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Characterization of bacteriophages PAA and PAM and evaluation of their antibiotic synergy against *Pseudomonas aeruginosa* PAZMYU isolated from urine sample

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Abstract

Antibiotic resistance has been attributed to both the overuse of current drugs and the lack of availability of newer drugs due to stringent regulatory requirements and reduced commercial incentives. Novel alternative therapies, such as phage treatments, have demonstrated promising outcomes in clinical trials, indicating their potential for treating recalcitrant infections in the future. The objective of this study was to isolate and characterize bacteriophages against a uropathogenic strain of *Pseudomonas aeruginosa* and evaluate the antimicrobial efficacy of combining sub-inhibitory concentrations of cefepime and meropenem with the isolated bacteriophages. The isolated phages were designated PAA and PAM, both exhibiting icosahedral heads and long non-contractile tails. Both phages maintained stability within Limited pH and temperature ranges. The optimal adsorption times for PAA and PAM were 10 and 20 min, respectively, with PAA demonstrating a short latent time and burst size of 47, while PAM exhibited a burst size of 83. The optimal multiplicity of infection for PAA was 1, and for PAM was 0.1. PAA demonstrated efficacy against 40% of the tested strains of *P. aeruginosa*, while PAM was effective against 64%. Both phages remained stable at subinhibitory concentrations of cefepime and meropenem and when employed with sub-inhibitory concentrations of cefepime and meropenem, exhibited synergistic effects against planktonic bacterial cells and demonstrated efficacy in both biofilm inhibition and eradication.

Keywords Antibiotic resistance, *Pseudomonas aeruginosa*, Synergistic effect, Biofilm

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Introduction

Antibiotic resistance has emerged as a major public health issue in the twenty-first century, posing a serious threat to the effective prevention and treatment of a wide range of infections caused by bacteria that have developed resistance and are no longer treatable with conventional medications [1]. Over many years, bacteria that cause both severe and minor illnesses have steadily developed varying degrees of resistance to new antibiotics that enter the market [2]. Given this fact, antibiotic resistance has developed into an escalating global healthcare catastrophe in this era [3]. It is a plausible basis for the dynamic "resistome" and a threat to global health, posing an inordinate burden on healthcare and highlighting the pressing need to address this issue [4]. In 2014, the World Health Organization (WHO) predicted that the 21st century could culminate in a post-antibiotic age, in which antibiotics will be ineffective [5]. According to a comprehensive analysis conducted in 2019, bacterial antimicrobial resistance was Linked to 4.95 million deaths worldwide, of which 1.27 million were directly brought about by resistance [6]. Pakistan has the 176th-highest age-standardized mortality rate per 100,000 people, which is correlated with AMR [7]. Pakistan has the fifth highest age-standardized death rate among the five nations in the GBD region of South Asia. According to the U.K commission assessment, drug-resistant diseases will be the cause of 10 million deaths annually by 2050, at an estimated cost of $3 trillion [8].

The most common healthcare-associated infections that account for more than 40% of all nosocomial infections are urinary tract infections (UTIs), which can be complicated or uncomplicated [9]. Uncomplicated UTI cases are mostly diagnosed in normal patients with a healthy urinary tract, and the major causative agent is *E. coli*, which accounts for 80% of the cases, and etiological agents of 7–15% of the uncomplicated UTI cases are *K. pneumoniae* and *P. aeruginosa* [10]. *P. aeruginosa* had been mostly found in the urine samples of patients with complicated UTI [11]. *P. aeruginosa*, a Gram negative bacterium has probably been the first pathogen to acquire MDR and XDR attributes with the advent of strains resistant to all anti-pseudomonal drugs except polymyxins [12]. MDR and XDR *P. aeruginosa* strains have been identified as high-risk clones associated with global clonal lineages [13]. These strains have accumulated a variety of resistance indicators through mutations, including those that upregulate AmpC beta-lactamase, affect the regions of topoisomerases that determine quinolone resistance, or by horizontal acquisition of resistance genes, such as aminoglycoside-modifying enzymes, ESBLs, or carbapenemases [14]. According to a report by the U.S. Centers for Disease Control and Prevention, *P. aeruginosa* infections concerning healthcare are anticipated to affect 51,000 individuals annually [15]. Of these infections, 13% are multidrug-resistant (MDR) and account for approximately 400 deaths annually [16]. This multidrug-resistant (MDR) bacterium has a high rate of fatalities because of its resistance to a broad spectrum of antibiotics [17]. Biofilm formation leads to recurrent infections resistant to standard antibiotics [18]. Owing to rising trends in antibiotic resistance in recent years, this pathogen is a frequent source of nosocomial infections, especially in immunocompromised hosts [19]. The range of viable treatments for such infections has gradually reduced as resistance to various classes of antibiotics has accumulated, giving rise to strains with multidrug-resistant (MDR) phenotypes [20]. Although resistance can be linked to a decline in fitness or virulence, some MDR strains exhibit an impressive capacity for infection and propagation in a clinical context and have the ability to quickly develop epidemics [21]. Hence, searching for alternative options is necessary.

One solution is to incorporate combination therapy that combines phages and antibiotics as a preventative measure against the potential emergence of resistance to individual antibacterial drugs [22]. Combination therapy employing phages and antibiotics has recently received extensive research attention as an intriguing treatment for bacterial infections [23]. This is due, in particular, to its enhanced efficacy in reducing phage and antibiotic resistance, in addition to the synergistic antibacterial effects produced by enhancement in phage replication when coupled with antibiotics [24]. Several studies over the past 20 years have discussed the use of phage-antibiotic combinations to combat *P. aeruginosa* [25]. In vitro sensitivity to a number of antibiotics significantly increased in two *P. aeruginosa* strains infected with phages Pf3 and Pf1 reported in previous study [26]. According to Knezevic et al., *P. aeruginosa* growth was inhibited when phages and subinhibitory concentrations of ceftriaxone were administered together [27]. Lin et al. reported that ciprofloxacin and phage PEV20 displayed the most significant synergistic effects [28]. Uchiyama et al. evaluated various combinations of phages and antibiotics against *P. aeruginosa* and concluded that combinations, including piperacillin and ceftazidime, had the most potent PAS [29]. In light of the problems with antibiotic resistance, these trials demonstrate continued attempts to investigate phage antibiotic synergism as a possible therapeutic option for *P. aeruginosa*-caused infections.

The objective of this study was to address the emerging challenge of antibiotic resistance in *P. aeruginosa* by isolating and characterizing bacteriophages from sewage samples against *P. aeruginosa*, determining the phage-antibiotic synergy with commercially available antibiotics, and evaluating the anti-biofilm potential of the

phage-antibiotic synergy phenomenon to treat resistant *Pseudomonas* infections.

Materials and methods

Location of study

This research was conducted in the Applied, Environmental, and Geomicrobiology Laboratory (AEG), Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

Isolation and evaluation of phenotypic traits (Biofilm assay and disk diffusion assay) of bacterial strain

P. aeruginosa PAZMYU, a bacterial strain previously isolated from the urine of a 67-year-old female patient, had its complete genome sequenced and deposited in NCBI under the accession number JAZHPT000000000.

The strain's capacity to form biofilms was evaluated using a modified microtiter plate assay based on a previously published protocol. In brief, a 24-hour culture of the bacterial strain was diluted in Muller-Hinton broth containing 1% glucose to achieve a final OD_{600} of 0.5. A 200 µL aliquot of this dilution was added to wells in a flat-bottomed polystyrene microtiter plate. Muller-Hinton broth with 1% glucose served as a negative control. The plates were incubated at 37 °C for 24 h. Following incubation, the wells were emptied aseptically, washed thrice with distilled water, air-dried, and fixed by heating at 60 °C for 60 min. The plate was then stained with 2% crystal violet for 15 min, washed, and air-dried. The biofilms were dissolved in 95% ethanol, and the OD was measured at 550 nm using a microplate reader [30]. The strain's biofilm formation ability was assessed based on the OD values using formulae from a previous study [31]. Additionally, biofilm formation by *P. aeruginosa* PAZMYU was observed microscopically on glass slides [32]. These were submerged in a 24-hour bacterial culture and incubated statically for 24 h at 37 °C. The slides were then washed, air-dried, stained with 0.1% crystal violet, and examined under an optical microscope (Nikon YS2-H) at 100× magnification.

The Kirby-Bauer disk diffusion method was employed [33] to assess the host bacterial strain's susceptibility to various antibiotics as mentioned in the following Table. Bacterial suspensions (OD600 = 0.5) were spread on Muller-Hinton agar plates. Antibiotic disks (Thermo Scientific Oxoid) were placed equidistantly on these plates using sterile forceps. The plates were incubated overnight at 37 °C. After incubation, the inhibition zones around each antibiotic disk were measured with a precise scale. Based on these measurements, the bacterial strain was classified as "susceptible," "resistant," or "intermediate" according to the Clinical Laboratory Standards Institute (CLSI) guidelines for antibiotic breakpoints (Table 1).

