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PHAGE-ANTIBIOTIC SYNERGISM AGAINST *PSEUDOMONAS AERUGINOSA* ISOLATED FROM DIABETIC WOUNDS

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Abstract

Diabetes mellitus is one of the most alarming diseases around the globe as well as in Pakistan.Diabetes is often associated with many other health complications among which few are notable,such as non-healing or delayed wounds. This is associated with the bacterial infection caused mostprominently by *Pseudomonas aeruginosa* which is a drug-resistant bacterial strain. In order to control or kill the bacterial strains, the synergism of antibiotics and bacteriophage is widely preferred as an effective strategy. It is believed that the combination ofantibiotics and phages is more effective than using individual drugs. Therefore, the current studywas aimed to investigate the synergistic impact of synergism of antibiotics and bacteriophages against *P. aeruginosa* isolates of diabetic patients. Samples were sourced from the wardrooms of Allied Hospital, Faisalabad. First, isolation and purification of *P. aeruginosa* was carried out. Then, the biochemical identification was performed. For bacteriophage isolation, samples were collected from sewage and were isolated using the double agar overlay method. Synergism was measured by using phages (1×10^6 PFU/ml) and different concentrations of antibiotics against *P. aeruginosa*. The results showed that the phage exhibited synergistic effects with both antibiotics.Upon conducting a comparison of optical density (OD) values; it was observed that the synergistic treatments exhibited a higher rate of bacterial killing. A higher rate of killing was observed in the phage and meropenem combination as compared to phage alone and ciprofloxacine and phage combination. PAS therapy therefore presents a new window and reinforces the view that it can act as an alternative treatment option for MDR*P. aeroginosa* infections in diabetic patients.

Keywords: Antibiotic, bacteria, diabetes, bacteriophages, wound, *Pseudomonas aeruginosa*

Introduction

In recent decades, the impact of diabetes has increased to an unprecedented level as the patients affected with diabetes mellitus are increasing day by day. It is now considered an intricate and seriously harmful disease as it leads to many further clinical complications(Otta S*et al*., 2109). During the early years of the previous decade, the spread of diabetes accounted for about 439 million people around the globe leading to a significant surge in mortality rates worldwide (Olokoba *et al.*, 2012). However, towards the end of the previous decade, the diabetic cases rose to a surprising level of 463 million adults alone in 2019 which depicted a prevalence level of 9% throughout the world (Sun *et al.*, 2022). According to American Diabetic Association, Diabetes mellitus (DM) is an illness caused by metabolic disarray. It is divided into two basic forms Type and Type II diabetes. Type I DM is caused by immunological insufficiency in pancreas leading to inadequate insulin production. Type II DM occurs when the body becomes insulin defiant or it does not react to the insulin formed (Uma and Sudarsanam, 2012). Major problem in diabetic patients is the non-healing or delayed healing of infections especially the foot wound often described as diabetic foot ulcer which in most cases leads to inevitable organ amputation and a large number of patient deaths (Wolcott *et al.*, 2010). Life time threat of a patient for foot ulcer might be up to 25% more in diabetic patients than in non-diabetic (Fincke *et al.*, 2010).

Pathogenic bacteria are the frequent cause of ulcers due to diabetes and their infection is provided by immunological deficits correlated to diabetes (Khan *et al.*, 2021). Different microorganisms colonize in the wounds of patients and sometimes more than one species of organisms proliferate to the wounds and may cause damage to the tissues, host response and inflammation in case of clinical infections (Lipsky *et al.*, 2012). Bacterial pathogens isolated from diabetic infections are Gram negative organisms such as *P. aeruginosa*, *Escherichia coli* and *Proteus* species and Gram positive organisms such as *Staphylococcus aureus, Staphylococcus epidermidis* (Zubair *et al.*, 2010).

This problem is often associated with bacterial pathogens among which *P. aeruginosa* are more dominant which is a gram-negative bacterium. These are motile (have a flagellum), non-sporulating and are involved in non-fermenting reactions (Nouraldin *et al.*, 2016). It is regarded as a highly opportunistic pathogen believed to be the cause of a large number of diseases and infections and its presence in diabetic patients mostly results in morbidity (Ertugrul *et al.*, 2012). These bacteria form a biofilm which serves an aggregation of microbial cells utilized as the inherent defense mechanism against antibiotic drugs and antimicrobial agents. These are among the most antibiotic-resistant pathogens isolated from diabetic patients and are continuously evolving in terms of resistance against many antibiotic drugs. Recent studies to perform the surveillance of *P. aeruginosa* have shown that they are major contributors to the increasing resistance against antibiotic drugs(Tacconelli *et al.*, 2018).

