Original Article

Journal of Microbiology and Related Research
Volume 9 Number 1, January - June 2023
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.9123.1

Isolation and Characterization of Pseudomonas Aeruginosa Bacteriophages

Jangili Pavan Kumar[1], Shaik Muzammil Pasha[2], Yemgdda Goutham Sudhan[3], Chand Pasha[4]

How to cite this article:
Jangili Pavan Kumar, Shaik Muzammil Pasha, Yemgdda Goutham Sudhan *et al.*/Isolation and Characterization of Pseudomonas Aeruginosa Bacteriophages/J Microbiol Relat Res. 2023;9(1): 09–16.

Abstract

Pseudomonas aeruginosa is a notorious, opportunistic pathogen and has ability of biofilm formation making it possible to survive in extreme conditions. As antibiotics and disinfectants have shown limited activity, P. aeruginosa phage based treatments are proposed as a promising way for biofilm control. P. aeruginosa isolates and PB1 like phage particles were isolated from sewage samples collected from sewage treatment plants. PB1 like phage was featured with large burst size of 151PFU/cell, host inactivation in 6 hrs, 80% of host range and had icosahedral head with a diameter of 85X80nm and long non contractile tail of 135nm. PB1 like phages are suitable for lysis of broad range MDR Pseudomonas biofilms, which could be used in phage therapy.

Keywords: Pseudomonas aeruginosa; Bacteriophages; PB1; Cell lysis; Biofilm.

INTRODUCTION

Antibiotics play an important role as therapeutic and prophylactic in healthcare clinics, veterinary, agriculture, food processing industries, etc. However, indiscriminate uses of antibiotics have led to development of resistance to most of the antibiotics (Reygaert, W. C., 2018). Additionally, the decline in research and development of new antibiotics has limited choice of weapons against pathogenic bacteria (Nathan, C, 2020). It is assumed that, by 2050, antibiotic resistance will result in 10 million deaths (Bassetti, M. *et al.*, 2017). In recent years, increasing morbidity and mortality due to infections with multidrug resistant pathogens have become a serious concern (De Oliveira et al., 2020). Because of the rapidly increasing cases of antimicrobial resistance, India too has recently declared K. pneumoniae, E. coli, A. baumannii, and P. aeruginosa as the 'critical priority' pathogens (DBT, 2021). The phage therapy through administration of lytic bacteriophages has proved successful in the war against these pathogens (Schooley *et al.*, 2017, Duplessis *et al.*, 2018, Qin *et al.*, 2021, Petrovic Fabijan *et al.*, 2020, Patil *et al.*, 2021, El Haddad *et al.*, 2019, Mulani *et al.*, 2019). Among the resistant pathogens, P. aeruginosa is a gram negative opportunistic pathogen, predominantly found in hospitals, animal farms, slaughter houses, soil, aquatic environment, and sewage water. P. aeruginosa is notorious for being the major cause of death by nosocomial infections,

Author Affiliation: [1-3]Student, [4]Assistant Professor, Department of Microbiology, Nizam College, Osmania University, Hyderabad 500001, Telangana, India.

Corresponding Author: **Chand Pasha,** Assistant Professor, Department of Microbiology, Nizam College, Osmania University, Hyderabad 500001, Telangana, India.

E-mail: cpasha21@gmail.com

Received on: 03.12.2022

Accepted on: 23.12.2022

© Red Flower Publication Pvt. Ltd.

especially in patients with severe wounds, causing sepsis in immune suppressed patients, chronic lung infections in patients with cystic fibrosis, and chronic obstructive lung disease, bladder catheter associated chronic infections in the urinary tract and ventilator associated serious pneumonia (Diggle *et al.*,2020). In most cases, treatment of P. aeruginosa is very challenging owing to its multiple mechanisms to resist antibiotics and ability to form antibiotic resistant bioflms (Ciofu *et al.*, 2019).

