

ISOLATION OF BACTERIOPHAGE AGAINST *PSEUDOMONAS AERUGINOSA* FROM MALE URINARY TRACT INFECTION (UTI) specimen.

Abstract

Bacteriophages were isolated from raw sewage and selected based on the size and clarity of plaque that were formed. The selected phages were further enriched and purified. The isolated bacteriophages were found to be able to lyse *Pseudomonas aeruginosa* isolate from raw sewage.

The virulent bacterial viruses of *Pseudomonas aeruginosa* were found to be of widespread occurrence in nature. Bacterial viruses were applied to control pathogenic bacteria in different fields and successfully. Therefore, this work aimed to study is to isolate bacteriophages capable of lysing *Pseudomonas aeruginosa* as a potential therapeutic alternative to antibiotics.

Keywords: *Pseudomonas aeruginosa*; PCR; sewage water; virulent bacteriophages.

Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans throughout their life span due to the uncontrollable factors of lead caused of *Escherichia coli*, *Proteus mirabilis* etc by humans activities, in which the infections target the kidneys, bladder, ureters and urethra. Symptoms of urinary tract infections include: pain during urination (dysuria), frequent urination (frequency), blood in urine (hematuria), turbid or cloudy urine, fever and abdominal pain.

“The organisms most commonly responsible for urinary tract infections (UTIs) are *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus faecalis*” (CDC 2021).

“Bacteriophages, also known as phages, are viruses that infect and replicate only in bacterial cells” (Ackermann, H.W 2006). “They are ubiquitous in the nature and are recognized as the most abundant microorganisms on earth” (Ackermann, H.W 2004). “They are extremely diverse

in size, morphology, and genomic organization” (Aksyuk *et al* 2009). “However, all consist of a nucleic acid genome encased in a shell of phage-encoded capsid proteins, which protect the genetic material and mediate its delivery into the next host cell” (Batinovic *et al* 2019). “Phages replicate within the bacterium following the injection of their genome into its cytoplasm” (McGrath 2007). “Electron microscopy has allowed the detailed visualization of hundreds of phage types, some of which appear to have "heads," "legs", and "tails". Despite this appearance, phages are non-motile and depend upon Brownian motion to reach their targets” (Simmonds, 2018). “Like all viruses, bacteriophages are very species-specific with regard to their hosts and usually only infect a single bacterial species or even specific strains within a species” (Guan, *Let al* 2016). “Once a bacteriophage attaches to a susceptible host, it pursues one of two replication strategies: lytic or lysogenic” (Bishop-Lilly *et al*, 2019). “During a lytic replication cycle, a phage attaches to a susceptible host bacterium, introduces its genome into the host cell cytoplasm, and utilizes the ribosomes of the host to manufacture its proteins” (Hampton, H. *Get al* 2020). “The host cell resources are rapidly converted to viral genomes and capsid proteins, which assemble into multiple copies of the original phage. As the host cell dies, it is either actively or passively lysed, releasing the new bacteriophage to infect another host cell. In the lysogenic replication cycle, the phage also attaches to a susceptible host bacterium and introduces its genome into the host cell cytoplasm” (Oechslin, F. 2018). “However, the phage genome is instead integrated into the bacterial cell chromosome or maintained as an episomal element where, in both cases, it is replicated and passed on to daughter bacterial cells without killing them” (McGrath *Set al* 2007). “Integrated phage genomes are termed prophages, and the bacteria containing them are termed lysogens” (Mangas, E. *Let al* 2019). “Prophages can convert back to a lytic replication cycle and kill their host, most often in response to changing environmental conditions” (Ptashne, 2006).

Phages were first used as antibacterial agents by the former Soviet Republic of Georgia during the 1920s and 1930s for treating bacterial infections. They had widespread use, including treatment of soldiers in the Red Army. However, they were abandoned for general use in the West for several reasons:

- Antibiotics were discovered and marketed widely. They were easier to make, store, and to prescribe.
- Medical trials of phages were carried out, but a basic lack of understanding raised questions about the validity of these trials.
- Publication of research in the Soviet Union was mainly in the Russian or Georgian languages and for many years, was not followed internationally.

