Research Article

iMedPub Journals
www.imedpub.com

Health Science Journal
ISSN 1791-809X

2021
Vol. 15 No. 11: 897

DOI: 10.36648/1791-809X.15.11.897

Isolation of *Pseudomonas Aeruginosaphages* from Residential Waste Waters

Stephen Chibuike Emencheta[1], Chinelo Charity Eze[1], Anthony Amaechi Attama[2], Damian Ejike Agbo[1] and Ebele Benedette Onuigbo[1]*

1 Department of Pharmaceutical Microbiology and Biotechnology, Nigeria

2 Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Nsukka, Enugu State, Nigeria

***Corresponding author:**
Ebele Benedette Onuigbo

ebele.onuigbo@unn.edu.ng

Tel: 234(0)8062995712

Department of Pharmaceutical Microbiology and Biotechnology, Nigeria

Citation: Emencheta SC, Eze CC, Attama AA, Agbo DE, Onuigbo EB (2021) Isolation of *Pseudomonas Aeruginosaphages* from Residential Waste Waters. Health Sci J. 15 No. 10: 897.

Abstract

Objective: This research was designed to isolate and test the lytic action of a bacteriophage specific to *Pseudomonas sp*.

Methods: Both *Pseudomonas sp* and bacteriophages were isolated from residential wastewaters. The isolated *Pseudomonas sp* was confirmed through bioChemical tests. Antibiogram was done with nine different antibiotics. Plague assay was done to determine lytic action of the bacteriophage on *Pseudomonas sp*. Host range of isolated bacteriophages was assessed.

Results: Cultural characteristics and bioChemical tests confirmed *Pseudomonas aeruginosa* which was sensitive to ciprofloxacin only. The Multi- Antibiotic Resistance Index (MARI) of the isolated strains was greater than 0.8. The formation of plagues (clear zone) on the lawn of *Pseudomonas sp* confirmed lytic action. The bacteriophage showed plaques on *Pseudomonas sp* isolated from other sites.

Conclusion: The isolation of *Pseudomonas aeruginosaphages* from residential waste waters is a promising molecular tool in combating the global antimicrobial resistance threat.

Keywords: Bacteriophage; *Pseudomonas aeruginosa*; Plaques; Lytic; Antibiotic; Resistance

Received with Revision on: October 16, 2021, **Accepted:** November 01, 2021, **Published:** November 07, 2021

Introduction

On the 13th of OCtober, 2020, World Health Organization (WHO) announced that one of the top ten global health threats is Antimicrobial resistance (AMR) [1]. It has been forewarned that by, 2050, antibiotic resistance will be responsible for the death of 10 million people [2]. In another study, AMR was projected to drain the global economy of US$100 trillion in the same period [3]. Among the ESKAPE (*EnteroCoCcus faecium*, *StaphyloCoCcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Enterobacteriaceae*) pathogens, *Pseudomonas aeruginosa* has been classified as one of the critical priority pathogens [4]. *Pseudomonas aeruginosa*, a frontline nosoComial pathogen is a motile, aerobic, Gram negative rod which produces water-soluble fluorescent yellow and blue pigments. It is versatile and is found widely in soil, water, plants and animals. It is implicated in about 18-61 % of deaths in the hospitals especially in immunoCompromised patients [5]. *Pseudomonas aeruginosa* uses different adaptive metabolic pathways, quorum sensing to regulate virulence factor production, resistance and the formation of biofilms [6]. It is the leading proven cause of mortality in cystic fibrosis patients. Recent trends of failures of first-line drugs in the treatment of *Pseudomonas aeruginosa* infection has led to the re-exploration of bacteriophages as an alternative in humans as well as in animals. Bacteriophages (phages) are natural eaters of bacteria and are ubiquitous in the environment. The use of host-specific bacteriophages has been promoted as a cost-effective and adaptable approach to control bacteria [7]. Phages have unique advantages over the antibiotics. They replicate only on the specific strain of bacteria, avoiding the imbalance of commensal gut flora (dysbiosis) often caused by broad-spectrum antibiotics. Additionally, they only replicate as long as the targeted bacterium is present and so are naturally self-limiting [7]. The first step in a bacteriophage lytic cycle is the adsorption of the phage on the specific cell surface receptor of the bacteria. This attachment determines phage host range. Some phages of Gram-negative bacteria interact with various specific lipopolysaccharides (LPS, endotoxin) components, some with outer membrane proteins (OMP) and others have complex adhesions that recognize specific receptors [8,9]. In this research, we attempted to isolate *Pseudomonas aeruginosabacteriophages* in residential areas for further characterization.