The extensive use of antibiotics is a major cause of developing resistance in *P. aeruginosa*. This is due to the over administration, self-medication, random prescription of inappropriate drugs, and extended use of antibiotics. So, phages were introduced as an alternate to antibiotics (Shahi and Kumar, 2016). However, studies have shown that there are a number of bacteriophages that have shown effectiveness in killing or controlling bacteria and biofilms. Bacteriophages are the smallest viruses present in nature. Bacteriophages can be lytic or lysogenic depending upon their activity inside the host cells. Lytic phages are virulent and are more effective in killing bacterial biofilms (Essoh *et al.*, 2015).

In order to control these drug-resistant pathogens and the wound infections caused by these pathogens, synergism of drugs and bacteriophages has been reported to be an effective clinical strategy in which two or more antibiotics and bacteriophages are used in a combination to generate an effect that is not a simple cumulative of the individual drugs or bacteriophages. It enhances the drug delivery to particular cells and increases the local drug concentrations due to the activity of bacteriophages. This strategy has been around for a while now as the past studies show the use of

rifampicin in combination with imipenem against a number of antibiotic-resistant wounds and infections(Tascini *et al.*, 2006). In more recent times, Ceftolaozane in combination with Tazobactam and bacteriophages has been employed to evaluate their synergistic impact to control the drug-resistant pseudomonas responsible for wound infection (Dietl *et al.*, 2018). Moreover, use of antibiotics and bacteriophages has also been utilized in a combination to eliminate bacterial biofilms (Nouraldin *et al.*, 2016).

Materials and Methods

Ethical approval

Sampling from humans needs ethical approval that was obtained from Institutional Biosafety Committee.

Sample Collection

A total of 25 pus samples from the wound of the diabetic patients were collected from Allied Hospital. Different sewerage water samples were also collected from sewerage systems of related Hospital.Collected samples of both pus and sewerage water were brought to Institute of Microbiology, University of Agriculture Faisalabad for conducting the study.

Isolation of *Pseudomonas aeruginosa*

Swab samples collected from diabetic patients werecultured first on a selective agar, Cetrimide agar and MacConkey agar; then, suspected colonies were sub cultured on Blood agar. All the inoculated plates were incubated at 37°C for 18-24 hours. Isolates were identified on colonial characteristics on these media (Banerjee *et al.*, 2017).

Identification and Biochemical characterization of *Pseudomonas aeruginosa*

Further identification were done by their Gram staining reaction and biochemical tests such as oxidase test, catalase test, citrate test, coagulase test, indole test, methyl red (MR) test, triple sugar iron test and Voges Proskauer test (VP)(Pal *et al.*, 2010).

Antimicrobial susceptibility test

To check antibiotic resistance against certain antibiotics, a disc diffusion method wasused. The test was performed by using commercially prepared discs and Muller-Hinton agar in accordance tounder the guidelines given by CLSI (clinical laboratory standards institute). Briefly, pure bacterial colonies were suspended in 0.85% Saline solution with turbidity adjusted to the 0.5 McFarland standards. Each inoculum was be spread as uniform lawn over the dried surface of 100 mm MH agar plates, this was followed by the placement of a maximum of six antimicrobial discs, and incubation at 37ºC for 24 h (Andrade *et al.,* 2023). Bacterial growth around each disc (i.e., zone of inhibition diameters) were then measured and recorded. In this study antibiotic susceptibility of Meropenem (10µg), Imepenem (10µg), Cholestin (10µg), Ampicillin (10µg), Sulphamethoxazole (100µg), Chloramphenicol (30µg), Tazobactam (110µg), Ciprofloxacin (5µg), Gentamicin (10µg) and Amikacin (30µg) were tested.