P. aeruginosa is a biofilm forming with multiple antibiotic resistances and degradation of various molecules (Hall-Stoodley *et al.* 2004; Harrison *et al.* 2008; Kumari *et al.*, 2009). P. aeruginosa is a principle microbe responsible for nosocomial infections (Driscoll JA *et al.*, 2007, Hancock RE & Speert DP, 2000). Phage therapy is a great option in combating P. aeruginosa infections (Fu *et al.*, 2010, Wright A *et al.*, 2009, Marza *et al.*, 2006). The infectiveness of antibacterial component, intervention of biofilm brought a Steve growth of phages as an alternative to prevent biofilm formation. Despite of several people reported phages of P. aeruginosa isolation, characterization but utilization for lysis of biofilm are limited. Here we described the characterization of PB1 phage and its possible exploring to block biofilm.

MATERIALS AND METHODS

Source of bacteria: P. aeruginosa was isolated from samples collected from sewage treatment plant, Necklace road Hyderabad, Telangana, India.

Serial dilutions of the sewage water were prepared, inoculated on LB Agar containing Citramide Plates and incubated at 37°C for 24 - 48 h. A single colony was transferred onto slant surface of Nutrient agar and incubated at 37°C for 48 h. The bacterial isolates were identified by growth on specific media, microscopy, arginine dehydrolase test, coagulase test, catalase test, oxidase test, H2S test and mannitol fermentation test.

Antibiotic susceptibility test:

Antibiotic susceptibility of Isolated P. aeruginosa strains was determined by Kirby Bauer Disc diffusion method. Eight different antibiotics were used in these tests are chloramphenicol, gentamicin, azithromycin, levofloxacin, doxycycline, ciprofloxacin, tobramycin and amikacin.

Isolation of the bacteriophages:

Bacteriophage enrichment:

Bacteriophage enrichment was done by taking 4ml of 0.2 µ filtered sample water suspension (phage source), 1ml of 10x Luria broth and 1ml of exponential growing P. aeruginosa and incubated at 37°C for 24 hr. Then the suspension was centrifuged at 15000 rpm for 5 mins and filtered through 0.2 µ syringe filter. The filtrate was mixed with pure culture and over layed using double agar layered based plaque assay method.

Detection of bacteriophages / Plaque assay:

In a sterile eppendorf, 0.2 µ syringe filtered 100 µl of bacteriophage source and 100 µl of exponential bacterial culture were added and incubated at 37°C for 15 min, then it was mixed with 7ml low melting agar (0.8%) and poured onto nutrient agar plate. Allowed the low melting agar to solidify for 30 min at room temperature and then plates were incubated inverted at 37°C for 24h.

Isolation of Pure phage:

Using a sterile scalpel, an isolated plaque was picked from the overlayed nutrient agar plate and suspended in 500 µl of phage buffer and diluted. A dilution was mixed with exponential bacterial culture; incubated and under gone double agar layered based plaque assay. Individual plaque obtained in this method was selected.

Host Inactivation studies:

Pure P. aeruginosa strains were inoculated separately into different flasks containing nutrient broth and incubated at 37°C for 24hr. Then the flasks were infected with 0.2µ filtered phages and incubated at 37°C with gentle shaking. The sample was collected from different flasks for every 1 hour, till 8 hours consecutively. The hourly samples of flasks were spread on the nutrient agar plates respectively for viability of host cells. The numbers of colonies in the hourly samples were counted by using colony counter. The time required to kill 90% of initial cells was measured.

Burst size determination:

An isolated plaque was picked into a sterile eppendorf containing 500 µl of phage buffer and then it was added to 500 µl of bacterial culture in a eppendorf and 100 µl of the mixture was undergone double layered agar based plaque assay.

Phage purification:

Centrifugation:

Phage lysate was made cell free by centrifuging at 5000rpm for 10 min and clear lysate was again centrifuged at 15000rpm for 5h to precipitate phages. The pellet was suspended in the phage buffer.

PEG:

By centrifugation of 15000rpm for 5min, the cells

were removed and the supernatant was collected. PEG 8000 was added to the supernatant solution to make 2% concentration and stirred at 4ºC overnight to precipitate the bacteriophages. Then the solution was centrifuged at 15000 rpm for 10 mins, bacteriophages were collected as pellet and suspended in phage buffer and dialyzed.

Transmission electron microscopy of phages:

One drop of the purified phage suspension was placed on a copper grid with carbon coated Formvar film for 10 mins at room temperature. 4% aqueous phosphotungstic acid was used for staining at pH 7. The sample was air dried overnight and examined with a Zeiss TEM 900 electron microscope; it was operated at 50 kV. The phage particles were visualized using the Image SP software and a CCD camera.

