EFFICACY OF BACTERIOPHAGE AGAINST MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATES

Faseeha Khalid, Abu Baker Siddique, Zeeshan Nawaz, Muhammad Shafique, Muhammad Asif Zahoor, Muhammad Atif Nisar and Muhammad Hidayat Rasool

Department of Microbiology, Government College University, Faisalabad, Pakistan

Abstract. *Pseudomonas aeruginosa*, an increasingly widespread opportunistic human pathogen, is the most common gram-negative bacterium found in nosocomial infections, the most serious of which include malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia. Infections due to *Pseudomonas aeruginosa* are being treated with different antibiotics but its emerging resistance to several antibiotic groups is leading to difficulty in treatment of infected patients. In order to combat infections of antibiotic resistant *P. aeruginosa* bacteriophage therapy is a potential substitute. This study isolated *P. aeruginosa*-lytic bacteriophages from sewage waste water and evaluated their efficacy *in vitro* and *in vivo* against multidrug resistant *P. aeruginosa* isolates from wounds of burn patients attending the Allied Hospital, Faisalabad, Pakistan. *In vivo* efficacy of bacteriophage was determined using rabbit as a wound model. Prevalence of multidrug (MDR) *P. aeruginosa* was 60% and among the 6 MDR isolates 4 could be lysed (clear or turbid plaques) by the isolated bacteriophage samples, which was also effective in attenuating MDR *P. aeruginosa* infected wound healing in rabbit. These results suggest the potential of bacteriophage therapy in the treatment of MDR *P. aeruginosa* infection.

Keywords: *Pseudomonas aeruginosa*, bacteriophage, burn wound, rabbit, sewage waste water

INTRODUCTION

Burn wounds provide appropriate environment for growth of bacterial infections by providing a larger area and an extended stay of person in the hospital allowing increased opportunity for acquiring nosocomial infection compared to other wounds and surgical interventions (Agnihotri *et al*, 2004; Seth *et al*, 2012). These infections are the main cause of mortality (Nikokar *et al*, 2013). There are about 75% of deaths in burn patients due to sepsis in developing countries, such as Pakistan (Atiyeh *et al*, 2005). The most common causes of sepsis are *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Olszak *et al*, 2015).

There are many factors contributing towards virulence of *P. aeruginosa* and these can be divided into cell-associated and secretory virulence factors (Shi and Sun, 2002; Hosseinidoust *et al*, 2013). *P. aeruginosa* can be treated with a number of different antibiotics, such as amikacin, carbenicillin, ceftizoxime, cephalothin,

Correspondence: Abu Baker Siddique, Department of Microbiology, Government College University, Faisalabad, Pakistan.
Tel: +92419201205
E-mail: absiddique@gcuf.edu.pk

gentamicin, and tetracycline, but its emerging resistance to several antibiotic groups is leading to difficulty in treating burn wound patients infected with *P. aeruginosa* (Estahbanati *et al*, 2002). The bacteria also exhibit intrinsic resistance to different types of chemotherapeutic agents and antibiotics, thereby making the selection of appropriate drugs for treatment more problematic (Breidenstein *et al*, 2011). Another cause of increasing resistance is the ability of *P. aeruginosa* to forming biofilm on biotic as well as abiotic surfaces, such as catheters, contact lenses artificial implants and endotracheal tubes (Parasion *et al,* 2014).

Owing to antibiotic resistance, bacteriophage therapy is thought to be an effective substitute for antibiotics to treat *P. aeruginosa* infection. Lytic bacteriophages only are used to treat bacterial infections as they are host specific and theoretically harmless to eukaryotic cells (Sulakvelidze *et al,* 2001). This study was conducted to evaluate the potential of lytic bacteriophages isolated from local sewage water in treating *P. aeruginosa* infection using a rabbit as model.

MATERIALS AND METHODS

Samples collection

Fifty sterile cotton swabs were collected from wounds of burn patients attending the Allied Hospital, Faisalabad, Pakistan (Agnihotri *et al*, 2004). Samples were inoculated on pseudomonas agar (Oxoid, Basingstoke, UK) and colonies were subjected to biochemical tests, *viz*. oxidase, catalase, indole and methyl red, to confirm their identification as *P. aeruginosa* (Naqvi *et al*, 2014).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates was carried out according to CLSI guidelines (CLSI, 2008), employing Kirby-Bauer disks (Oxoid) containing amikacin (30 μg), cefepime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin + piperacillin (10/100 μg), piperacillin + tazobacam (100/10 μg), and tobramycin (10 μg).