Table 1 List of antibiotics used to assess the host strain's susceptibility by disk diffusion assay

Antibiotics	Concentration (µg)
Vancomycin	30µg
Penicillin	10units
Piperacillin	100µg
Cefazolin	30µg
Cefixime	5µg
Ceftazidime	30µg
Cefipime	30µg
Meropenem	10µg
Imipenem	10µg
Doxycycline	30µg
Tobramycin	10µg
Chloramphenicol	30µg
Gentamicin	10µg
Azithromycin	15µg
Clindamycin	2µg
Fusidic acid	10µg
Linezolid	30µg
Ciprofloxacin	5µg
Sulphamethoxazole-trimethoprim	25µg
Nitrofurantoin	300µg

Isolation, purification and morphological characterization of bacteriophages

For the isolation of bacteriophages, domestic sewage water samples were collected from Abbottabad located in Khyber Pakhtunkhwa province and Murree located in Punjab province of Pakistan and processed according to the previously described protocol [34]. Briefly, 20 mL of sewage wastewater was centrifuged at 10,000 rpm for 5 min and filtered through a 0.22 µm syringe filter. Subsequently, it was mixed with 10 mL of 24-hour-old culture of *P. aeruginosa* PAZMYU and 20 mL of 2× nutrient broth and incubated for 24 h at 37℃. After 24 h, 1 mL of sample was drawn, centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a "Filter Bio-Company" syringe filter with 0.22 µm pore size. The filtrate was serially diluted and tested for the presence of bacteriophages by double layer agar (DLA) assay as previously described, and plaques with different morphologies were observed. An isolated plaque was quadrant-streaked on nutrient agar seeded with host bacteria and incubated for 24 h; this procedure was repeated thrice to obtain pure phage. From the final quadrant-streaked plate of phage, the layer of soft agar containing pure bacteriophages was scraped and suspended in 10 mL of SM buffer (200 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl with pH 7.5). It was then vortexed for 1 min and incubated in a shaking incubator at 35℃ for one hour. After incubation, it was centrifuged for 10 min at 10,000 rpm, the supernatant was filtered, and the pelleted soft agar was discarded.

0.25 ml of this supernatant was mixed with 0.75 mL of 75% glycerol and stored at −80°C.

Phages isolated and purified in this study through the aforementioned protocol were designated PAA (isolated from the sample collected from Abbottabad) and PAM (isolated from the sample collected from Murree).

Transmission electron microscopy (TEM) was employed at the National Institute of Biotechnology and Genetic Engineering (NIBGE), located in Faisalabad, Pakistan, to examine the morphology of both phages [35]. A 300-mesh copper grid was spotted with 10 µL of the phage filtrate diluted in SM buffer at a ratio of 1:10 and allowed to dry. The grid was then stained with 2% uranyl acetate and blotted with filter paper. Subsequently, it was observed under an electron microscope at a magnification of 100kx using an electron microscope (JOEL JEM 1010).

Thermal and pH stability of PAA and PAM

Filtrate of PAA and PAM was subjected to one hour of incubation at varying temperatures (4 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C) to assess the thermal stability of the phages. Following incubation, the filtrates underwent a double layer agar assay to determine the number of stable phages and were compared with the control (phage filtrate at room temperature).

The filtrate of the phages was diluted in SM buffer with varying pH (pH2-pH10) at a ratio of 1:100 and incubated for one hour to assess the pH stability of both phages. Double layer agar assay was utilized to assess the PFU of stable phage at various pH levels following incubation.

Evaluation of adsorption time, one step growth and optimal multiplicity of PAA and PAM

The evaluate the adsorption time of PAA and PAM previously reported protocol with some modifications was employed [36], briefly, 1 mL of the host bacterial broth culture was added in 100 mL of nutrient broths and filtrates of PAA and PAM were added in these broth separately at MOI 1 and incubated for 30 min and after every 5 min 1 mL of the samples were drawn, centrifuged, filtered and subjected to double layer agar assay to found out the number of free phages in filtrates. The time, where no free phages were found in filtrate was designated as optimal adsorption time.

To evaluate latent time and burst size of PAA and PAM, one step growth experiment was employed reported previously with some modifications [37], where, 1 mL of the host bacteria was infected with the filtrate of PAA and PAM separately at MOI 1 and allowed to adsorb phages for their optimal adsorption time. After adsorption time, 10 µL of the sample was taken out to find out the number of uninfected bacterial cell by spread plate method following dilution while the rest of the sample was centrifuged, hence after, supernatant was subjected to double layer agar assay to find out the number of unadsorbed phages while the pellet was inoculated in 100 mL nutrient broth and incubated for an hour at 37 °C in shaking incubator at 180 rpm and after every 10 min, 1 mL of the sample was drawn, centrifuged, filtered, diluted and subjected to double layer agar assay. The time where no increase in Phage PFU was observed was latent period and the time point where phage PFU start increasing was rise phase and the burst size was calculated by dividing the average number of released phages to the number of infected bacterial cells.

The multiplicity of infection (MOI) is the measure of the proportion of phages to host bacterial cells in a given medium. The ideal MOI of the PAA and PAM was found out by infecting 1 mL of 24 h old host bacterial culture at MOI 10, 1, 0.1, 0.01 and 0.001 of both phages in 100 mL nutrient broth and incubating for 24 h. Later, 1 mL the samples were collected, centrifuged, diluted and subjected to double layer agar assay and the MOI where maximum titer of the PAA and PAM was observed was regarded as optimal MOI.

Evaluation of host range of PAA and PAM

Host range of PAA and PAM was found out against the already isolated and characterized 25 strains of *P. aeruginosa* [38] isolated from urine samples. For the host range determination 10 µL of the phage filtrates were spotted on the Muller-Hinton agar plates seeded with tested bacterial strains and incubated overnight at 37 °C for 24 h. The strains on which bacteriolytic zones of phages were observed, double layer agar assay was performed to confirm either its phage based bacteriolytic activity or phage hydrolytic enzymes activity which were present in phage filtrate. On the basis of PFU of the PAA and PAM against these tested strains, efficiency of plating (EOP) was calculated by dividing the titer of phage against tested strains to the titer of the phage to host bacterial strains [39].

Biofilm formation Inhibition and biofilm eradication potential of PAA and PAM

100 µL of the host bacterial culture in its algorithmic state diluted in 2× nutrient broth (OD_{600} 0.5) was added in the wells of flat bottomed polystyrene microtiter plate followed by the adding 100 µL PAA and PAM filtrates diluted in SM buffer to achieve different MOIs (10, 1, 0.1, 0.01, and 0.001) and for positive control 200 µL of the host bacteria diluted in nutrient broth in 1:1 was used while for negative control 200 µL of nutrient broth was used. The microtiter plate was incubated for 24 h in static incubator at 37 °C. Following incubation, the planktonic cells were aseptically removed from the microtiter plate wells and wells were washed thrice with distill water, air dried, biofilm was fixed by baking microtiter plate at 60°C

for 60 min and microtiter plate was stained for 15 min with 2% crystal violet, washed and air dried. Biofilm from air dried plate was solubilized in 95% ethanol and OD at 550 nm was checked through microplate reader and OD of the phage treated wells at different MOIs was compared with each other and with the OD of the control and percentage of biofilm formation inhibition by phage at each MOI was calculated by dividing observed OD to the OD of control and multiplying it with 100 [40].

Biofilm eradication potential of PAA and PAM was evaluated by treating preformed biofilm of the host bacteria with phage filtrate with varying PFU. Biofilm was formed by incubating 200 µL of the host bacterial broth culture in the wells of microtiter plate at 37 °C in static incubator, for 24 h. After incubation, planktonic cells were aspirated aseptically from the wells and the wells were washed with autoclaved distill water thrice under aseptic condition, wells were added with 200 µL of the PAA and PAM diluted in nutrient broth at different PFUs (2.6×10^8 PFU/mL, 2.6×10^9 PFU/mL and 2.6×10^{10} PFU/mL) and incubated at 37 °C for 24 h. For control 200 µL of the nutrient broth was added in the wells with preformed biofilm. After incubation, Liquid from the wells was aspirated and wells were washed and above mentioned protocol based on crystal violet staining was employed to assess the biofilm reduction and the percentage of reduction in comparison to control was calculated by dividing OD of the phage treated preformed biofilm at each PFU to the OD of control and multiplying it to 100.

Evaluation of the synergy of PAA and PAM with antibiotics against planktonic and biofilm embedded host bacterial cells

Determination of minimum inhibitory concentration (MIC) of antibiotics

To examine phage-antibiotic synergy, cefepime, and meropenem were selected. The stock solution of these antibiotics was prepared in normal saline (0.9% NaCl) with the final concentration of 512 µg/mL, based on the potency of antibiotic, concentration of antibiotics powder needed to achieve final concentration of 512 µg/mL was calculated by employing previously reported formulae [41]. These stock solutions of antibiotics were serially diluted two-fold until achieving 0.125 µg/mL. To determine MIC, micro-dilution method was employed [42] by following CLSI guidelines, briefly, 100 µL of antibiotics dilutions were sequentially added in the wells of microtiter plate followed by the addition of *P. aeruginosa* PAZMYU culture diluted in 2× Muller-Hinton broth and allowed to incubate for 24 h at 37 °C. The lowest concentration of antibiotics where visible growth inhibition of host bacteria was observed was considered as MIC. Based on MIC (Table 2), three sub-inhibitory concentrations of selected antibiotics (1/4th, 1/8th, and 1/16th) were chosen to proceed phage-antibiotics synergy study.