Determination of host range:

The host range of obtained phages was determined by P. aeruginosa strains. 1ml of pure P. aeruginosa strains, were spread on nutrient agar plates respectively. 50 µl of phages were sprayed on the nutrient agar plates with pure culture. These plates were incubated at 37ºC for 24 hrs. Then plates were observed for plaques.

Biofilm formation studies:

For the quantification of biofilm formed, overnight cultures of P. aeruginosa, was diluted to 106 CFU/ml into fresh LB broth supplemented with 1% glucose. 100µl of culture was diluted with 100µl of the same medium and added to 800µl of LB broth placed into each well of 24 well plates. The media changed for every 12 hrs without shaking. Half the volume of old medium in the culture was replaced with fresh medium. The development of biofilms was monitored up to 96 hrs. Biofilms formed were fixed with 200µl of methanol for 15 mins, followed by the addition of crystal violet and incubated for 15 mins. The wells were then washed with water and dried for 2 hrs at room temperature. 200µl of ethanol (95%) was added to dissolve the stain. The absorbance of eluted stain was measured at 570nm in a spectrophotometer. The absorbance at 570nm is a direct indication of the amount of biofilm formed. The biomass was represented as O.D. at 570nm.

Bacteriophage treatments:

In this study, the ability of bacteriophages to act on biofilms was determined. To accomplish this, biofilms formed by P. aeruginosa, after 12, 24, 36, 48, 60, 72, 84 and 96 hrs were infected with P. aeruginosa phage. About 1×10^9 phage particles suspended in 0.5 mL were added to the different aged biofilms and incubated for 4 hrs, immediately after incubation with the phages, the biofilms were quantitated by staining with crystal violet and measuring the absorbance at 570 nm.

Statistical analysis:

Experiments were repeated thrice in triplicates (n = 9) and average values with standard deviation was provided.

RESULTS

Isolation and identification of P. aeruginosa:

Five strains of P. aeruginosa were isolated from sewage samples, collected from sewage treatment plants. P. aeruginosa were identified by growth on Citramide agar, microscopy, Biochemical characteristics and the results were presented in Table 1.

Five strains were identified as P. aeruginosa based as white colonies on Citramide agar. They were gram negative, rod shaped and 1-3 x 0.4-0.7 µm in size. In the biochemical tests, it was Arginine dehydrolase postive, coagulase negative, catalase

Table 1: Identification of P.aeruginsa by growth on specific media, microscopic morphology and biochemical tests.

Strain	Growth on specific media	Microscopy morphology	Biochemical Tests					
			Arginine dehydrolase test	coagulase test	catalase test	Oxidase test	H2S test	Mannitol fermentation test
P. aeruginosa 1	white colonies on Citramide agar	Gram negative, rod shaped and 1-3 x 0.4-0.7 µm in size.	positive	Negative	positive	positive	Negative	Positive
P. aeruginosa 2	white colonies on Citramide agar	Gram negative, rod shaped and 1-3 x 0.4-0.7 µm in size.	positive	Negative	positive	positive	Negative	Positive
P. aeruginosa 3	white colonies on Citramide agar	Gram negative, rod shaped and 1-3 x 0.4-0.7 µm in size.	positive	Negative	positive	positive	Negative	Positive

table cont.....

P. aeruginosa 4	white colonies on Citramide agar	Gram negative, rod shaped and 1-3 x 0.4-0.7 µm in size.	positive	Negative	positive	positive	Negative	Positive
P. aeruginosa 5	white colonies on Citramide agar	Gram negative, rod shaped and 1-3 x 0.4-0.7 µm in size.	positive	Negative	positive	positive	Negative	positive

positive, oxidase positive, H2S negative and mannitol fermentation positive.

Antibiotic susceptibility test:

Results showed that P. aeruginosa was resistant to majority of antibiotics. High rate of resistance was against doxycycline, azithromycin, chloramphenicol and ciprofloxacin. It was intermediately susceptible to gentamycin, levofloxacin, tobramycin and amikacin. It was not highly susceptible to any of the antibiotics.