Isolation and identification of bacteriophages

Sewage samples were collected from different areas of Faisalabad for isolation of bacteriophages. Aliquots of municipal waste water collected from 10 different areas and 5 samples collected from hospital waste water at different intervals (Olszak *et al*, 2015) were overlaid on LB soft agar (Oxoid) to isolate bacteriophages (Henry *et al*, 2013). which were enriched by incubating for 48 hours with *P. aeruginosa* MDR3 as host organism (Boulanger, 2009). Spot assay was performed to check the efficacy of each enriched sample against multidrug resistant (MDR) strains of *P. aeruginosa.* The most efficacious sample was purified by repeated agar overlay method. Then a plate containing clear plaques of similar size was prepared by adding 5 ml of SM buffer (50 ml of 1 M Tris HCl, 2 g of $MgSO_4$, 5 ml of 2% gelatin, 5.8 g of NaCl, and distilled water to make 1,000 ml) onto the plate and incubating at 37°C with shaking incubator for 4 hours. Then the SM buffer was centrifuged at 1,500*g* and filtered through a 0.22 μm membrane filter. Aliquot of 100 μl from each strain of MDR *P. aeruginosa* culture equal to 0.5 McFarland unit in SM buffer was spread on a nutrient agar plate. After drying, 10-20 μl of plaque solution were spotted and the plate was incubated for 18-24 hours at 37°C and observed for the presence of clear zone of cell lysis.

In vivo lytic potency of bacteriophage preparation

In vivo lytic activity was determined

Table 1
Antibiogram of multidrug resistant (MDR) *Pseudomonas aeruginosa* isolates from burn wounds of patients attending the Allied Hospital, Faisalabad, Pakistan.

Strain ID	FEP	CAZ	CRO	TOB	CIP	TZP	AK	PRL
MDR1	R	R	R	R	S	R	R	S
MDR2	IR	S	R	R	R	R	S	R
MDR3	IR	IR	R	R	R	R	IR	R
MDR4	S	R	R	R	S	R	S	R
MDR5	R	R	R	R	S	R	IR	IR
MDR6	R	IR	R	R	R	R	R	R

AK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; FEP, cefepime; PRL, piperacillin; TOB, tobramycin; TZP, piperacillin-tazobactam. IR, intermittent resistant; R, resistant; S, susceptible.

using rabbit as model. Exponentially growing culture of *P. aeruginosa* strain MDR3 was adjusted to a concentration of 3×10^8 cell/ml, centrifuged at 1,500*g* for 15 minutes and the pellet was suspended in phosphate-buffered saline to induce infection in the rabbits. A deep wound was aseptically induced on shaved flank. Group 1 rabbit (three rabbits per group) was treatment group (100 µl aliquot of 3×10^8 CFU bacterial suspension) MDR3 applied to wound and after 30-45 minutes application of 2×10^8 PFU bacteriophage sample MDR3, group 2 prophylactic group (application of bacteriophage followed by bacterial challenge, group 3 positive control (application of bacterial suspension), and group 4 negative control (application of saline) (Rasool *et al*, 2016). Animals were monitored for 24-48 hours development of abscesses and inflammation were recorded.

The experiment was conducted following the approved guidelines of the Institutional Ethical Review Committee, Government College University, Faisalabad, Pakistan (Reference no. GCUF/ERC/16/03, dated 14/03/2016).

RESULTS

Antimicrobial susceptibilities of *P. aeruginosa* clinical isolates

Out of 50 wound swab samples from burn patients 15 (30%) were positive for the presence of *P. aeruginosa*, of which all were resistant to CRO, TOB and TZP and 50% sensitive CIP (Fig 1). Six (40%) strains showed resistance against amikacin, cefepime, ceftazidime, ceftriazone, tobramycin, ciprofloxacin, piperacillin-tazobactam, and piperacilin and classified as multidrug resistant (MDR) (Table 1).