Table 2 MIC breakpoints and concentrations to be used of selected antibiotics

Antibiotics	Antibiotics' MIC	Selected concentrations for PAS(µg/ml)		
		1/4	1/8	1/16
Cefepime ≥ 16 µg/mL	16 µg	4 µg	2 µg	1 µg
*Meropenem ≥ 4 µg/mL	2 µg	0.5 µg	0.25 µg	0.125 µg

Evaluation of effect of sub-inhibitory concentrations of antibiotics on PAA and PAM phage stability and plaque morphology

The stability of phages with sub-inhibitory concentrations of antibiotics was examined by mixing 100 µL of phage filtrates (PAA and PAM) with the 900 µL of sub-inhibitory concentrations of antibiotics and incubated at 37 °C for 2 h. After, phage filtrates diluted in antibiotics solution were subjected to double layer agar assay to assess the PFU of stable phages and plaque morphology and compared with control which was phage dilution in SMbuffer.

Effect of different MOI of PAA and PAM on Colony Forming Unit (CFU) reduction of host bacteria

To evaluate the minimal MOI of both phages that can eradicate the host bacterial population completely, 100 µL host bacterial diluted in 2× nutrient broth with the final CFU 3.1×10^7/mL was treated with 100 µL both phages separately at different MOIs (10, 1, 0.1, and 0.01) in the wells of microtiter plate for 24 h at 37 °C and for control 100 µL of host bacteria added with 100 µL of nutrient broth was used while for negative control 200 µL of nutrient broth was used. Following the incubation, 100 µL of the samples were drawn from phage treated wells and control wells, serially diluted and spread on MacConkey agar plates and incubated overnight at 37 °C. After incubation reduction in host bacterial CFU after treated with phages at different MOI was compared with control. The MOI of both phages, where no complete eradication of host bacteria was observed was selected to assess their potential effect with the sub-inhibitory concentrations of antibiotics.

Synergistic effect of PAA and PAM with Sub-inhibitory concentrations of antibiotics

To assess the synergistic effect of phages on planktonic cells, phages were individually mixed with host bacteria in 2× Muller-Hinton Broths so that the host bacteria's final CFU was 2.8×10^8/mL and the MOI of PAA and PAM was 0.1 and 1, respectively. To compare the combined effects of antibiotics and phages to their antibacterial effects alone 100µL of the host bacteria harboring phages were further combined with 100µL of

sub-inhibitory doses of antibiotics in microtiter plates and microtiter plate was incubated for 24 h at 37 °C and three controls were used in this experiment: phage + bacteria, antibiotics + bacteria, and bacteria alone. After the incubation period, 100 µL of the samples were taken from each well, diluted, and spread on MacConkey agar to find out the reduction in CFU of host bacteria at various treatment.

The fractional inhibitory index for synergistic study of two components should be less or equal to 0.5. The fractional inhibitory index of phages and antibiotics used in combination was less than 0.5, which was calculated by following previously reported formulae [43].

Synergistic effect of phage with antibiotics on biofilm formation Inhibition and biofilm eradication

To assess the synergistic effect of phages on the inhibition of biofilm of host bacteria, phages were individually mixed with host bacteria in 2× Muller-Hinton broths so that the host bacteria's final CFU was 2.8×10^8/mL and the MOI of PAA and PAM were 0.1 and 1, respectively. 100 µL of the host bacteria harboring phages were further combined with 100 µL of sub-inhibitory doses of antibiotics in microtiter plates and microtiter plate was incubated for 24 h at 37 °C and three controls were used in this experiment: phage + bacteria, antibiotics + bacteria, and bacteria alone. After the incubation period, the liquid from the microtiter plate was aspirated and by following the above mentioned protocol in Sect. 2.7 was employed to assess the combine effect of phages and antibiotics on biofilm formation inhibition of host bacteria in contrast to control [44].

Biofilm eradication potential of PAA and PAM in combination with sub-inhibitory concentrations of cefepime and meropenem was evaluated by treating 24 h old, preformed biofilm of the host bacteria with PAA and PAM filtrates (2.6×10^8 PFU/mL) added with selected sub-inhibitory concentrations of cefepime and meropenem. Three controls were used in this experiment: preformed biofilm treated with phage alone, preformed biofilm treated with sub-inhibitory concentrations of antibiotics alone, and preformed biofilm treated with nutrient broth. After incubation, liquid from the wells was aspirated and wells were washed and above mentioned protocol based on crystal violet staining was employed to assess the biofilm reduction by antibiotics and phage combination in comparison to phage and antibiotics alone.

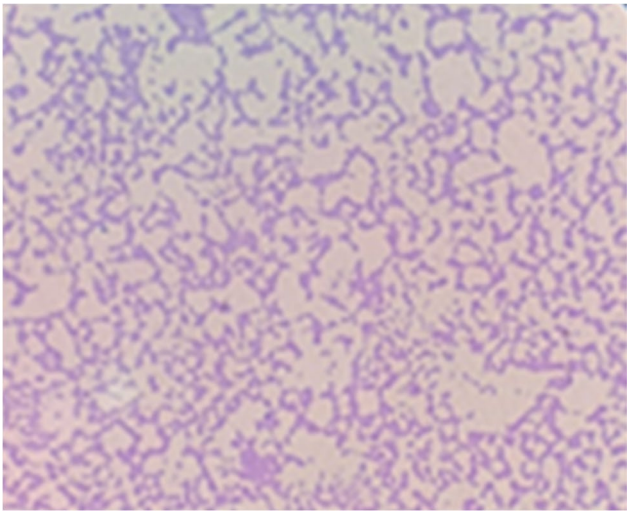

Fig. 1 Optical microscopy of biofilm produced by *P. aeruginosa* PAZMYU which was observed at 100× magnification

Statistical analysis

All the experiments were performed in triplicate and standard deviation from the mean value of each data set was calculated. One-way ANOVAs were employed as statistical analysis tests in this investigation. $P < 0.05$ was the significance threshold.

Results

Isolation and evaluation of phenotypic traits (biofilm assay and disk diffusion assay) of bacterial strain

P. aeruginosa PAZMYU strain was isolated in our previous study from urine sample and its whole genome sequence was submitted in NCBI under accession number JAZHPT000000000. The biofilm formation potential of *P. aeruginosa* PAZMYU was evaluated by microtiter plate assay. *P. aeruginosa* PAZMYU was a strong biofilm producer with the OD measured at 550 nm was 0.814 in contrast to negative control (OD_{550} 0.0543). Optical microscopy examination of biofilm of *P. aeruginosa* PAZMYU on glass slides provided additional evidence of the strain's biofilm formation (Fig. 1).

The antibiotics resistance of *P. aeruginosa* PAZMYU strain against 20 antibiotics (Table 1)` from different class of antibiotics was assessed by disk diffusion assay. Against *P. aeruginosa* PAZMYU, the zones of inhibition for cefipime, chloramphenicol, and meropenem were 9 mm, 19 mm, and 34 mm, respectively. No zones of inhibition were seen for the remaining tested antibiotics. *P. aeruginosa* PAZMY was sensitive to meropenem and chloramphenicol, as indicated by the CLSI zone diameter breakpoints for Enterobacteriales. These results suggest that *P. aeruginosa* PAZMYU was multi-drug resistant.

Isolation, purification and morphological characterization of bacteriophages

From the domestic wastewater sample collected from Abbottabad, and Murree, two phages active against *P. aeruginosa* PAZMYU were isolated and named as PAA and PAM, and TEM analysis of both phages revealed that, both are siphophages with icosahedral capsid and long