Bacteriophage isolation and Enrichment

Sewerage water samples were collected from different hospitals of Faisalabad division for isolation of bacteriophage against *P. aeruginosa*. After collection sewage water was centrifuged at 12000rpm for 10 minutes. Then supernatant was collected and filtered through 0.22 micron syringe filter. Then for enrichment of bacteriophage, 0.6ml of filtered supernatant, 0.3ml of fresh bacterial culture and 1ml of nutrient broth were mixed in 1.5ml of Eppendorf tube and then incubated for 24 hours.After incubation, again centrifugation of above mixture was performed at 8000 rpm for 10 minutes. Separate the supernatant and again filter through syringe filter paper of size 0.22-micron(Alharbi *et al.*, 2023). This lysate was checked for bacteriophage through spot assay.

Spot test

This technique is performed to evaluate whether bacteriophages are present in phage lysate or not. For this purpose, we poured 5 ul of phage lysate on agar plate having lawn of bacterial growth. Incubated it at 37ºC for 24 hours (Jensen *et al.*, 2015). After incubation clear zones were present which showed presence of bacteriophages.

Purification of Bacteriophage

Double agar overlay method was used for the purification of isolated bacteriophages (Kropinski *et al.*, 2009). Soft agar media was prepared firstly by adding 0.8 grams soft agar powder in 100ml of distilled water. For support to bacteriophages, we added salts like MgSo4 and $CaCl_2$ in above solution. Placed them in autoclave at a temperature of 121°C for 15-20 minutes. Then place the molten soft agar inthe water bath at 45°C. After that, 1.5ml of phage lysate was mixed with 1.5ml of *P. aeruginosa* culture. Incubated the mixture at 37°C for 1 hour so that adsorption of phages with bacteria occurred. After that, mix 3ml of soft agar with this mixture. Gently swirled the mixture and poured it on the solidified Petri-plates of nutrient Agar. The plates were incubated the plates for 24 hours at 37°C. Petri-plates were examined for plaque formation.

Bacteriophage Stock Preparation

The phage buffer was prepared to preserve and store the isolated phages against *P. aeruginosa.* After the isolation of phages, plaques were removed by using sterile needle. Placed it in an Eppendorf tube with 100µl SM buffer and 10µl chloroform. Then, phage was stored at 4°C (Yuan *et al.*, 2019).

Minimum Inhibitory Concentration

MIC values of antibiotics (Meropenem and Ciprofloxacin) were determined by microdilution method (Lutz et al., 2012). 100µl of sterile water was added in up to 10^{th} well. Then 100µl of 10% gentamicin was added in 1^{st} well and was mixed properly. After that, 100µl solution from 1^{st} well was added in second well and procedure was repeat until 10^{th} well (two fold dilution). Same procedure was followed for the chloramphenicol in another row of microtitration plate. Plate was covered and incubated for 24 hours. After required incubation, OD were checked at 600 nm by spectrophotometer.

Phage-antibiotic synergism against *P. aeruginosa*

Phage antibiotic synergism was evaluated by microtitration plate method (Jo *et al.*, 2016). 96 well microtitration plate was used.Each antibiotic (gentamicin and chloramphenicol) was serially diluted (1:2) and the phages were serially diluted (1:10) from 10^6 PFU/ml. 90ul of broth was added in all selected wells of micro titration plate. Took first as positive control by adding 100ul of broth and 10ul of bacterial culture. In 2^{nd} row added 100ul of phage and 10ul of bacteria. In the 3rd row 100ul of meropenem (10%) (A1) and 10ul of bacteria was added.

In row 4^{th} added 100ul of ciprofloxacin (10%) (A2) and 10ul of bacterial culture. In 5^{th} row 50 ul of phage and 50 ul of meropenem (A1) with 10 ul of bacteria were added. In row 6^{th}added 50ul of phage and 50 ul of ciprofloxacin (A2) with 10ul of bacteria. In 7^{th} row only added 110ul of broth so that it will be 200 (negative control).Incubated the plate at 37°C for 24hrs.After 24 of incubation, OD was checked at 600nm by spectrophotometer.Results were presented as a percentage decrease in the optical density of treated wells in comparison to control wells (positive controls), where only bacteria were introduced, for the effectiveness of action of phage, antibiotic, and phage plus antibiotic.

Statistical analysis

The statistical analysis for the effectiveness of phage antibiotic synergism were determined by applying one way ANOVA by comparing values of optical densities. The significance of the test was determined at $p \leq 0.05$.