Lytic activity of bacteriophage samples from sewage water

Out of 15 sewage water samples, 12 were positive for the presence of bacteriophage. When the enriched bacteriophage samples were tested for lytic activity against the 6 MDR *P. aeruginosa* strains, bacteriophage samples Eff7, Eff10 and Eff11 were the most effective against all MDR strains. Three MDR *P. aeruginosa* strains (MDR 1, 2 and 4) showed clear lytic zones when tested with bacteriophage samples Eff10, two (MDR 3 and 6) produced turbid lytic zones and MDR 5

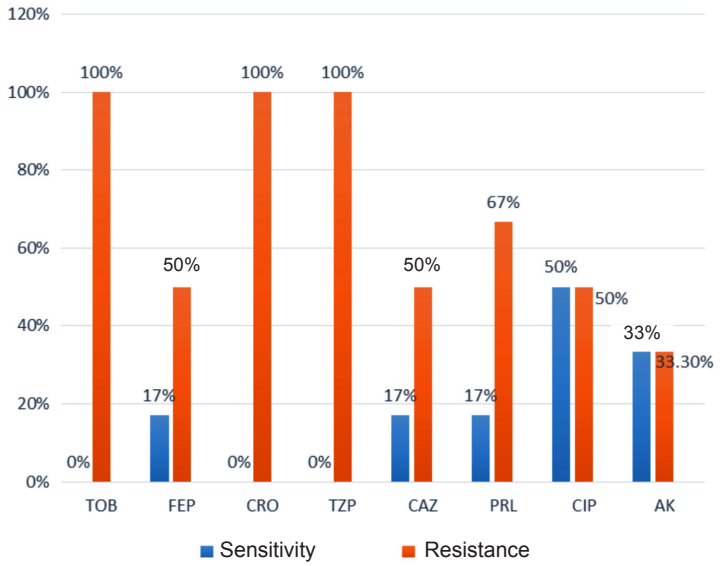

Fig 1–Antibiotic susceptibility of *Pseudomonas aeruginosa* isolates from burn wounds of patients attending the Allied Hospital, Faisalabad, Pakistan. Intermittent resistant isolates are not included. AK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriazone; FEP, cefepime; PRL, piperacillin; TOB, tobramycin; TZP, piperacillin-tazobactam.

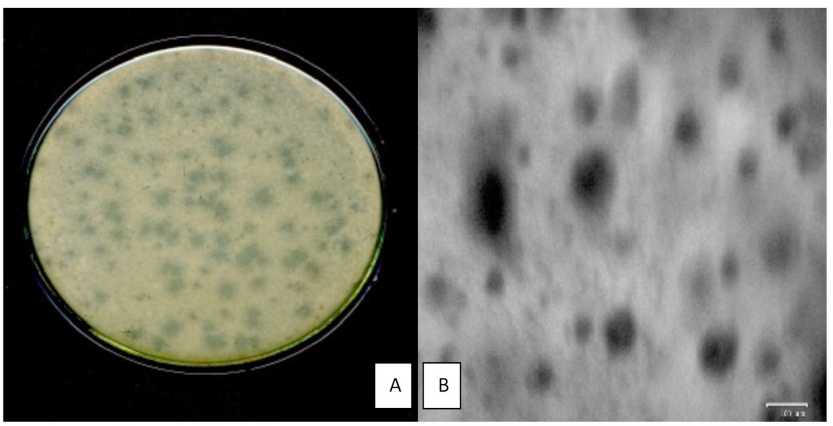

Fig 2–Plate showing plaques of various diameters obtained by agar overlay method (A) and viewed using a Cell Imager (Bio-Rad, Hercules, CA).

was completely resistant. It has been reported that divalent cations can improve the efficacy of bacteriophage lytic activity (Chhibber *et al*, 2014). There is a significant effect of 5M $CaCl_2$ on improvement of plaque morphology as well as PFU, while no significant effect of 5M $MgSO_4$ on plaque morphology and PFU was observed. Mitomycin C is used to convert lysogenic to lytic stage of bacteriophage to enhance its bacteriocidal efficiency (Chhibber *et al*, 2014). However, treatment with 1.5 M mitomycin C has no significant effect on the lytic effect of the bacteriophage preparations.

In addition to plaque assay, the effect of the bacteriophage preparations on growth of MDR *P. aeruginosa* strains was tested. After 1 hour of incubation with approximately10^8 PFU/ml of bacteriophage, growth of MDR3 began to decline and reached a minimum after 6 hours. All MDR strains were tested against each of the isolated bacteriophage samples and found to be susceptible to at least three different bacteriophage samples (Fig 2). The growth inhibition kinetic pattern was similar in MDR strains producing variable zones.