“The use of phages has continued since the end of the Cold War in Russia, Georgia and elsewhere in Central and Eastern Europe. The first regulated, randomized, double-blind clinical trial was reported in the *Journal of Wound Care* in June 2009, which evaluated the safety and efficacy of a bacteriophage cocktail to treat infected venous ulcers of the leg in human patients. The FDA approved the study as a Phase I clinical trial. The study's results demonstrated the safety of therapeutic application of bacteriophages, but did not show efficacy. 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” (Kutter, 2010). “Shortly after that, another controlled clinical trial in Western Europe (treatment of ear infections caused by *Pseudomonas aeruginosa*) was reported in the journal *Clinical Otolaryngology* in August 2009. The study concludes that bacteriophage preparations were safe and effective for treatment of chronic ear infections in humans. Additionally, there have been numerous animal and other experimental clinical trials evaluating the efficacy of bacteriophages for various diseases, such as infected burns and wounds, and cystic fibrosis associated lung infections, among others” (Kutter, 2010).

Materials and method

Sample collection

50ml each of raw sewage samples were collected from two septic tanks at the Department of works, Akwa Ibom State University. The samples were analyzed in the Microbiology Laboratory of Akwa Ibom State University, Ikot Akpaden, Mkpato Enin.

Study Population and Sample

Two samples were used for the research project. The first sample involved sewage collection from the Works Department of Akwa Ibom State University. The sewage was used for isolation of phages because it is known that sewage is one of the natural reservoirs for numerous bacteriophages. Secondly, urine samples infected with UTI were collected from Emmanuel Hospital, Eket, Akwa Ibom State. The aim of the urine collection was to isolate *Pseudomonas aeruginosa* which in turn were used together with the sewage for phage development.

Isolation of *Pseudomonasaeruginosa* from raw sewage

Five milliliters (5 ml) of sewage samples were cultured on CLED agar plates without dilution and incubated at 37 ° C for 48h. A single colony of each isolate was inoculated on MacConkey agar and incubated at 37 ° C for 48h.

Characterization of *Pseudomonas* species

Isolates forming round, flat, and colorless colonies on MacConkey agar indicated that the organism is a lactose non-fermenter were suspected to be *Pseudomonas* species. The isolates were characterized using Gram staining alongside biochemical tests such as catalase test, citrate test, coagulase test, nitrate test, and oxidase test.

Gram staining

This is a staining technique that helps to differentiate bacteria into Gram-positive and Gram-negative cells. A grease free dry slide was taken. The inoculating loop was sterilized on a flame of a basin burner and a loopful of the test organism was transferred by sterile loop and a smear was made at the center. The smear was allowed to air -dry and the smear was heat fixed by passing the slide 3-4 times through the flame quickly with the smear slide facing up. The slide was placed on the staining rack and covered with crystal violet. This was left for 1min and carefully rinsed under running tap water. The slide was flooded with Gram's iodine solution and allowed for 1 min. the slide was rinsed in a gentle running water tap and then flooded with decolorizing agent (ethyl alcohol) for 20-30 seconds. The slide was rinsed gently under running tap water and drain completely. The smear was counter-stained with safranin and was allow to stand for about 30 seconds to 1min. the slide was rinsed under running tap water. It was air-dried and observed under microscope using 100X objective lens. Gram-positive cells were stained purple under the microscope while Gram-negative were stained red as described by Cheesebrough (2002).

Catalase test

This test was carried out to identify the various isolates with the ability to produce catalase enzymes that breakdown hydrogen peroxide into water and oxygen. A small amount of bacteria colony was transferred to a surface of clean, dry glass slide using a wire loop or sterile wooden stick. A drop of 3% H₂O₂ was placed on to the slide and mixed. A positive result is rapid evolution of oxygen (within 5-10sec.) as evidenced by bubbling.