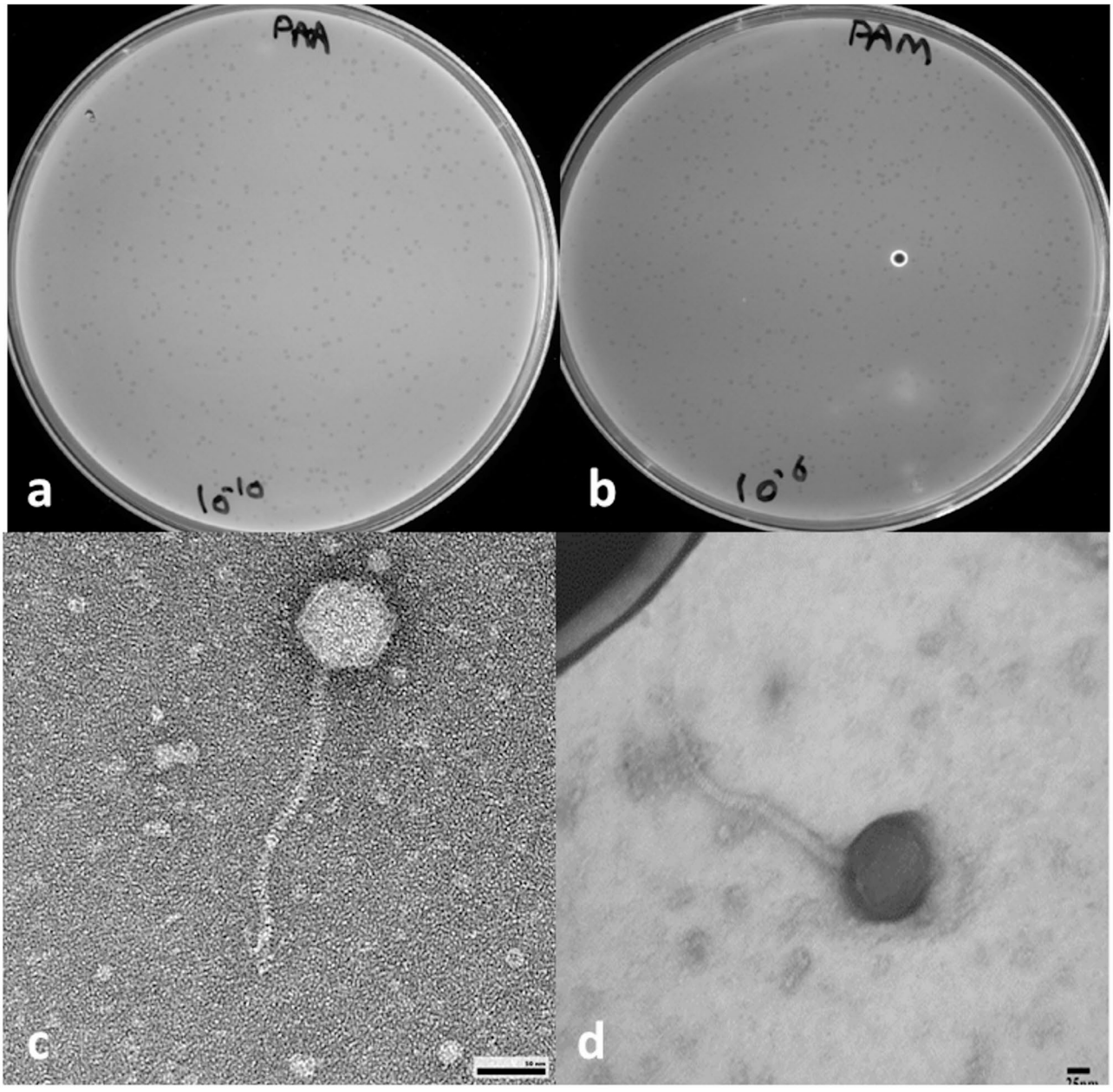

Fig. 2 Plaques of PAA (**a**) and PAM (**b**) produced against *P. aeruginosa* PAZMYU. Transmission electron microscopy of PAA (**c**), and PAM (**d**)

non-contractile tail (Fig. 2c and d). The mean diameter of the icosahedral capsid of PAA and PAM was 61.56 nm and 55.55 nm, respectively, while the mean length of the non-contractile tail of PAA and PAM was 219.7 nm and 160.356 nm, respectively. Both phages produces small, clear plaques against *P. aeruginosa* PAZMYU (Fig. 2a and b).

Thermal and pH stability of PAA and PAM

To evaluate the thermal stability of PAA and PAM, filtrates of both phages were incubated at different temperature range (4 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C) for an hour and phage stability was assessed by double layer agar assay. PAA phage appeared stable between 4 and 35 °C and at 40 °C and 45 °C, 0.7 log and 3.1 log reduction in its PFU was observed while at 50 °C, complete inactivation of PAA was observed (Fig. 3a). PAM appeared as stable between 4 and 35 °C, while at 40 °C, 45 °C and 50 °C, 1log, 1.1 log, and 2.9 log reduction in its PFU was observed (Fig. 3a). PAA and PAM appeared stable at pH 6, pH 7, and pH 8, and above and below to this pH range reduction in the PFU of both phages was observed and PAA was completely inactivated at pH 2, pH 3 and pH 4. The inactivation of PAM

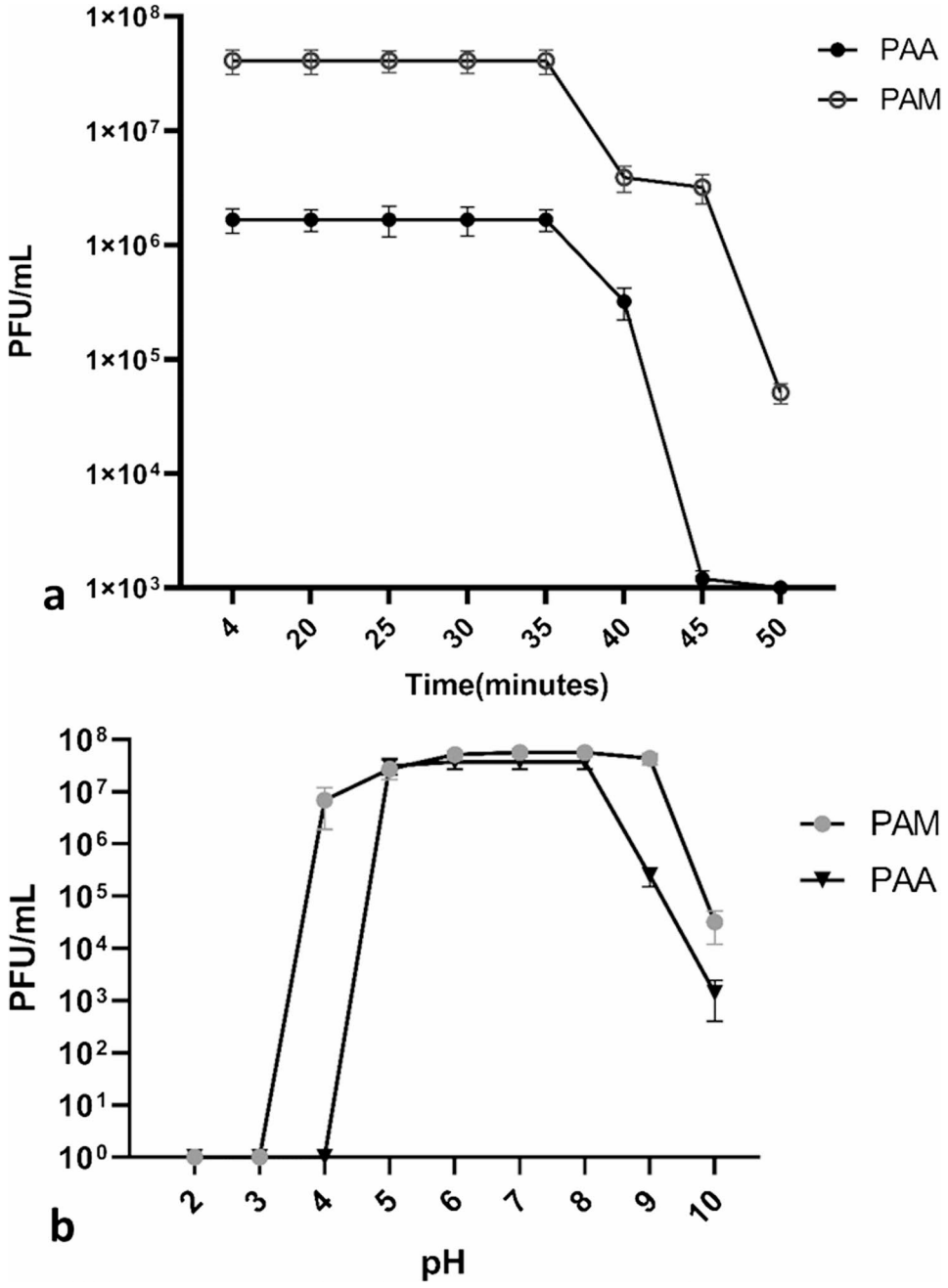

Fig. 3 Thermal stability of PAA and PAM (**a**), and stability of PAA and PAM at different pH range (**b**). The data points represent mean values and the error bars represent standard deviation of three replicates

was observed at pH 2 and pH 3 while 0.59 log reduction in its PFU was observed at pH 4 (Fig. 3b).