In vivo efficacy of bacteriophage samples

Antibacterial effect of bacteriophage samples *in vivo* was evaluated using rab-

bit as an experimental model. Wounds of treatment group (challenged with 2×10^8 CFU *P. aeruginosa* MDR3 followed by 2×10^6 PFU bacteriophage sample Eff10, prevention group (treated with bacteriophage sample followed by bacteria) and negative control group (treated with normal saline) are significantly better healed than those of positive control group (challenged with bacteria only) (data not shown).

DISCUSSION

This study was conducted because of the increase in infection rate in burn patients admitted at hospitals, rise in antibiotic resistant *P. aeruginosa* strains and the need for alternate ways of treating bacterial infection. There is a growing interest in employing bacteriophage therapy to combat the multidrug resistance *P. aeruginosa* infection (Sulakvelidze *et al*, 2001). The increased mortality rate in burn patients was found to be due to infection rather than hypovolemia and inhalation injury (Atiyeh *et al*, 2005).

In our study, *P. aeruginosa* was detected in nearly a third of burn wounds, with 40% of the bacterial isolates being multidrug resistant. Vinodkumar *et al* (2008) in India reported multidrug resistance in two thirds of *P. aeruginosa* isolates from septicemic neonates. Similarly, Saaiq *et al* (2015) reported in Islamabad, Pakistan *P. aeruginosa* infection in 35.3% of cases with infected burn wounds.

In the present study bacteriophages were isolated from sewage water collected from different areas of in and around Faisalabad, of which 80% were found to be positive for the presence of *P. aeruginosa*-lytic bacteriophages. In a similar study, Vinodkumar *et al* (2008) isolated *P. aeruginosa* bacteriophages from municipal sewage in Karnataka, India. Hospital effluents are considered to be rich sources for the presence of bacteriophages (Parasion *et al*, 2014). All the five samples collected from hospital effluent during this study were found to be positive for bacteriophages. This might be due to the presence of large amounts of bacteria, thereby providing a wide host range for bacteriophages. *P. aeruginosa* bacteriophages have been isolated from hospital sewage in Braga, Portugal (Pires *et al*, 2011).

Divalent salts are used in enhancing adsorption as well as attachment of bacteriophages on the bacterial cell (Jensen *et al*, 2015). Salts also help in effective intracellular progression of bacteriophages. Two divalent salts were used in this study to assess their effect on plaque morphology. $CaCl_2$ was found to be effective in enhancing plaque morphology while $MgSO_4$ did not show any significant effect. Positive effect of $CaCl_2$ on plaque morphology has previously been documented (Chhibber *et al*, 2014).

In vivo efficacy of isolated *P. aeruginosa* bacteriophages tested on wounds in the rabbit was just as effective as in mice as an animal model has been revealed (Golkar *et al*, 2013). Olszak *et al* (2015) reported bacteriophages significantly increase the lifespan of *Galleria mellonella* larvae. Bacteriophages applied to wound prior to bacterial challenge show a significant effect on wound healing rate. Morello *et al*, (2011) reported 100% survival of animals treated with bacteriophages prior to bacterial infection. Application of specific bacteriophages proved effective against immunosuppressed mice before experimental exposure with *Staphylococcus aureus* (Morello *et al*, 2011). Saussereau *et al* (2014) reported 64.6% of *P. aeruginosa* isolates from patients are susceptible to bacteriophages and activity of bacterio-

phages is not affected by age and gender of patients as well as duration of *P. aeruginosa* colonization and antibiotic treatment. Phage therapy has proved effective against immunocompromised patients experiencing bacterial infection (Zimecki *et al*, 2009).

In summary, this study demonstrate a high level of the occurrence of *P. aeruginosa* in wounds of burn patients attending the Allied Hospital, Faisalabad with nearly half of the bacterial isolates being MDR. *P. aeruginosa*-lytic bacteriophages isolated from local sewage water samples were effective against MDR *P. aeruginosa* both *in vitro* and *in vivo* (animal model). Further studies will be needed to determine if lytic bacteriophage therapy can be used against MDR *P. aeruginosa* and other pathogenic bacteria.

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