© Copyright iMedPub | **This article is available in:** http://www.hsj.gr/

Methodology

Study area and sample collection

The study was conducted in Nsukka, Enugu State, Nigeria. Residential waste water was obtained from residences in Nsukka community. A total fifty-eight samples were collected from different residences over a period of three weeks. A 10 ml sample was collected in sterilized Bijou bottles and taken to the research laboratory for bacteriological analysis. Samples were randomly collected.

Isolation of *pseudomonas sp* form the waste water

Fifty-eight waste water samples were diluted 10-fold with distilled water. A 0.1 ml of the diluted samples was dropped at the centre of different petri dishes containing MacConkey agar. A spreader was used to spread the sample on the surface under aseptic conditions. The inoCulated plates were incubated at 37 °C for 24 h in an auto Clave.

Purification of isolated *Pseudomonas sp*

The overnight culture was purified by picking a colony from each plate and subculturing using the streak plate method on MacConkey agar and incubating for 24 h at 37°C. Distinct colonies from each colony were then subcultured on cetrimide agar and incubated for 24 h at 37°C to identify the *Pseudomonas sp*. Pure *Pseudomonas sp* isolates were then stoCked in double strength nutrient agar for bioChemical tests.

BioChemical tests

Citrate and Oxalase tests were carried out on 24 h broth culture to confirm presence of *Pseudomonas aeruginosa*.

Antibiogram of pseudomonas isolates

Suspension of the bacterial culture was swabbed on the surface of the Mueller Hinton agar plates with the aid of sterile swab sticks and the antibiotic disks (Aztreonam, chloramphenicol, gentamicin, tetracycline, sulphamethoxazole/trimethoprim, amoxicillin/clavulanic acid, meropenem, ciprofloxacin, ceftriaxone) were placed on the surface of the swabbed Mueller Hinton agar plates and was incubated at 37°C for 24 h. The zone of inhibition was measured and recorded.

Multiple antibiotic resistance Index (MARI)

MARI index was determined by following the proCedure described by Krumperman [10]. A MAR index for an isolate is calculated as: Number of antibiotics to which isolate is resistant/ Total number of antibiotics against which isolate was tested.

Isolation of bacteriophages

The *Pseudomonas sp* phage was isolated from the residential waste water by centrifuging at 15,000 rpm for 10 min to separate the phage from other particles. The supernatant was filtered through 0.45µm syringe filter units. The filtrates were stored in the freezer.

Phage enrichment and filtration

The pure stoCked isolates of *Pseudomonas sp* was inoCulated into 10 ml nutrient broth and incubated overnight. A 0.4 ml culture was introduced into a sterile test tube and 0.5 ml of 5x Luria Bertani broth and 0.04 ml $CaCl_2$ was added into the same test tube. Then 0.2 ml of phage filtrate was added and the test tube was incubated for 48 h. After incubation, the mixture was centrifuged for 15 min at 15,000 rpm. The supernatant was filtered using 0.45µm syringe filter unit and stored at 4°C.

Spotting and plaque assay

A suspension of the 24 h bacterial culture was swabbed on the surface of the Mueller Hinton agar plates. A drop of the enriched phage was spotted on the lawn of the bacterial isolate. The plate was incubated at 37°C for 24 h. After incubation, the plate was observed for the presence of plaques. A sterile needle was used to pick the plaques containing the phages and put inside the SM buffer and stored at 4°C.

Phage purification

This was done by preparing 5x LB broth. A 0.5 ml volume was collected and put into a sterile bottle and 1.2 ml of the stored phage was withdrawn and added into the sterile bottle. Then 0.04 ml of sterile calcium chloride was also added and the mixture incubated for 48 h. After incubation, the medium was centrifuged at 15,000 rpm for 20 min and the supernatant collected and passed through the membrane syringe filter under aseptic condition. The filtrate was inoCulated into SM buffer and adequately stored at 4°C.

Host range of the phage

The host range of the phage was determined based upon the ability to form plaques on the lawns of 40 isolates of *P. aeruginosa*. For this, 20 ml aliquots of nutrient broth were inoCulated with single colonies of each *P. aeruginosa* isolate from different sites and incubated overnight at 37°C. A 10µl aliquot of each phage to be tested was spot inoCulated onto each *P. aeruginosa* lawn. After the spots were dried the plates were incubated in an inverted position for 24 h at 37°C and next day they were checked for plaques.