Citrate test

Two (2) grams of Sodium Citrate, 5g Sodium Chloride, 1g Dipotassium Phosphate, 1g Ammonium Dihydrogen Phosphate, 0.08g Bromothymol Blue, 0.2g Magnesium Sulphate and 15g agar was mixed together, and 1000ml sterile distilled water was dispensed in the same mixture. The pH was adjusted to 6.9 and gentle heat was applied to dissolve agar. About 3-4 ml was collected in test tubes and sterilized at 121°C for 15 minutes in an autoclave. The tubes were inoculated and incubated at 37°C for 24-48 hours. The color changes in the medium that was observed. Color change of medium to blue indicate citrate positive. No color change of the medium indicates citrate negative.

Coagulase test

This test was performed to indicate the presence of various isolates with the ability to produced enzyme known as “Coagulase” capable of coagulating human blood plasma. The slide method was used (Cheesebrough, 2002). Some drop of normal saline was placed on a clean grease free slide. The test organism was added to it and mixed properly, followed by a drop of plasma and

mixed gently. Clumping/agglutination reaction within 5-10 seconds indicated a positive Coagulase test, while no agglutination indicated Coagulase negative.

Indole Test

Peptone broth was prepared by weighing (15) ml of peptone broth in a test tube and sterilized in an autoclave at 121°C for 15 minutes at 15lbs pressure. A loop full of bacteria culture was inoculated in broth and incubated for 24-48 hours at 37°C. Two (2) drops of Kovac's reagent were dispensed into the test tubes and mixed together after sterilization. (Ajiboye and Emmanuel, 2021).

Methyl Red Test:

The bacteria culture was inoculated into a fresh sterile broth medium and incubated at 37°C for 48 hours. A sterile pipette was used to dispense 5 drops of Methyl red reagent into the broth culture and colour change was observed (Ajiboye and Emmanuel, 2021).

Oxidase test

This test was carried out to identify the various isolates with the ability to produce the enzyme "oxidase". The filter method was employed. The test organism was picked out and smeared on a filter paper, and then some drops of tetramethyl-P-Phenylenediaminedihydrochloride was placed on the filter paper. A change in colour to sleep purple or blue indicated a positive test, and no colour change indicated a negative test.

Voges- Proskauer Test

"Preparation of 5 % α – naphthol in ethanol and 40 % Sodium hydroxide in deionized water was done. MR-VP broth was also prepared and 5 ml dispensed in different test tubes and sterilization

was done at 121°C for 15 minutes using an autoclave. The medium was allowed to cool to room temperature. Inoculum from fresh culture media was introduced in different test tube and this was incubated together with the control at 37°C for 48 hours. About 2.5 ml of culture was dispensed in a sterile cultures tube and 5 drops of methyl red reagent was added. The test organism was also compared with the control and colour change was observed” (Ajiboye and Emmanuel, 2021).

Molecular identification of *Pseudomonas* isolate

DNA Extraction

PCR Amplification

16S rDNA amplification was carried out using universal primers, 27F (AGAGTTTGATCCTGGCTCAG) and 1392R (GGTTACCTTGTTACGACTT). The reaction mix was prepared in a final volume of 20 µL containing 4 µL Taq buffer (5×), 1.2 µL of MgCl₂, 4 µL of dNTPs, 0.5 µL of each primer, 0.2 µL of Taq polymerase, 2 µL of extracted DNA and 6.6 µL of nuclease free H₂O.

DNA Sequencing and Phylogenetic Analysis

Sanger sequencing was performed at the Center of Innovation (USMBA, Fez-Morocco) using a ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems). Preliminary identifications were performed based on sequence assembly and by search in the NCBI database. Strains were attributed to a particular genus when the sequence similarity with a strain type was at least 98% and to a given species when sequence similarity was at least 99.5%.