Evaluation of adsorption time, one step growth and optimal multiplicity of PAA and PAM

The adsorption time of PAA and PAM was evaluated by incubating host bacteria and phages at MOI 1 for 30 min, the optimal adsorption time of PAA was 10 min while at 5 min of incubation 98.5% of the phages were adsorbed on host bacterial surface receptors. The adsorption time of PAM was 20 min, and the percentage of phages adsorbed to host bacterial surface receptors at 5 min, 10 min and 15 min of incubation were 86%, 97.5% and 99.6% (Fig. 4a). Replication time of PAA and PAM was evaluated by employing one step growth experiment. The initial CFU of the host bacteria was 2.3×10^7/mL, initial PFU of PAA and PAM was adjusted to 2.6×10^7/mL by diluting their filtrates in SM buffer. No free phages were observed after absorption time, while the number of infected cells by PAA and PAM were 1.9×10^7/mL and

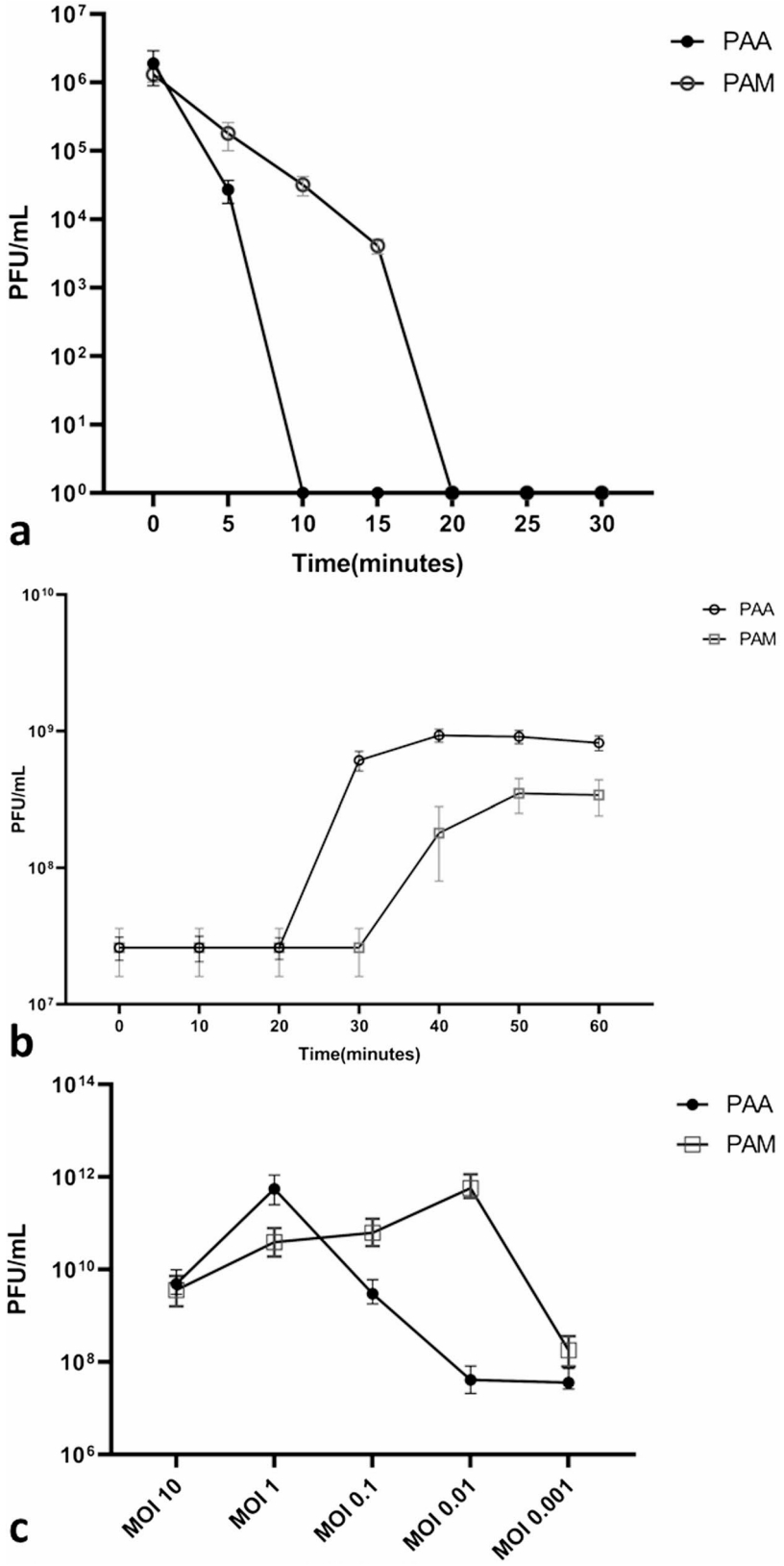

Fig. 4 Evaluation of optimal adsorption time (**a**), latent time and burst size (**b**), and optimal MOI of PAA and PAM (**c**). The data points represent mean values and error bars represent standard deviation of three replicates

4.2×10^6/mL. The latent time of PAA and PAM was 30 and 40 min and the burst size of PAA and PAM was 47 and 83 virions per infected cells (Fig. 4b). The optimal MOI of PAA was 1 and PAM was 0.01 (Fig. 4c).

Evaluation of host range of PAA and PAM

The host range of PAA and PAM against 25 pre-isolated uro-pathogenic strains of *P. aeruginosa* was evaluated. PAA was active against 10 tested strains of *P. aeruginosa* with the EOP ranged between 0.2 and 0.619 while PAM was active against 16 tested strains with the efficiency of plating between 0.14 and 0.93 (Table 3).

Biofilm formation Inhibition and biofilm eradication potential of PAA and PAM

Biofilm inhibition potential of PAA and PAM was evaluated by adding host bacterial broth culture and PAA and PAM phages at different MOIs in the wells of microtiter plate and incubated the microtiter plate for 24 h at 37 °C. After incubation, the percentage of biofilm was calculated and compared to control. The biofilm inhibition by PAA at MOI 10, 1, 0.1, 0.01 and 0.001 was 100%, 51%, 46%, 41% and 35%. The biofilm inhibition by PAM at MOI 10, and 1 was 100% while at MOI 0.1, 0.01, and 0.001 were 42%, 36% and 29% (Fig. 5a). The percentage of preformed biofilm eradicated by PAA when 10^8, 10^9 and 10^{10} PFU/mL of the phage was used was 22%, 54% and 76% and the percentage of reduction of pre-formed biofilm by PAM at 10^8, 10^9 and 10^{10} PFU/mL was 30%, 60% and 100% (Fig. 5b).

Table 3 Host range determination of PAA and PAM against uro-pathogenic strains of *P. aeruginosa*

Lab ID	Activity of PAA	EOP	Activity of PAM	EOP
PA P1	-	0	-	0
PA P2	+	0.61	+	0.19
PA P3	-	0	+	0.82
PA P4	-	0	+	0.58
PA P5	+	0.36	+	0.88
PA P6	-	0	-	0
PA P7	+	0.22	+	0
PA P8	+	0.49	+	0.14
PA P9	-	0	-	0
PA P10	-	0	+	0.76
PA P11	-	0	+	0.21
PA P12	+	0.59	+	0.57
PA P13	+	0.28	+	0.51
PA P14	-	0	-	0
PA P15	-	0	+	0.47
PA P16	-	0	-	0
PA P17	+	0.39	+	0.93
PA P18	-	0	-	0
PA P19	+	0.2	-	0
PA P20	-	0	-	0
PA P21	-	0	+	0.23
PA P22	-	0	-	0
PA P23	-	0	+	0.53
PA P24	+	0.46	+	0.29
PA P25	+	0.25	+	0.81

Evaluation of the Synergy of PAA and PAM with Antibiotics against planktonic and biofilm embedded host bacterial cells

Determination of minimum inhibitory concentration (MIC) of antibiotics

Cefipime and Meropenem were selected to carry out phage antibiotics synergy study. MIC of these antibiotics was determined and *P. aeruginosa* PAZMYU was resistant to cefipime and sensitive to meropenem with the MIC 16 µg and 2 µg. MIC breakpoint for the resistance of the tested pathogen belonging to Enterobacterales for cefepime is ≥ 16 µg/mL and for meropenem is ≥ 4 µg/mL. For phage-antibiotics synergy the selected sub-inhibitory concentrations of cefepime were 4 µg, 2 µg and 1 µg and the selected sub-inhibitory concentrations of meropenem were 1 µg, 0.5 µg and 0.125 µg.

Evaluation of effect of sub-inhibitory concentrations of antibiotics on PAA and PAM phage stability and plaque morphology

The stability of PAA and PAM was assessed by diluting phage filtrates in antibiotic dilutions in a 1:10 ratio to achieve the sub-inhibitory concentrations of particular antibiotics after adding phage filtrates. Following a two-hour incubation period, the stability of the phages at sub-inhibitory antibiotic concentrations was assessed using the double layer agar assay. The results were compared with a control group (phages were diluted 1:10 in SM buffer). No discernible increase in phage plaque size was seen when sub-inhibitory antibiotic concentrations were used, and both phages remained stable at sub-inhibitory concentrations of cefepime and meropenem.