Results and Discussion

Fifty-eight samples were collected from different residential waste water. Eight pure isolates of *Pseudomonas sp* on cetrimide agar showed fluorescent yellow and greenish blue pigmented colonies. The colonies had a sweet smell. The bioChemical tests were catalase and oxidase positive with no gas production in all eight isolates. Identification was based on the colonial morphology, oxidase positivity, the presence of blue-green and yellow pigments. *P. aeruginosa* is common in soils and water, especially in urban and rural communities with congested living conditions. It has been found also in deionised and distilled water due to their simple nutritional requirements and versatile adaptation mechanisms. This adaptation mechanism enables them to withstand oxidative, heat and salt stress as well as equips them with virulence and resistance factors [11]. Sinks, water baths, showers, hot tubs, and other wet areas need to be decontaminated regularly since Pseudomonas thrives in

moist environments especially in houses inhabited by persons with low immunity. The isolated strains of *P. aeruginosa* were screened for Aztreonam (ATM), Chloramphenicol (CPL), Gentamicin (GTN), Tetracycline (TTE), Amoxicillin/Clavulanic acid (AMC), Sulphamethoxazole/Trimethoprim (SXT), Ciprofloxacin, Meropenem (MEM) and Ceftriaxone (CRO). All the strains were susceptible to ciprofloxacin (CIP) according to CLSI, 2020 [12] as shown in **Table 1**. The CLSI, 2020 states that antibiotics with minimum inhibitory concentration greater than 1 µg/ml and with IZD<17 mm show resistance. There was 100 percent susceptibility of the strains to ciprofloxacin. There was 100 percent resistance to amoxicillin/clavulanic acid, meropenem, ceftriaxone and sulphamethoxazole/trimethoprim. Inotherwords, these isolates are from high risk sources of antibiotic resistance. This observed resistance of *Pseudomonas aeruginosa* from different residential waste water to all the above antibiotics including gentamicin may have oCcurred through production of multicellular biofilms making it difficult for targeted antibiotics to act. It has also been reported that the MexAB-OprM efflux pump of *Pseudomonas aeruginosa* has the widest antibiotic specificity and confers resistance to macrolides, aminoglycosides, sulfonamides, fluoroquinolones, tetracyclines and many β-lactams [13]. The deletion of the outer membrane protein has been assoCiated with increased resistance to imipenem and meropenem. Most recent researches have shown that methylation of 16S rRNA confers high-level resistance against aminoglycosides in *P. aeruginosa* [14]. The overexpression of chromosomally encoded cephalosporinase, AmpC is equally prevalent in *P. aeruginosa*. Multiple antibiotic resistance index is helpful in analyzing health risk, as well as to check the antibiotic resistance. Organisms which have MAR indices greater than 0.8 confirm the presence of multidrug resistant genes originating from the environment where there is an abuse of these drugs and also that the plasmids contain one or more resistance genes each encoding a single antibiotic resistance phenotypes [15]. The widespread of multidrug resistant *Pseudomonas aeruginosa* in the community reveals that there is proliferation of patent vendors who prescribe and dispense antibiotics illegally. The resistance to many antibiotics could also be connected to indiscriminate use of antibiotics in poultry and consumption of such poultry has transferred the resistance to humans [15]. This is worrisome because the multidrug resistant isolates were from residential areas and not from the hospitals. There is serious need for antimicrobial stewardship for fostering and monitoring judicious use of antimicrobials to preserve their effectiveness [16,17].

Following phage enrichment and purification, two phages, δ274 and Ψ102 were isolated from a total of fifty-eight residential samples. Only bacteriophages from residential (R3) and (R4) waste waters showed distinct plaques on the bacterial lawn of *Pseudomonas aeruginosa* isolates with approximately 5 ± 0.42 mm diameter. It is possible that some phages present in the waste waters were temperate phages. In many lytic phages, host lysis is achieved by a single-gene lyses protein or through holin-endolysin system [7]. Phages can also produce enzymes when biofilms are present to dissolve the biofilm matrix produced by *P. aeruginosa* [18]. Gupta and Prasad [19] successfully purified an endolysin from phage P-27/HP and showed its ability (about 99.9%) to decolonize the spleens of treated mice from *S. aureus* 27/HP. Witzenrath et al [20] demonstrated the therapeutic potential of endolysin Cp-l in the systemic treatment of fatal *S. pneumoniae*-induced pneumonia in mice. Some plaques showed regrowth of the bacteria which may be a sign of growing resistance. Nevertheless, the discovery of phages in the same waste water which the MDR Pseudomonas were found shows the enormous potentials of bacteriophages but requires further purification and standardization [21-24]. This is a welcome development in drug development research especially in this time of AMR threat.

