistant pathogenic bacteria (Shlezinger *et al*., 2017; El-Dougdoug *et al*., 2019). In the current study phage (VB_ PseM-EB-E44) targeted to *P. aeruginosa -S-EB3* was isolated after a screening of 100 samples of Wound in concordance with previous studies (Edlund *et al*., 2015). Phage VB_ PseM-EB-E44 belongs to *Myoviridae*.

Growth kinetics of the isolated phages exhibited typical growth kinetics of most bacteriophages. Phage VB_ PseM-EB-E44 gave burst sizes of (60) PFU/cell respectively with latent periods of (15) minutes. This value is similar to those obtained in another study with *Pseudomonas aeruginosa* phage (Kokjohn and Sayler, 1991). Where the average burst size of such phage was approximately 67 PFU/ infected cell and latent period 25 min. This phage-displayed a narrow spectrum of lytic activity. This could be attributed to the ability of the tested strains to develop resistance against these phages (Petty *et al*., 2007; León and Bastías, 2015). The host range of the isolated phage was determined against 20 bacterial isolates.

This phage established a narrow spectrum of lytic activity. A narrow host range could be overcome using a cocktail of phages (Goodridge and Bisha, 2011). In addition to that cross infectivity of phage against different species and genera was investigated in the current study, and no lytic activity was shown. The stability of phages under stressful environmental conditions promotes the application of phages as a bio-control agent in dental therapy.

The vB_ PseM-EB-E44 phage was stable at temperatures ranging from 10 to 60°C, and pH range between pH 3 and 9 for 1h. Previous studies showed stability of phage against *P. aeruginosa* under a wide range of temperature degrees (4–60°C) (Yu, *et al*., 2017), These findings make the isolated phage highly potent to be used in clinical settings where they can be combined with alkaline disinfectants, that are commonly used in the treatment of endodontic infections. The stability of phages under stressful environmental conditions promotes the application of phages as a bio-control agent in dental therapy.

CONCLUSION

Health concerns related to chronic wound infection have been exacerbated by antibiotic-resistant *P. aeruginosa* Hence, bacteriophage could be proposed as an alternative strategy to mitigate the causative bacterial pathogens. In this study, virulent phage was isolated for antibiotic-resistant *P. aeruginosa*, vB_PseM-EB-E44 belonged to family *Myoviridae* morphologically. The phage has a narrow host range, low latent period, strong pH and thermal resistance. More importantly, our findings demonstrate the efficacy of phage VB_PseM-EB-E44 for the inhibition of multidrug-resistant *P. aeruginosa* growth *in vitro* respectively. This research forms the basis for the therapeutic application of phage to manage *P.aeruginosa* infection.

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Supplementary data

Table S1. Biochemical characterization of *Pseudomonas aeruginosa* clinically isolated from patients with chronic wound

Morphological characteristics and Biochemical tests	*P. aeruginosa*
Motility	+
Gram reaction	(-ve)
Shape	rods
Citrate utilization	+
Indole	-
Methyl reduction	-
Oxidase	+
Catlase	+
Urease	-
Voges proskaur	-
Glucose	+
Mannitol	-
Sucrose	+
Lactose	-
Starch	+
Hemolysis (Beta-hemolysis)	+

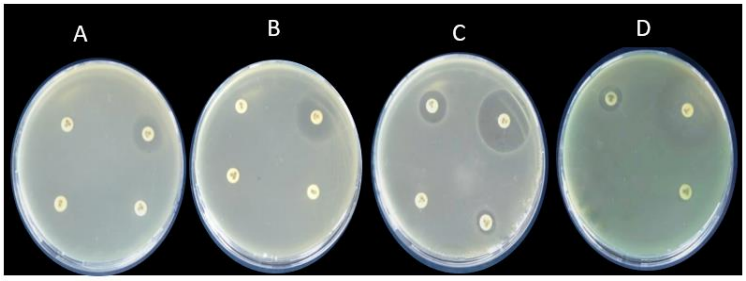

Fig. S1. Antibiotic susceptibility test showing zones of clearance surrounding Antibiotic Discs: Multidrug resistance *P. aeruginosa-S-EB3* (**A-D**), against a selection of 15 antibiotic