Effect of different MOI of PAA and PAM on Colony Forming Unit (CFU) reduction of host bacteria

The effect of varying MOIs of PAA and PAM on host bacterial CFU reduction was evaluated. It was observed, that at MOI 10 of each phage, no viable bacterial cells were determined i.e. 0 cells as compared to control 1.6×10^{13} CFU/mL. PAM at MOI 1, reduced the number of bacteria to 5.3×10^5 CFU/mL whereas complete reduction was observed in the case of PAA at MOI 1. Decreasing the MOI to 0.1 and 0.01, PAA reduced the bacterial count to 3.2×10^6 CFU/mL and 1.7×10^8 CFU/mL and PAM reduced the bacterial count to 2.8×10^8 CFU/mL and 4.3×10^9 CFU/mL (Fig. 6). Conclusively, the sub-inhibitory MOI of each phage to obtain viable bacterial cells was deduced from this data as MOI 0.1 for PAA and MOI

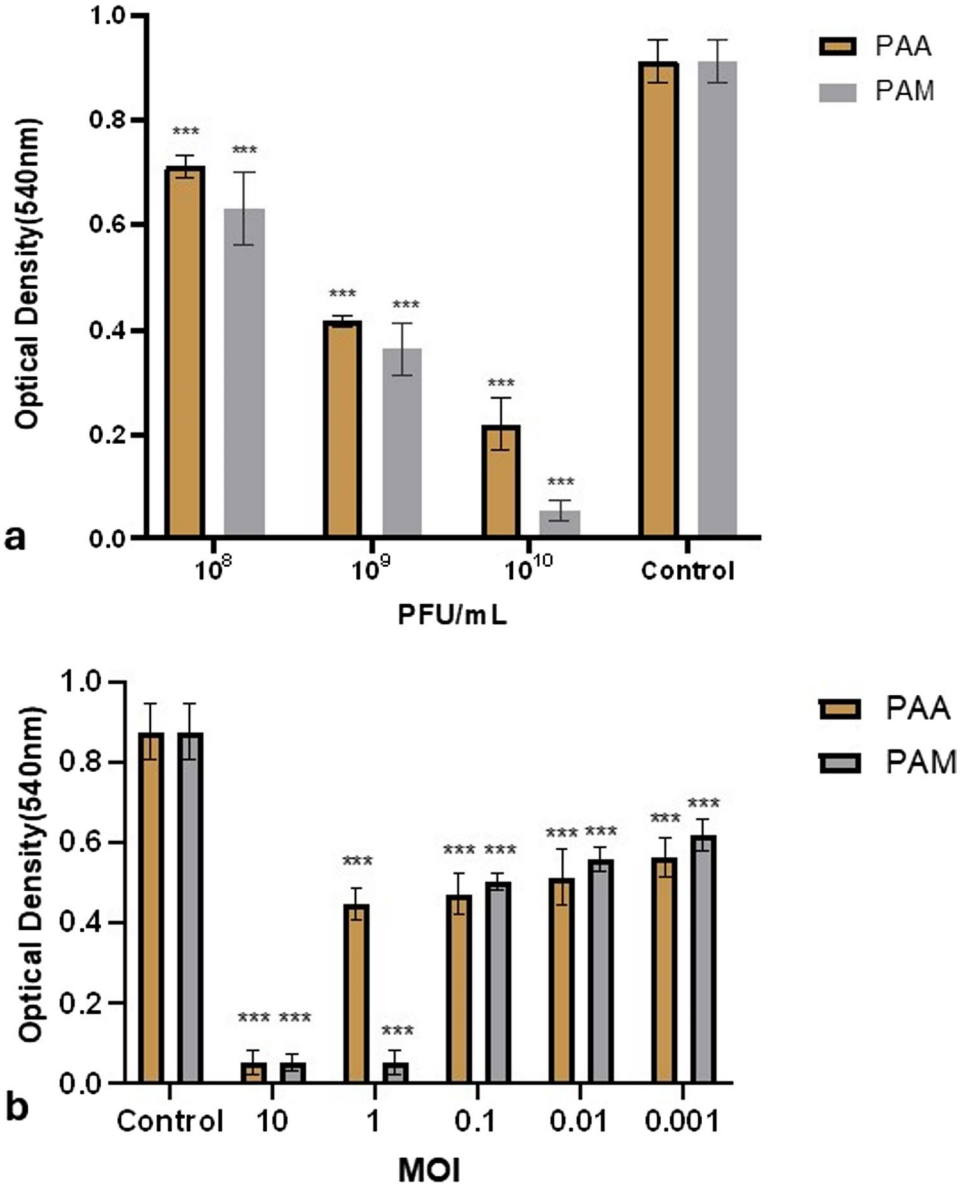

Fig. 5 Biofilm formation inhibition of *P. aeruginosa* PAZMYU by PAA and PAM (**a**) and biofilm eradication potential of PAA and PAM (**b**). In these graphs, control denotes the host bacteria's biofilm in the absence of any phage therapy. One way ANOVA was conducted followed by dunnett's test for multiple comparison of control with mean values in experimental group *** represents a p value of < 0.001 ** represents $p < 0.01$ * represents $p < 0.05$

1 for PAM, which was further used with antibiotics for phage antibiotics synergy.

Synergistic effect of PAA and PAM with Sub-inhibitory concentrations of antibiotics

To configure the synergistic effects of PAA and PAM in combination with antibiotics, the contribution of each individual component was assessed, and the synergistic effects were evaluated for all sub-inhibitory concentrations of antibiotics with PAA at MOI 0.1 and PAM at MOI 1. PAM at MOI 1 caused 7.4 log reduction in host bacterial CFU, while PAA caused 6.6 log reduction in CFU of the host bacteria. Cefepime at the concentration of 4 µg, 2 µg and 1 µg caused 3.5 log reduction, 1.8 log reduction and 0.7 log reduction in CFU of host bacteria and when these concentrations of cefipime were used with PAA and PAM at the concentration of 4 µg, CFU of the host bacteria was reduced to 0, while at the rest of the tested concentrations, the number of host bacterial cells reduction was higher than the sum of the effects of both components alone (Fig. 7a), which was the indicative of synergistic effect of both components at all the tested combination.

The synergistic effect of meropenem at the concentration of 1 µg, 0.5 µg and 0.125 µg with PAA and PAM was evaluated. Meropenem cause 4.43 log reduction, 2.7 log

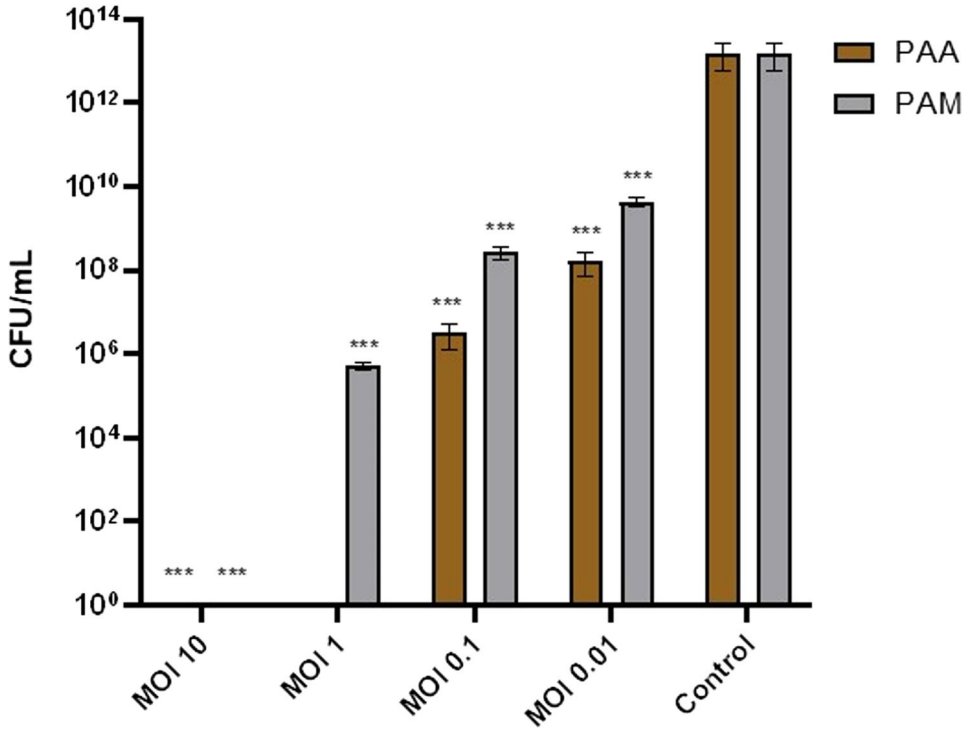

Fig. 6 Evaluation of different MOIs of PAA and PAM on host bacterial colony forming unit reduction after 24 h of incubation. In this graph, control denotes the host bacterial growth in the absence of any phage therapy. One way ANOVA was conducted followed by dunnett's test for multiple comparison of control with mean values in experimental group *** represents a p value of < 0.001 ** represents $p < 0.01$ * represents $p < 0.05$

reduction and 0.59 log reduction in host bacterial CFU when used alone at the concentration of 1 µg, 0.5 µg and 0.125 µg. When meropenem was used with PAM at the concentration of 1 µg and 0.5 µg, complete eradication of host bacterial cells was observed. Meropenem at the concentration of 1 µg with PAA caused complete eradication of host bacteria while at the rest of the concentrations of meropenem with PAA and PAM showed synergistic effect (Fig. 7b).

Synergistic effect of phage with antibiotics on biofilm formation Inhibition and eradication of Pre-formed biofilm

The ability of PAA and PAM combined with cefepime and meropenem at specific doses to prevent the formation of biofilms was assessed. Meropenem has a greater capacity to prevent biofilm formation than cefepime at every tested concentration. Complete inhibition of biofilm was observed when cefepime and meropenem at the concentration of 4 µg and 1 µg was employed with PAA and PAM. The percentage of biofilm formation inhibition when cefepime was employed at the concentration of 2 µg and 1 µg with PAA was 75.8% and 65% and when same concentrations of cefepime were employed with PAM the percentage of biofilm formation inhibition of host bacteria was 70.5%% and 56% compared to cefepime which caused 27%, 22% and 6% inhibition of biofilm at the concentration of 4 µg, 2 µg and 1 µg (Fig. 8a). The percentage of biofilm formation inhibition when meropenem was used at the concentration of 0.5 µg and 0.125 µg with PAA was 83.4% and 76.8% while when meropenem at the same concentrations were employed with PAM, the percentage of biofilm formation inhibition was 75% and 67.63% (Fig. 8b).