On the basis of the lytic spectra data of δ274 and Ψ102 screened against eight different *Pseudomonas aeruginosa* isolates from abbatoir and residential waste waters and done in quadruplicates, there were differences in the number of distinct plaques as shown in **Table 2**. These differences in the number of plaques against specific *Pseudomonas* strains for δ274 and Ψ102 could be attributed to their different phenotypes, hence the need to characterize the phages. Though, for phage therapy to be useful, it has to be done under in-vivo conditions mimicking real life situations and challenging the models with MDR isolates. In-vitro tests may not be a reliable indicator of its activity in-vivo. It has been said that phages are inhibited by blood, serum, bile, white cells and tissue debris. Ψ102 was ineffective against RES 144 and ABA 41 which could have resulted from a failure to be adsorbed to the cell surfaces of the *Pseudomonas* strains. This is usually determined by the host density in the environment and adsorption rate constant of the phage. A way to solve this setback is the use of phage coCktails, as this widens the range of action [25].

Table 1 Antibiogram showing inhibition zone diameter (IZD) of different antibiotic disks.

Bacterial Isolate	Inhibition zone diameter (mm)								
Pseudomonas aeruginosa strain from residential waste water	ATM 30 µm	CPL 30 µm	GTN 10 µm	TTE 30 µm	SXT 25 µm	AMC 30 µm	MEM 10 µm	CIP 5 µm	CRO 30 µm
BS201	12(R)	5(R)	4(R)	0(R)	0(R)	0(R)	0(R)	24(S)	0(R)
BS221	10(R)	5(R)	5(R)	2(R)	0(R)	0(R)	0(R)	24(S)	0(R)
BS432	7(R)	8(R)	5(R)	10(R)	0(R)	0(R)	0(R)	24(S)	0(R)
BS453	11(R)	8(R)	6(R)	2(R)	0(R)	0(R)	0(R)	24(S)	0(R)
BS442	3(R)	2(R)	5(R)	3(R)	0(R)	0(R)	0(R)	24(S)	0(R)
BS113	10(R)	11(R)	10(R)	9(R)	0(R)	0(R)	0(R)	24(S)	0(R)
BS151	11(R)	5(R)	10(R)	1(R)	0(R)	0(R)	0(R)	24(S)	0(R)
BS376	12(R)	12(R)	9(R)	6(R)	0(R)	0(R)	0(R)	24(S)	0(R)

© Under License of Creative Commons Attribution 3.0 License

Table 2 Lytic spectra of two bacteriophage isolates determined on eight *Pseudomonas* host strains from abbatoir and residential waste water.

Lysis by bacteriophages		
P. aeruginosa strains	δ274	Ψ102
ABA5	++	+
ABA1	+	+
RES141	++	++
RES144	++	-
ABA31	++	++
ABA41	++	-
ABA42	+	+++
ABA61	+	++

References

1 World Health Organization (2020) Antimicrobial Resistance. WHO, Geneva.

2 Sharma S, Datta S, Chatterjee S, Dutta M, Samanta J, et al. (2021) Isolation and Characterization of a lytic bacteriophage against Pseudomonas aeruginosa. Sci Rep 11: 193093.

3 Mogasale VV, Saldanha P, Pai V, Rekha PD, Mogasale V (2021) A descriptive analysis of antimicrobial resistance patterns of WHO priority pathogens isolated in children from a tertiary care hospital in India. Sci Reports 11: 5116.

4 WHO (2017) publishes list of Bacteria for which new antibiotics are urgently needed. WHO, Geneva.

5 Zhang Y, Li Y, Zeng J, Chang Y, Han S, et al. (2020) Risk factors for mortality of inpatients with Pseudomonas aeruginosa bacteremia in China: Impact of Resistance profile in the mortality. Infec Drug Res 13: 4115-4123.