Table 2: Antibiotic susceptibility test of P. aeruginosa as zone of inbition (mm) with 100µ concentration.

Bacteria	Strain	Chloram-phenicol	Gentamicin	Azithro-mycin	Levo-floxicin	Doxy-cycline	Cipro-floxacin	Tobra-mycin	Amikacin
P. aeruginosa 1	HN1	7.04	10.24	2.85	11.45	2.56	8.3	10.28	15.36
P. aeruginosa 2	HN2	7.01	10.11	2.24	11.25	2.21	7.6	10.19	15.12
P. aeruginosa 3	HN3	6.98	9.98	2.48	10.89	1.99	8.1	10.54	14.89
P. aeruginosa 4	HN4	7.03	10.02	2.59	11.56	2.24	7.9	10.98	15.19
P. aeruginosa 5	HN5	7.01	10.22	2.78	11.94	2.17	7.3	10.33	14.94

Bacteriophage enrichment:

Phage enrichment filtrate contained numerous phages and formed plaques of varying sizes specific to P. aeruginosa strains.

Detection of bacteriophages/plaque assay:

In Plaque assay, after incubation, bacteriophage plaque formation was determined and plaques were counted as plaque forming units (PFU). P. aeruginosa plaques were round as in fig. 1.

Fig. 1: Plaque assay of P. aeruginosa showing plaques

Phage purification:

Plaque was purified and used in plaque assay method which produced plaques specific to P. aeruginosa on nutrient agar plates. Single and isolated plaque was selected for pure phage.

Host Inactivation Studies:

The number of colonies in hourly samples were counted using colony counter. The viable cell count was more till 1 hour, from 2nd hours, the number of viable cells started decreasing in a descending order. 90% of P. aeruginosa cells were inactivated in 6 hours.

Burst size determination:

Plaques were observed on the nutrient agar plates. The plaque with largest burst size was the bacteriophage with higher affectivity. P. aeruginosa phage produced the burst size of 151 PFU per cell.

Bacteriophage isolation and purification:

The P. aeruginosa bacteriophages were isolated from sewage samples, phages were like lambda phages in morphological appearance, having icosahedral head with a diameter of 85 x 80nm and long non contractile tail of 135 nm based on TEM. It is PB1 like phage in Fig. 2.

Determination of Host range:

Host range was determined using five P.

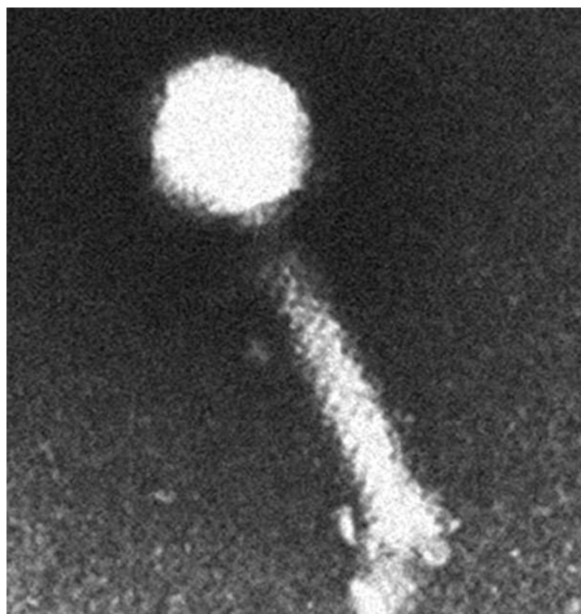

Fig. 2: Transmission electron microscopy image of P. aeruginsa phage.

aeruginosa strains. The P. aeruginosa bacteriophage had 80% of host range as it infected four out of five P. aeruginosa strains.

Effect of phage treatment on the bacterial biofilms:

P. aeruginosa biofilms obtained were treated with the phages for 2–4 hrs. Immediately after incubation with phage, the biofilms were stained by crystal violet assay. The phage reduced the biomass of P. aeruginosa bacterial biofilms in a time dependent manner. Upon phage treatment for 4 hrs the O.D. decreased in biofilms of 12h (0.34± 0.04 to 0.17± 0.05), 24 h (0.75±0.03 to 0.38±0.08),36h(1.09± 0.04 to 0.55± 0.06), 48 h (1.34±0.01 to 0.72±0.02), 60h(1.72± 0.02 to 0.91± 0.03), 72 h (2.06±0.037 to 1.08±0.017), 84h(2.42± 0.01 to 1.25± 0.04) and 96 h (2.76±0.05 to 1.45±0.026).