Synergistic effect of cefepime and meropenem at their sub-inhibitory concentration with PAA and PAM on eradication of preformed biofilm was evaluated. PAA when employed with cefepime at the concentration of 4 µg, 2 µg and 1 µg reduced 69.1%, 62.7% and 46% of preformed biofilm while PAM when employed at the same concentrations of cefepime caused 75.7%, 69.5% and 57% reduction in preformed biofilm (Fig. 8c).

Meropenem when used with PAA at the concentration of 1 µg, 0.5 µg and 0.125 µg caused 76%, 67% and 53% reduction in preformed biofilm while PAM when employed with meropenem at the concentration of 1 µg, 0.5 µg and 0.125 µg caused 83.7%, 72% and 64% reduction in preformed biofilm compared to control (Fig. 8d).

Discussion

Multi-Drug Resistant (MDR) *P. aeruginosa* causes disease with an elevated mortality rate as a result of resilience to many antibacterial drugs [45]. With varying degrees of virulence, this bacterium causes significant organ damage, and the development of its biofilm causes recurrent

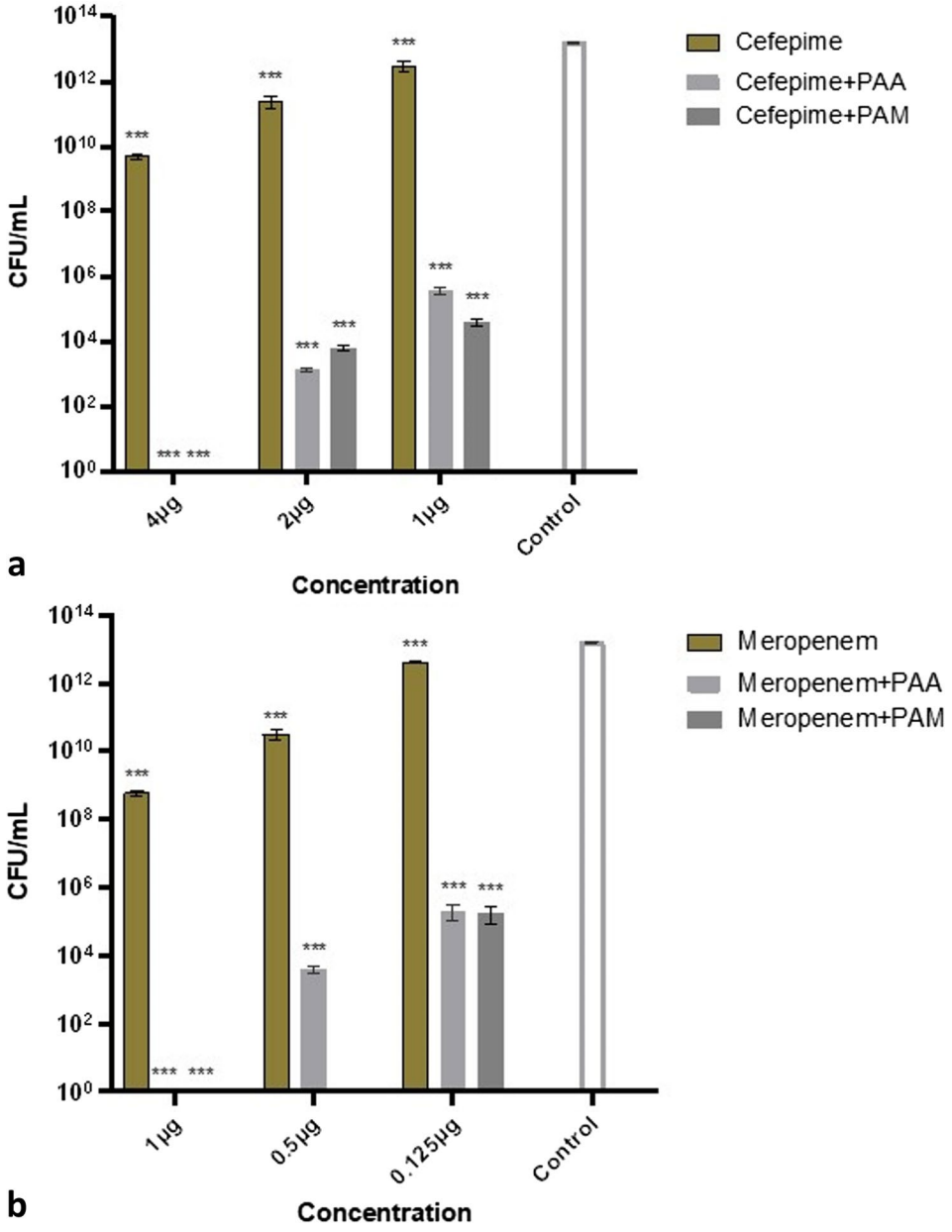

Fig. 7 Effect of cefepime (**a**) at its sub-inhibitory concentrations with PAA and PAM on host bacterial CFU reduction, and effect of meropenem (**b**) at its subinhibitory concentrations with PAA and PAM on host bacterial CFU reduction. In these graphs, control denotes the host bacterial growth in the absence of any phage or antibiotic therapy. Two way ANOVA was conducted followed by dunnett's test for multiple comparison of control with mean values in experimental group *** represents a p value of < 0.001 ** represents $p < 0.01$ * represents $p < 0.05$

infections that are antibiotic-resistant [46]. One of the unique therapeutic approaches that have been suggested as a result of antibiotics' incapability to remove *P. aeruginosa* biofilm is phage therapy [47]. Utilization of phage therapy is beneficial as phages appear to be equally efficient against bacteria that are susceptible to antibiotics and resistant to them, have low intrinsic toxicities, and can effectively dislodge bacterial biofilms [48]. They also tend to cause minimum disruption of normal flora. Importantly, research has shown that using antibiotics in addition to this complementary therapy increases its effectiveness.

This research aimed to isolate, characterize bacteriophages from domestic wastewater against multi-drug resistant uro-pathogenic *P. aeruginosa* (biofilm forming) and evaluate their synergistic effect with sub-inhibitory concentrations of meropenem and cefepime. From the sample of sewage waste, potent bacteriophages known as PAA and PAM were isolated. In a previous study, N4 like bacteriophage, from wastewater in Kerala, India was

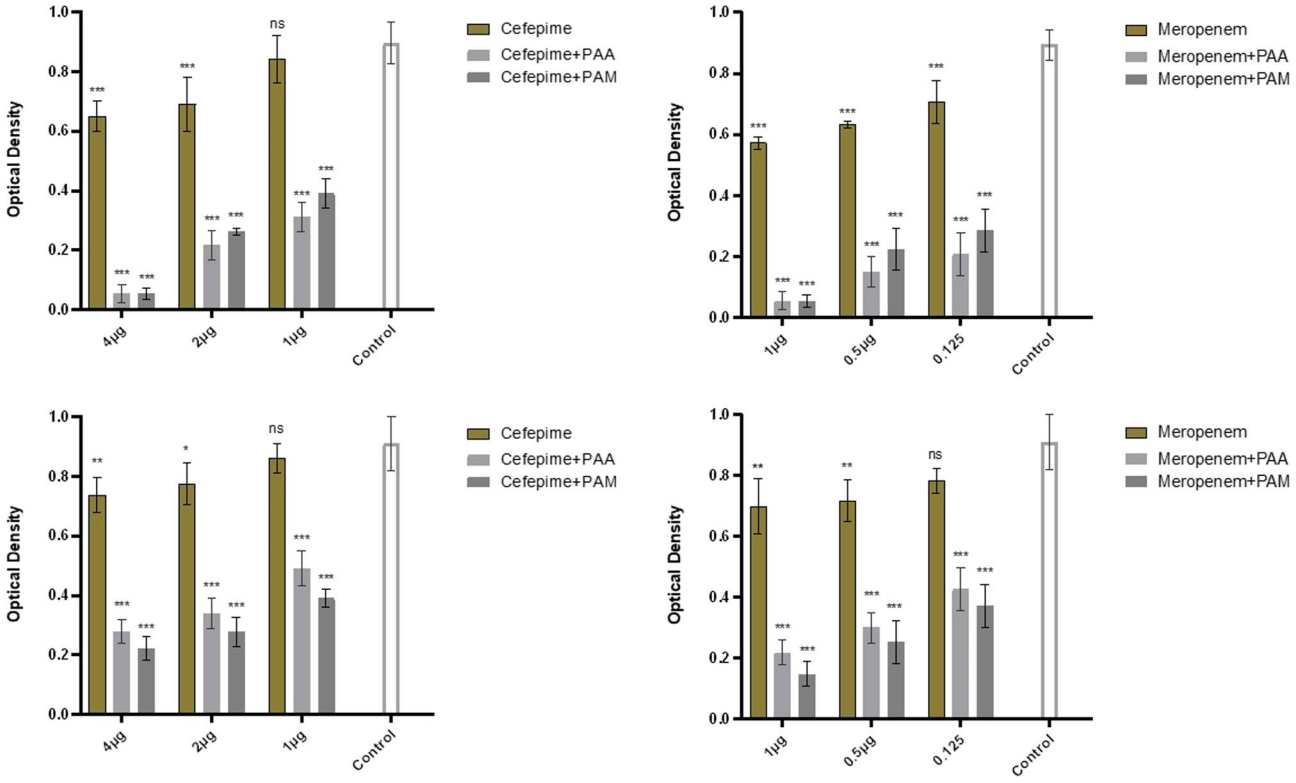

Fig. 8 Effect of cefepime in combination with PAA and PAM on biofilm inhibition (**a**), effect of meropenem in combination with PAA and PAM on biofilm inhibition (**b**), effect of cefepime in combination with PAA and PAM on biofilm eradication (**c**), and effect of meropenem in combination with PAA and PAM on biofilm eradication. In these graphs, control denotes the host bacteria's biofilm in the absence of any phage or antibiotic therapy. Two way ANOVA was conducted followed by dunnett's test for multiple comparison of control with mean values in experimental group *** represents a p value of < 0.001 ** represents $p < 0.01$ * represents $p < 0.05$