RESULTS

Prevalence of *P. aeruginosa* in collecting samples

When the samples were cultured on selective media out of 25 samples 10 (40%) were positive for *P. aeruginosa* on the basis of colony characteristics. While rest of 15 (60%) were other bacteria (Fig. 1).

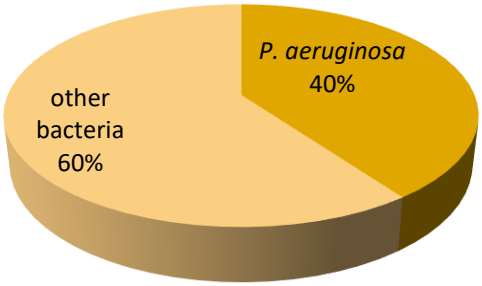

Fig.1 Prevalence of *P. aeruginosa*

Isolation *P. aeruginosa*

P. aeruginosa was formed blue green fluorescence colonies on the cetrimide agar (Fig.2a). *P. aeruginosa* was formed smooth, flat and colorless colonies.(Fig.2b). *P. aeruginosa* was formed mucoid-type colonies on blood agar (Fig.2c).

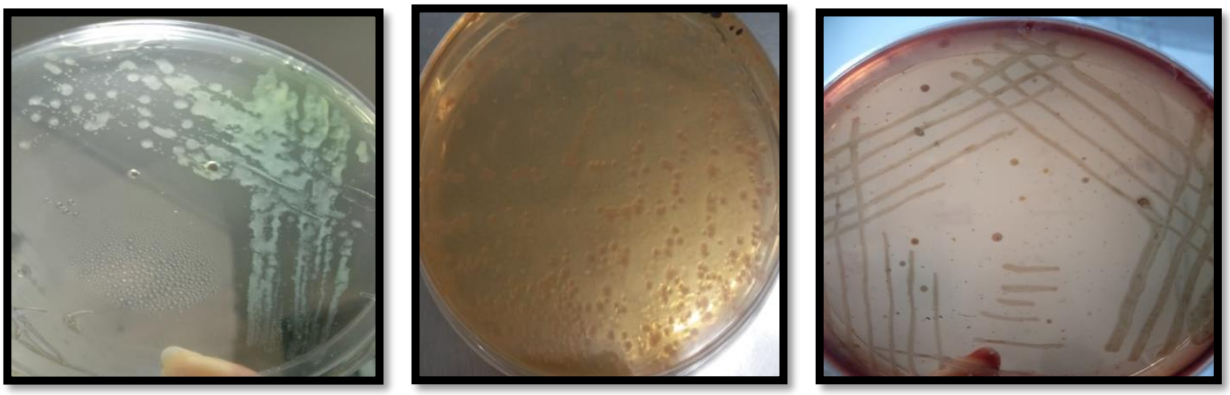

Fig.2a *P. aeruginosa* on Cetrimide AgarFig.2b *P. aeruginosa* on MacConkey agar Fig.2c *P. aeruginosa* on Blood Agar

Microscopic identification

On Gram staining *P. aeruginosa* had appeared as pink colored Gram-negative short rods (coccobacilli) (Fig.3).

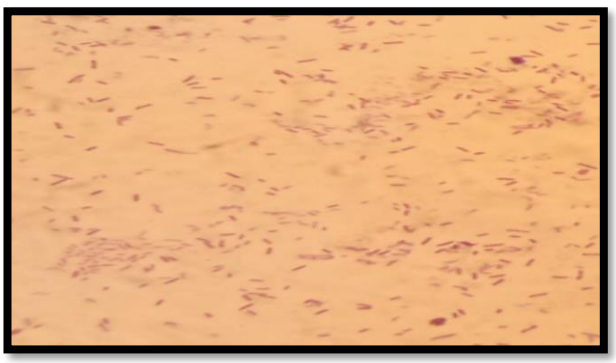

Fig.3*P. aeruginosa* under microscope

Biochemical Identification

Different biochemical tests were performed to confirm *P. aeruginosa* from collected samples.