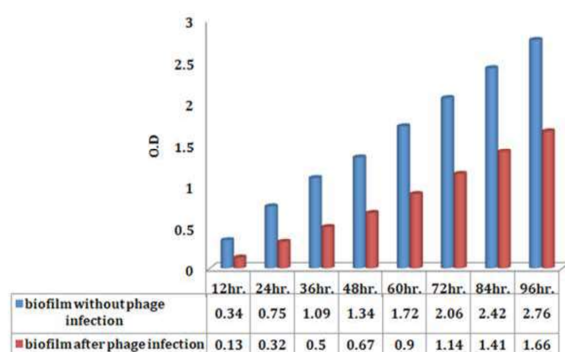

Fig. 3: Impact of P. aeruginosa phage on the biofilms by crystal violet staining.

DISCUSSION

Pseudomonas form biofilm on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems have become problematic (Hall-Stoodley *et al.* 2004). Biofilms are difficult to deal than the single bacterial infections as they require high doses of antibiotics, longer periods of time for treatment. This leads to increase in antibiotic resistance among the microbes in biofilms. Biofilm formation leads to less penetration of antibiotics (Nair *et al.* 2016). Pseudomonas are prevalent in sewage (Piracha *et al.*, 2014), hospital (Remold SK *et al.*, 2011), lakes (ZehraChegini *et al.*, 2020), etc. and generally responsible for nosocomial infections (Diggle *et al.*, 2020). The antibiotics and disinfectants are decomposed by P. aeruginosa forcing us to think for alternatives (Harper D.R. *et al.*, 2014). Lytic bacteriophages are most effective alternatives to handle resistant and biofilm forming P. aeruginosa (Harper D.R. and Enright M.C. *et al.* 2011). Sewage is known to be rich source of P. aeruginosa and its bacteriophages (Eman M. Marei *et al.*, 2020). In this study, P.aerugonisa was isolated from samples, collected from sewage treatment plant. Eman M. Marei, (2020) isolated P. aeruginosa from Cystic fibrosis patients (Essoh C *et al.*, 2015). Phages will be more in the environment of their corresponding bacteria (Xie *et al.*, 2005). P. aeruginosa phages were also isolated from sewage samples (Essoh C *et al.*, 2015; Chegini *et al.* 2020). P. aeruginosa was found to be multidrug resistant (Pallavali *et al.*, 2017; sonika Sharma *et al.*, 2021; Sharahi JY *et al.*, 2020) and the same is confirmed in the present study. Isolates of P. aeruginosa revealed 50%, 63.9%, 16.7%, 8.3%, and 16.7% resistance to imipenem, cefepime, amikacin, tobramycin, gentamicin, respectively (Hosu MC *et al.*, 2021). Brzozowski *et al.* 2020 reported lower reistanceto imipenem and cefepime and higher resistance to tobramycin, amikacin, and gentamicin. P. aeruginosa lytic phages have been used in several studies to cure infections caused by MDR strains of P. aeruginosa. phage in this study infects and destroys five MDR P. aeruginosa bacterial strains. The lytic phage PAXYB1 isolated from waste water was used to kill P. aeruginosa strain PAO1 and other clinical isolates (Yu X *et al.*, 2017). The lytic phage AZ1 was isolated against the MDR clinical strain of P. aeruginosa-2995 and this phage activity was determined against P. aeruginosa 2995 in both planktonic cells and the biofilm (Jamal M *et al.*, 2017). In another work, bacteriophages were isolated from sewage and tested against MDR-bacterial isolates