isolated against several MDR clinical *P. aeruginosa* isolates [49]. In another research, Phage SALSA was derived from the clinical isolate SM01, which was collected from sewage at the Aachen-Soers wastewater treatment plant in Germany [50]. Electron miocrographs have revealed that these phages show resemblance with the phages of siphoviradae possessing icosahedral head and long non-contractile tail. Nouran Rezk in 2022 reported that 58% of the examined clinical isolates of the antibiotic-resistant *P. aeruginosa* were susceptible to infection by ZCPA1 phage, a member of the Siphoviridae family [51]. Guillermo Santamaría-Corral et al. 2024 reported, Phage vB_PaeP-F1Pa as a promising candidate for phage therapy against MDR and XDR *P. aeruginosa* infections as it effectively inhibited bacterial growth and disrupted preformed biofilms [52]. The isolated phages in this study were stable at narrow pH and temperature range and both phages were inactivated at pH 2 and 3 and previous study had reported the maximum stability of TSE1-3 phage when incubated under mesophilic conditions [53] and inactivation of phages at lower pH was reported in previous study [54]. PAA and PAM showed maximum stability between pH 6- pH 8 and similar results were reported earlier [55]. Israa M Abd-Allah et al. 2022 reported, F2 phage (isolated sipho-virus) was fully inactivated at highly acidic conditions (pH 1) and showed only minimal lytic activity at pH 2, however, it retained activity between pH 3 and 11. Maximum stability between pH 6 and 8, it's likely due to a combination of biochemical compatibility, capsid protein structure, DNA protection and phages enzymatic activity [56]. The stability of PAA and PAM was effected when temperature was increased to 40°C and inactivation of PAA was observed at 50°C and previous study reported the adverse effect of temperature on stability of D10 phage [57]. Similar was reported in a study conducted by Sophie Camens et al. 2021, phages PA4 and PA8 were viable between 4 and 50 °C and rendered inactivity above 60 °C. Bacteriophages, Like all biological entities, are composed of proteins and nucleic acids that are thermally sensitive, usually maintaining stability at temperatures below 40 °C. Initial denaturation begins at 45 °C leading to destabilization of capsid and tail proteins and ultimately complete loss of infectivity at higher temperatures [58]. The optimal adsorption time of PAA was 10 min while more than 95% of the phages were adsorbed within 5 min of incubation and similar findings were reported in previous study. The optimal adsorption time of PAM was 20 min while more than 95% of the

phages were adsorbed to host surface receptors within 10 min of incubation and previous study reported 76% of the phage adsorption in 20 min of incubation [59, 60]. Compared to PAM, PAA had higher adsorption rate which ultimately reduced its latent time which was in accordance with previous study [61]. PAM having latent time higher than PAA have increased burst size in contrast to PAA and previous study had reported the direct relation of latent time on burst size [62]. Bacteriophages are highly specific and can target a specific bacterial species or strains [63], the host range of PAA and PAM was determined against 25 strains of *P. aeruginosa*, PAM showed high efficiency against 56% and PAA showed high efficiency against 20% of *P. aerouginosa* strains against which both phages were active. EOP values between 0.5 and 1 represents high efficiency, while the values between 0.2 and 0.5 and 0.001-0.2 represent medium and low efficiency [63]. PAM showed high efficiency against 56% and PAA showed high efficiency against 20% of *P. aerouginosa* strains. In a study conducted by Zhixin Shi et al. 2024, researchers isolated a novel lytic phage, Phage_Pae01, from hospital sewage. It demonstrated broad-spectrum antibacterial activity, effectively targeting 83.6% of clinical *P. aeruginosa* isolates, including those resistant to multiple drugs and carbapenems [64].

The increase in phage production with the sub-inhibitory concentrations of antibiotics is referred as phage-antibiotic synergy [65]. Utilization of combination of phage with antibiotics can cause increase in phage plaque size and phage can potentiate the effect of antibiotics by prolongation or restoration of the antibacterial activities of antibiotics being used [66]. In this study, to determine phage-antibiotics synergy, the stability of PAA and PAM in sub-inhibitory concentrations of cefepime and meropenem was evaluated, both phages appeared stable but no effect of the sub-inhibitory concentrations of both antibiotics and phages on plaques size of PAA and PAM was observed and our findings accord with previous research that showed no increase in MR-5 phage plaque size in the presence of β-lactam and the quinolone antibiotics [67]. In contrast to our findings, previous study has reported increase in plaque size of T4 phage in the presence of sub-inhibitory concentrations of cefotaxime [68]. Cefepime and meropenem are β-lactam antibiotics and β-lactam antibiotics cause bacterial cells filamentation by binding to penicillin binding protein. Cell filamentation is due to the inhibition of bacterial septum formation where bacterial cells grow without division. If the antibiotics are depleted, bacterial cells resume their replication but before the depletion of antibiotics, phage lyse bacterial cells with no significant increase in phage replication cycle with no ultimate increase in phage plaque size but it can be beneficial because of early clearance of host bacterial cells. Phage-antibiotics synergy study was proceeded with the MOI of 0.01 for PAA and 1 for PAM which efficiently eradicated host bacteria completely, after 24 h of incubation. Similar findings were reported by Ling et al. that the shorter latency period and inhibited host growth demonstrate that the phage load at MOIs of 0.01 was more successful against the host bacterium *Aeromonas salmonicida* than those at higher MOIs [69]. The synergistic effect of cefepime and meropenem on the 1/4th, 1/8th and 1/16th of their MIC was evaluated with PAA and PAM against planktonic cells, biofilm formation inhibition and eradication of pre-formed biofilm. All the tested concentrations of both antibiotics with PAA and PAM showed synergistic effect. The combine effect of antibiotics and phage on host bacterial CFU reduction and biofilm inhibition and eradication was higher than the sum of the effect of phage and antibiotics when employed alone [70]. Previous study reported reduced growth of *P. aeruginosa* when ceftriaxone was employed with phage. Phage combination with ceftazidime and piperacillin showed synergistic effect against *P. aeruginosa*. Previous studies based on ant-biofilm potential of phage against *P. aeruginosa* showed promising results and combination of phages with antibiotics is getting more attentions because of their synergistic effects [71]. Another study reported that meropenem had no anti-biofilm effect but when employed with phage the reduction in biofilm was observed [72]. Biofilm was effectively prevented when meropenem was employed with pB3074 phage. Increased anti-biofilm activity PA-56 was observed when used in combination with ciprofloxacin or meropenem against *P. aeruginosa* [73]. A stronger penetration of both antibiotics and phages into the biofilm has been proposed as the explanation for the antibacterial synergy between phages and antibiotics. According to reports, phages may employ depolymerases to break down the biofilm matrix, which facilitates their entry into the biofilm's deeper layers [74]. Additionally, it has been suggested that phages can enter the biofilm's lowermost regions by moving via its vacuum spaces. Phages begin multiplying in the deeper layer of the biofilm as a result of this occurrence, attaining large titers and disrupting the biofilm matrix [75]. Following this interruption, the administration of antibiotics leads to an improved bacterial reduction as a result of these drugs' more profound absorption.

Conclusion

In conclusion, phage treatments, either independently or in combination with currently available antibiotics, are likely to be increasingly utilized for infection treatment as their safety and efficacy are established. Phage therapy represents a method for reducing antibiotic resistance to commonly employed treatment regimens, thereby facilitating the continued use of antibiotics currently

available in the market. This investigation examined the combined effects of phages PAA and PAM with cefipime and meropenem. These antibiotics exhibited a synergistic relationship with the phages, demonstrating significant eradication of *P. aeruginosa* planktonic cells and biofilm at sub-inhibitory concentrations.

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Authors' contributions
"Conceptualization, SK and AN.; methodology, AN, SZ and NAK.; software, MB, MIK and MS; validation, AM,.; formal analysis, AN, NAK.; investigation, AN and NAK, AK and AM.; data curation, AN, NAK, NK and SK.; writing—original draft preparation, AN, AAS and SK.; writing, review and editing, AN, AAS, MB and SK.; visualization, SK.; supervision, SK.; project administration, SK, AN and AAS.; funding acquisition, SK and AN. All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors approve publication of the manuscript.

Competing interests
The authors declare no competing interests.

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