6 Diggle SP, Whiteley M (2020) Microbe Profile: Pseudomonas aeruginosa: opportunistic pathogen and lab rat. Microbiol 166: 30-33.

7 Attama AA, Agbo IS, Eke IE, Onuigbo EB, Ogbonna JC (2017) Bacteriophage: Clinical applications. In Book: Antimicrobial research: Novel bioknowledge and educational programs. In: Mendez-Vilas A (editor). Formatex Research Center

8 Brzozowska E, Lesniewski A, Sęk S, Wieneke R, Tampé R, et al. (2018) Interactions of bacteriophage T4 adhesin with selected lipopolysaccharides studied using atomic force microscopy. Sci Rep 8: 10935.

9 Kulikov EE, Golomidova AK, Prokhorov NS, Ivanov PA, Letarov AV (2019) High-throughput LPS profiling as a tool for revealing of bacteriophage infection strategies. Sci Rep 9: 2958.

10 Krumperman PH (1983) Multiple antibiotic resistanceindexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. Appl Environ Microbiol 46: 165-170.

11 Moradali MF, Ghods S, Rehm BHA (2017) Pseudomonas aeruginosa lifestyle: A paradigm for adaptation, survival and Persistence. Front Cell Infect Microbiol.

12 Clinical and Laboratory Standards Institute (2020) Performance Standards for Antimicrobial Susceptibility Testing. (30th edn), CLSI Supplement M100-S12, Wayne, PA.

13 Poole K (2001) Multidrug efflux pumps and antimicrobial resistance in Pseudomonas aeruginosa and related organisms. J. Mol Microbiol Biotechnol 3: 255-264.

14 Hirsch EB, Tam VH (2010) Impact of multidrug-resistant Pseudomonas aeruginosa infection on patient outcomes Exp Rev Pharmacoeconom outcomes Res 1094: 441-451.

15 Afunwa RA, Ezeanyika J, Afunwa EC, Udeh AS, Oli AN, et al. (2020) Multiple Antibiotic Resistance Index of Gram-negative Bacteria from bird droppings in two commercial poultries in Enugu State, Nigeria. Open J Med Microbiol 10.

16 Majumder AA, Rahman S, Cohall D, Bharatha A, Singh K, et al. (2020) Fighting Antimicrobial resistance and antimicrobial stewardship: fighting antimicrobial resistance and protecting global public health. Infect Drug Res 13: 4713–4738.

17 Murray AK (2020) The Novel Coronavirus COVID-19 outbreak: global implications for antimicrobial resistance. Front Microbiol 11: 1020.

18 Holger D, Kebriaei R, Morrisette T, Lev K, Alexander J, et al. (2021) Clinical Pharmacology of Bacteriophage Therapy: A focus on Multidrug-Resistant Pseudomonas aeruginosa infections. Antibiotics 10: 556.

19 Gupta R, Prasad Y (2011) P-27/HP endolysin as antibacterial agent for antibiotic resistant Staphylococcus aureus of human infections. Curr Microbiol 63: 39-45.

20 Witzenrath M, Schmeck B, Doehn JM, Tschemig T, Zahlten J, et al. (2009) Systemic use of the endolysins Cpl-1 rescues mice with fatal pneumococcal pneumonia. Crit Care Med 37: 642-649.

21 Qin J, Wu N, Bao J, Shi X, Ou H, et al. (2020) Heterogeneous Klebsiella pneumonia co-infections complicate personalized bacteriophage therapy. Front Cell Infect Microbiol 10: 608402.

22 Fabijan AP, Lin RCY, Ho J,Maddocks S, Zakour NLB(2020) Safety of bacteriophage therapy in severe Staphylococcus aureus infection. Nat Microbiol 5: 465-472.

23 Patil A, Banerji R, Kanojiya P, Koratkar S, Saroj S (2021) Bacteriophages for ESKAPE: Role in pathogenicity and measures of control. Expert Rev Anti Infect Ther 19: 845-865.

24 El Haddad L, Harb CP, Gebara MA, Stibich MA, Chemaly RF (2019) Systematic and critical review of bacteriophage therapy against multidrug-resistant ESKAPE organisms in humans. Clin Infect Dis 69: 167-178.

25 Domingo-Calap P, Delgado-Martinez J (2018) Bacteriophages: Protagonists of a post-antibiotic era. Antibiotics 7: 66.