(P. aeruginosa, S.aureus, K. pneumoniae and E. coli) from patient with septic wound infections. These phages displayed perfect lytic activity against the MDR bacteria causing septic wounds and concluded that phages are therapeutic options for treating septic wounds (Pallavali RR *et al.*, 2017). P. aeruginosa phage isolated from sewage of Ilam had significant effects against a variety of clinical and general laboratory strains of P. aeruginosa (Azizian R *et al.*, 2015). In present study, 90% of P. aeruginosa cells were inactivated in 6 hours whereas in study of Sonika Sharma *et al.*, (2021), 90% of P. aeruginosa cells inactivated in 7 hours. P. aeruginosa phage burst size is 151 PFU per cell. P. aeruginosa phages with larger burst sizes are phage PAXYB1 (burst size approximately 141 PFU/cell) and phage PPA-ABTNL (burst size approximately 110 PFU/cell) (Yu X *et al.*, 2017, Cao Z *et al.*, 2015), phage ø/PSZ1 (burst size approximately 100 PFU/cell) and phage ø/PSZ2 (burst size approximately 100 PFU/cell) (El Didamony G *et al.*, 2015).

In this study, Phages were PB1 like phage in morphological appearance, having icosahedral head with a diameter of 80nm X 75nm and long non contractile tail of 130nm based on TEM. The phage PPaMa 1/18 had large, icosahedral head with diameter of 90 nm × 75 nm and long contractile tail of 215nm in length and SL2 phages were with head of 120 nm diameter and tail of 170 nm length. (Jurczak-Kurek A *et al.*, 2016). The phage DRL P1 had icosahedral head with diameter of 197.47nm and tail of length 94.54 (Sonika Sharma *et al.*, 2021). PAK-P1 like phages showed morphology of 130 nm tail and a 67–70 nm head (Essoh C et al., 2015). The P. aeruginosa bacteriophage in this study had 80% of host range as it infected four out of five P. aeruginosa strains. PPaMa1/18 phage had the broadest host range with 85.7% infectivity of P. aeruginosa isolates (Majdani R, Shams Ghahfarokhi E., 2022). The cluster of phages which belonged to six genera also had 80% host range as they infected 16 out of 20 P.aureginosa isolates (Essoh C. *et al.*, 2015). The effect of phage treatment on the bacterial biofilms was tested by crystal violet staining as reported by Sonika Sharma *et al.*, (2021), whereas the anti-biofilm activity was tested using safranin staining by P. Gupta *et al.*, (2017). In this study, the biofilms were degraded by 50% due to phage infectivity for 4hrs, where as 55%, 41%, and 33% of biofilms were degraded after 12 h, 24 h, and 48 h of incubation with phage, respectively (Sonika Sharma *et al.*, 2021).

CONCLUSION

P. aeruginosa is a multi drug resistant, opportunistic pathogen forming biofilms and phage therapy is belived to be only solution. P. aeruginosa isolates and PB1 like phage particles were isolated from sewage samples collected from sewage treatment plant. PB1 like phage was featured with large burst size of 151PFU/cell, host inactivation in 6 hrs, 80% of host range and had icosahedral head with a diameter of 85 x 80nm and long non contractile tail of 135nm. PB1 like phages are suitable for lysis of broad range MDR Pseudomonas biofilms hence can be used in phage therapy.

REFERENCES

1. Azizian R, Nasser A, Askari H, Kalani MT, Sadeghifard N, Pakzad I, *et al.* (2015). Sewage as a rich source of phage study against Pseudomonas aeruginosa PAO. Biologicals, 43, 238–241.
2. Bassetti, M. *et al.* (2017). Antimicrobial resistance in the next 30 years, humankind, bugs, and drugs: A visionary approach. Intensive Care Med, 43, 1464–1475.
3. Brzozowski M, Krukowska Z, Galant K, Jursa-Kulesza J, Kosik-Bogacka D. (2020). Genotypic characterisation and antimicrobial resistance of Pseudomonas aeruginosa strains isolated from patients of different hospitals and medical centres in Poland. BMC Infect Dis, 20, 693.
4. Cao Z, Zhang J, Niu YD, Cui N, Ma Y, Cao F, *et al.* (2015). Isolation and characterization of a "phiKMV-like" bacteriophage and its therapeutic effect on mink hemorrhagic pneumonia. PLoS One, 10(1), e0116571.
5. 5. Ciofu, O. &Tolker-Nielsen, T. (2019). Tolerance and resistance of Pseudomonas aeruginosabioflms to antimicrobial agents: How P. aeruginosa can escape antibiotics. Front Microbiol, 10, 913.
6. Harper D.R. & EnrightM.C. (2011). Bacteriophages for the treatment of Pseudomonas aeruginosa infections. Journal of Applied Microbiology, 111, 1–7.
7. DBT.(2021).http://dbtindia.gov.in/latest-announcement/indian-priority-pathogen-list-jointly-developed-dbt-and-who-india-office.
8. De Oliveira, D. M. P. *et al.*(2020). Antimicrobial resistance in ESKAPE pathogens. ClinMicrobiol Rev, 33, e00181-19.
9. Diggle, S. P. & Whiteley, M.(2020). Microbe Profile: Pseudomonas aeruginosa: Opportunistic pathogen and lab rat. Microbiology (Reading), 166, 30–33.
10. Driscoll JA, Brody SL, Kollef MH. (2007). The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. Drugs, 67,