Table 1. Biochemical tests

Biochemical test for *P. aeruginosa*	**Results**
Catalase test	Positive
Citrate test	Positive
Methyl red test	Negative
Triple sugar iron test	Negative
Voges Proskaur test	Negative
Indole test	Negative

Antibiotic susceptibility test

Antimicrobial susceptibility tests have been performed to determine the susceptibility pattern of *P. aeruginosa* to various groups of commonly used antibiotics. In current study antibiotic susceptibility of Meropenem, Imepenem, Cholestin, Ampicillin, Sulphamethoxazole, Chloramphenicol, Tazobactam, Ciprofloxacin, Gentamicin and Amikacin were checked (Fig.4).

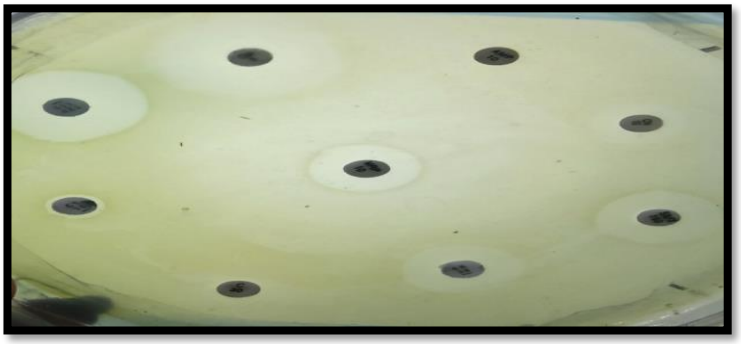

Fig 4. Antibiotics Sensitivity pattern of *P. aeruginosa*

Antibiotic susceptibility test was performed to evaluate the resistance of *P. aeruginosa* to different drugs. The high number of *P. aeruginosa* isolates showed of resistance to Chloramphenicol (90%), Cholisetin (85%), Gentamicin (80%), Ampicillin (80%), Sulfamethoxazole (75%), Amikacin (70%), Tazobactam (70%) and Ciprofloxacin (65%) respectively. While intermediate number showed resistance to Imipenem (60%). P. aeruginosa isolates were only susceptible to Meropenem (60%) that can be used as drug of choice against *P. aeruginosa* infection (Fig.5).

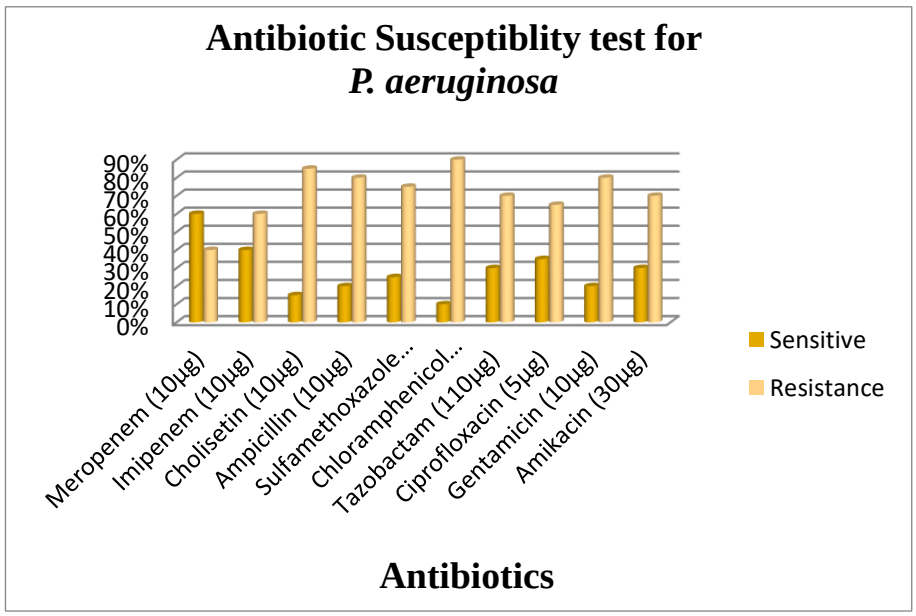

Fig.5 Percentages of sensitive and resistance strain of MDR *P. aeruginosa* to avalible antibiotics

Bacteriophage isolation and Purification

The lytic phages have been isolated against *P.aeroginosa*by using double-agar- overlay method. Clear plaques were observed (Fig.6).