Phage Development

45ml of raw sewage was measured out into a graduated measuring cylinder and decanted into a 250ml Erlenmeyer flask containing 5ml of deca broth and 5ml of cultured *Pseudomonas aeruginosa*. The mixture was then incubated at 37 °C for 24 hrs to allow development of bacteriophages. After incubation, the sewage culture was centrifuged at 2500 rpm for 10min and the supernatant was filtered through a 0.45µm filter to separate the lysed bacteria from the phage sample. The phages remained in the filtrate due to its considerable lower size.

Spot Test

The spot test was carried out to determine the presence of phages in the filtrate obtained after filtration.

Plaque Assay

Soft agar overlay method (Double layer agar method) was followed to determine the number of phage particles in the lysate. Normal Nutrient agar was melted and cooled to 45⁰c. This agar was poured into petri dishes and allowed to solidify. Soft Nutrient agar was melted, 3ml of the agar was placed into each of the 3 tubes and held at 45⁰c. 0.1ml of *Pseudomonas aeruginosa* culture was added into each tube and labeled as 1, 2, 3. To the first test tube, 10µl of phage suspension was added from step 2 above, then mixed and spread on one of the normal agar plate prepared earlier. The procedure was repeated with second test tube using 50µl of phage suspension. The content of the third tube was spread without adding phage suspension. This plate was served as control. The soft agar was allowed to solidify in all the plates. When the soft agar solidified, the plates was inverted and incubated at 37⁰c. Development of plaques (clear zones where viruses have infected and lysed the bacterial cells) was then examined periodically for growth.

Results

Sample Processing and Isolation

The sample was processed (i.e shaken vigorously to mix) and was prepared for the serial dilution. Of 10^{-1} to 10^{-5} was carried out for the samples; after which 1ml from dilution factors 10^{-3} to 10^{-5} was poured aseptically into the petri dishes (using pour plating method/technique) and appropriate medium (Nutrient agar) was poured into the petri dishes and was allowed to solidify. Thereafter, the petri dishes were incubated at 37°C for 48 hours.

Colony purification

After 48hours of isolation, colonies with discrete growth were picked and subcultured in a fresh nutrient agar medium to obtain a pure culture (that is, 24 hours culture).

Table 1: Biochemical test for *Pseudomonas aeruginosa*

Test	Gram Stain	Catalase	Citrate	Nitrate	Oxidase	Coagulase
Results	-	+	+	+	+	+

(+) = Positive

(-) = Negative

Table 2 : Biochemical Test and Identification of *Pseudomonas aeruginosa*

Characteristics	Shape (Cocci/Diplococci/Rods)	Motility (Motile / Non- Motile)	Capsule (Capsulated/Non- Capsulated)	Spore (Sporing/Non- Sporing)	Flagella (Flagellated/Non- Flagellated)
<i>Pseudomonas aeruginosa</i>	Rods	Motile (Unipolar)	Non-Capsulated	Non-Sporing	Single Flagella

Table 3 : Sugar Fermentation of *Pseudomonas aeruginosa*

Fermentation	Fructose	Glucose	Lactose	Maltose	Sucrose
<i>P.aeruginosa</i>	-	-	-	-	-

(-) = Negative

Table 4 : Gram-Staining isolate under light microscopic view

Isolate	Shape	Margin	Elevation	Size	Colour	Opacity	Consistency	Surface	Texture	Gram reaction
S ¹	Circular	Entire	Flat	Small	Creamy	Opaque	Brittle	Dull	Rough	+ cocci
S ²	Irregular	Undulate	Raised	Moderate	Creamy	Opaque	Sticky	Shiny	Rough	- cocci
S ³	Irregular	Undulate	Flat	Moderate	Creamy	Opaque	Sticky	Dull	Rough	+ cocci
S ⁴	Circular	Entire	Flat	Moderate	Creamy	Opaque	Brittle	Shiny	Smooth	-
S ⁵	Irregular	Undulate	Flat	Moderate	Creamy	Opaque	Sticky	Shiny	Rough	+ cocci
S ⁶	Circular	Entire	Flat	Small	Creamy	Opaque	Sticky	Shiny	Smooth	+ cocci
S ⁷	Irregular	Undulate	Flat	Small	Creamy	Opaque	Sticky	Dull	Rough	+ cocci
S ^{8*}	Circular	Undulate	Flat	Moderate	Creamy	Opaque	Sticky	Shiny	Smooth	+ cocci
S ^{9*}	Circular	Entire	Flat	Moderate	Creamy	Opaque	Sticky	Shiny	Smooth	- Rod
S ^{10*}	Circular	Entire	Flat	Small	Creamy	Opaque	Sticky	Shiny	Smooth	+ cocci
S ¹¹	Irregular	Undulate	Flat	Small	Creamy	Opaque	Sticky	Dull	Rough	+ cocci
S ¹²	Irregular	Undulate	Raised	Small	Creamy	Opaque	Sticky	Shiny	Rough	+ cocci
S ¹³	Irregular	Undulate	Raised	Moderate	Creamy	Opaque	Brittle	Dull	Smooth	+ cocci