351–368.

11. Duplessis, C. *et al.*(2018). Refractory Pseudomonas bacteremia in a 2-year-old sterilized by bacteriophage therapy. J Pediatric Infect Dis Soc, 7, 253–256.

12. El Didamony G, Askora A, Shehata AA.(2015). Isolation and characterization of T7-like lytic bacteriophages infecting multidrug resistant Pseudomonas aeruginosa isolated from Egypt. CurrMicrobiol, 70, 786–791.

13. El Haddad, L., Harb, C. P., Gebara, M. A.,Stibich, M. A. & Chemaly, R. F.(2019). A Systematic and critical review of bacteriophage therapy against multidrug-resistant ESKAPE organisms in Humans. Clin Infect Dis, 69, 167–178.

14. Eman M. Marei.(2020). Isolation and characterization of Pseudomonas aeruginosa and its virulent bacteriophages. Pak J BiolSci, 23, 491-500.

15. Essoh C, Latino L, Midoux C, Blouin Y, Loukou G, Nguetta S-PA, *et al.* (2015). Investigation of a Large Collection of Pseudomonas aeruginosa Bacteriophages Collected from a Single Environmental Source in Abidjan, Cote d'Ivoire. PLoS One, 10(6), e0130548.

16. Fu, W. *et al.*(2010). Bacteriophage cocktail for the prevention of bioflm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. Antimicrob Agents Chemother, 54, 397–404.

17. Hall-Stoodley, L., Costerton, J.W. & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol, 2, 95–108.

18. Hancock RE & Speert DP.(2000). Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. Drug Resist Updat, 3, 247–255.

19. Harper DR, Parracho HM, Walker J, Sharp R, Hughes G, Werthén M, Lehman S, Morales S.(2014). Bacteriophages and bioflms. Antibiotics, 3, 270–84.

20. Harrison, J.J., Turner, R.J., Joo, D.A., Stan, M.A., Chan, C.S., Allan, N.D., Vrionis, H.A., Olson, M.E. *et al.* (2008). Ammonium cations exert synergistic bactericidal and antibiofilm activity against Pseudomonas aeruginosa. Antimicrob Agents Chemother, 52, 2870–2881.

21. Hosu MC, Vasaikar S, Okuthe GE, Apalata T.(2021). Molecular detection of antibiotic-resistant genes in Pseudomonas aeruginosa from nonclinical environment: public health implications in Mthatha, Eastern Cape province, South Africa. Int J Microbiol,8861074.

22. Jamal M, Andleeb S, Jalil F, Imran M, Nawaz MA, Hussain T, *et al.*(2017). Isolation and characterization of a bacteriophage and its utilization against multi-drug resistant Pseudomonas aeruginosa-2995. Life Sci, 190, 21–28.

23. Jurczak-Kurek A, Gąsior T, Nejman-Faleńczyk B, Bloch S, Dydecka A, Topka G, *et al.*(2016). Biodiversity of bacteriophages: morphological and biological properties of a large group of phages isolated from urban sewage. Sci Rep, 6, 34338.

24. Kumari, S., K. Harjai and S. Chhibber. (2009). Characterization of Pseudomonas aeruginosa PAO specific bacteriophages isolated from sewage samples. Am J Biomed Sci, 1, 91-102.

25. Marza, J.A., Soothill, J.S., Boydell, P. and Collyns, T.A. (2006). Multiplication of the rapeutically administered bacteriophages in Pseudomonas aeruginosa infected patients. Burns, 32, 644–646.

26. Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S. & Pardesi, K. R.(2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A Review. Front Microbiol, 10, 539.

27. Nair, S., Desai, S., Poonacha, N., Vipra, A. & Sharma, U. (2016). Antibiofilm activity and synergistic inhibition of Staphy lococcus aureus biofilms by bactericidal protein P128 in combination with antibiotics. Antimicrob Agents Chemother, 60, 7280–7289.

28. Nathan, C.(2020). Resisting antimicrobial resistance. Nat Rev Microbiol, 18, 259–260.

29. P. Gupta, A. Sarkar, P. Sandhu, A. Daware, M.C. Das, Y. Akhter & S. Bhattacharjee.(2017). Potentiation of antibiotic against Pseudomonas aeruginosa biofilm: a study with plumbagin and gentamicin. Journal of Applied Microbiology, 123, 246—261.

30. Pallavali RR, Degati VL, Lomada D, Reddy MC, Durbaka VRP.(2017). Isolation and in vitro evaluation of bacteriophages against MDR-bacterial isolates from septic wound infections. PLoS One,12(7), e0179245.

31. 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 Ter, 19, 845–865.

32. Petrovic Fabijan, A. *et al.*(2020). Westmead Bacteriophage Terapy Team. Safety of bacteriophage therapy in severe Staphylococcus aureus infection. Nat Microbiol, 5, 465–472.

33. Piracha, Z.Z., U. Saeed, A. Khurshid & W.N. Chaudhary.(2014). Isolation and partial characterization of virulent phage specific against Pseudomonas aeruginosa. Global J Med Res, 14, 1-8.

34. Qin, J. *et al.* (2021). Heterogeneous Klebsiell apneumoniae co-infections complicate personalized bacteriophage therapy. Front Cell Infect Microbiol, 10, 608402.

35. Raheleh Majdani & Elham Shams Ghahfarokhi. (2022). Isolation and characterization of lytic bacteriophages against Pseudomonas aeruginosa isolates from human infections in the north-west of Iran. Iran J MicrobiolApr, 14(2), 203–213.

36. Remold SK, Brown CK, Farris JE, Hundley TC,

Perpich JA, Purdy ME.(2011). Differential habitat use and niche partitioning by Pseudomonas species in human homes. MicrobEcol, 62, 505.

37. Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiol, 4, 482–501.

38. Schooley, R. T. *et al.*(2017). Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection. Antimicrob Agents Chemother, 61, e00954-e1017.

39. Sharahi JY, Ahovan ZA, Maleki DT, Rad ZR, Rad ZR, Goudarzi M, Shariati A, Bostanghadiri N, Abbasi E, Hashemi A. (2020). In vitro antibacterial activity of curcumin-meropenem combination against extensively drug-resistant (XDR) bacteria isolated from burn wound infections. Avicenna J Phytomed,10, 3.

40. Sonika Sharma, Sibnarayan Datta, Soumya Chatterjee, Moumita Dutta, Jhuma Samanta, Mohan G.Vairale, Rajeev Gupta, VijayVeer & Sanjai K. Dwivedi. (2021). Isolation and characterization of a lytic bacteriophage against Pseudomonas aeruginosa. Scientific Reports,11, 19393.

41. Wright, A., Hawkins, C.H., Anggard, E.E. &Harper, D.R. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. Clin Otolaryngol, 34, 349–357.

42. Xie H, Zhuang X, Kong J, Ma G & Zhang H. (2005). Bacteriophage Esc-A is an efficient therapy for Escherichia coli 3-1 caused diarrhea in chickens. The Journal of General and Applied Microbiology, 51, 159-163.

43. Yu X, Xu Y, Gu Y, Zhu Y, Liu X. (2017). Characterization and genomic study of "phiKMV-Like" phage PAXYB1 infecting Pseudomonas aeruginosa. Sci Rep, 7, 13068.

44. Zahra Chegini, Amin Khoshbayan, Majid Taati Moghadam, Iman Farahani, Parham Jazireian & Aref Shariati. (2020). Bacteriophage therapy against Pseudomonas aeruginosabioflms: a review. Ann ClinMicrobiolAntimicrob,19, 45.