S ⁻¹⁴	Circular	Entire	Raised	Moderate	Creamy	Opaque	Brittle	Dull	Smooth	- cocci
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(+) = Positive

(-) = Negative

Table 5: Result showing molecular identification of *Pseudomonas aeruginosa*

Isolate code	Gene size	Taxonomic affinity	Accession number	Similarity (%)
S 1	1537bp	<i>Pseudomonas aeruginosa</i> strain NBAII AFP-7	HM439964.1	100%
S 2	1316bp	<i>Pseudomonas aeruginosa</i> strain WES2	MN960116.1	100%

Double Agar Overlay Plaque Assay

All the plates show positive for the isolation of Bacteriophages. Round, clear and transparent plaques were observed in all plates, indicating that the sample collected at works department Akwa Ibom State University contained bacteriophages that were infectious against *Pseudomonas aeruginosa*. The phages produce clear, medium sized (1.0 – 3.1mm in diameter) plaques with well-defined edges in bacterial lawn, showing that the isolated phages have lytic effect against *Pseudomonas aeruginosa*.

Fig 1: Plates showing Bacteriophages from cultured plates of nutrient Agar and CLED agar.



A

B

C

D

E

G

Results of Bacteriophages after cultured from nutrient agar for plate A, B, and C while D, E and G plates from CLED agar they were significance growth of Bacteriophage on both media.

UNDER PEER REVIEW

Discussion

The study's findings showed that both lytic phage isolates could be used as biological control agents against urinary tract infection.

Bacteriophage ineffective to *Pseudomonas aeruginosa* can be isolated from raw sewage. The most probable reason is using the cultured enrichment for phage isolation. In conclusion, the bacteriophages isolated from raw sewage were able to infect *Pseudomonas aeruginosa*. All the 14 isolated phages are virulent phages which produced round, clear and medium sized (1.0 – 3.0mm in diameter) plaques. Sampling site provided favorable condition for the growth of phages. Based on the number of phages isolated, higher number of host bacteria population was present in C,D, E than A and B.

“As discussed earlier, for 70 years antibiotics have saved countless lives and enabled the development of modern medicine, but it is becoming clear that the success of antibiotics may have only been temporary and we now anticipate a long-term, generational and perhaps never-ending challenge to find new therapies to combat antibiotic-resistant bacteria. As the search for new conventional antibiotics has become less productive and there are no clear strategies to improve success, a broader approach to address bacterial infection is needed” (Czaplewski *et al.*,2016).

CONCLUSION

The bacteriophages isolated from raw sewage taken from works department Akwa ibom State University were able to infect *Pseudomanas aeruginosa*. These can be used for phage therapy and are a promising tool for the control of *Pseudomanas aeruginosa*. The findings of this study may expand the knowledge of phages infecting *Pseudomanas aeruginosa*, and provide new phages that may control *Pseudomanas aeruginosa* infection. Phages can be readily obtained from sewage, indicating that sewage is a natural reservoir of phages. According to their morphology, these are members of the Podoviridae family and have an icosahedral head and an extremely short, almost invisible tail.

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