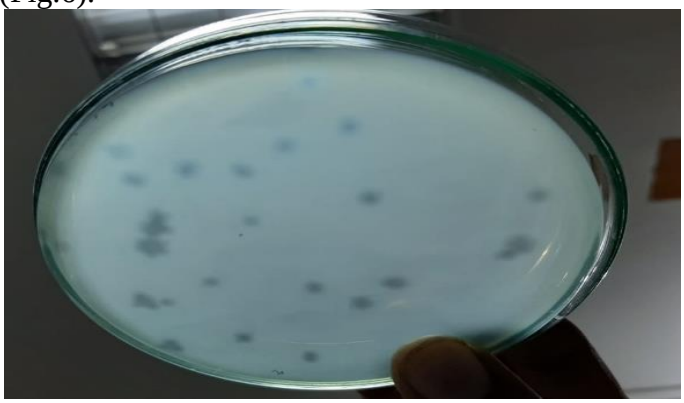

Figure.6 phage plaques showing lytic activity

Minimum inhibitory concentration

MIC of Meropenem and ciprofloxacin were determined using microdilution method. *P.aeroginosa* isolates were uniformly susceptible to Meropenem and Ciprofloxacin with MIC 4.5µg/ml and 0.5µg/ml respectively.

Phage Antibiotic Synergism

For the comparison of the activity of phage and antibiotic against *P. aeroginosa* was measured by micro titration plate method. Activity of Meropenem and phage separately was compared with the activity of phage and Meropenem in combination. Same ciprofloxacin and phage activity was compared with activity of phage and ciprofloxacin in combination. OD values were measured with spectrophotometer.

Table 2: ANOVA table of Phage, meropenem, ciprofloxacin and their synergistic treatments:

Parameters	Positive control	Phage	Meropenem (A1)	Ciprofloxacin (A2)	Phage +A1	Phage +A2	P-value
OD	1.13 ± 0.24^{a}	0.28 ± 0.1^{bc}	0.62 ± 0.16^{abc}	0.80 ± 0.22^{ab}	0.08 ± 0.01^{c}	0.15 ± 0.06^{bc}	0.003

The results showed that the optical density (OD) of the positive control and meropenem is higher ($P\leq0.05$) than the OD of the phage plus meropenem and phage plus ciprofloxacin groups. However, phage OD was also higher than phage plus meropenem and phage plus ciprofloxacin groups. The mean values of all treatment groups vary significantly from each other depicting a strong importance of this experiment with maximal results in synergistic therapy using antibiotic and phage combination.

Mean Comparison(a)

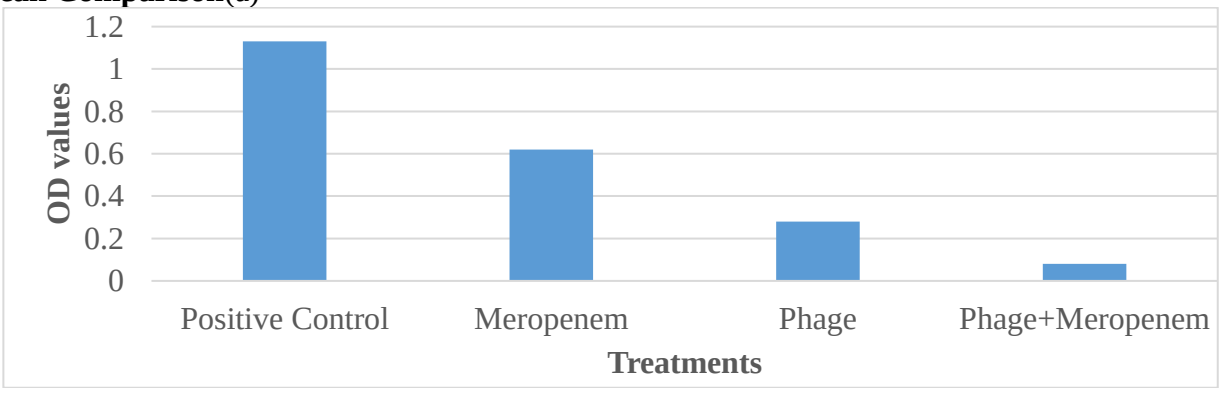

Fig. 7a Graphical presentation of mean comparison of OD values of different treatments. The results showed that treating bacterial cultures with a combination of both phage and antibiotic (right panel)

had greater inhibitory effect than treating bacteria alone with antibiotic (left panel) or phage (middle panel)

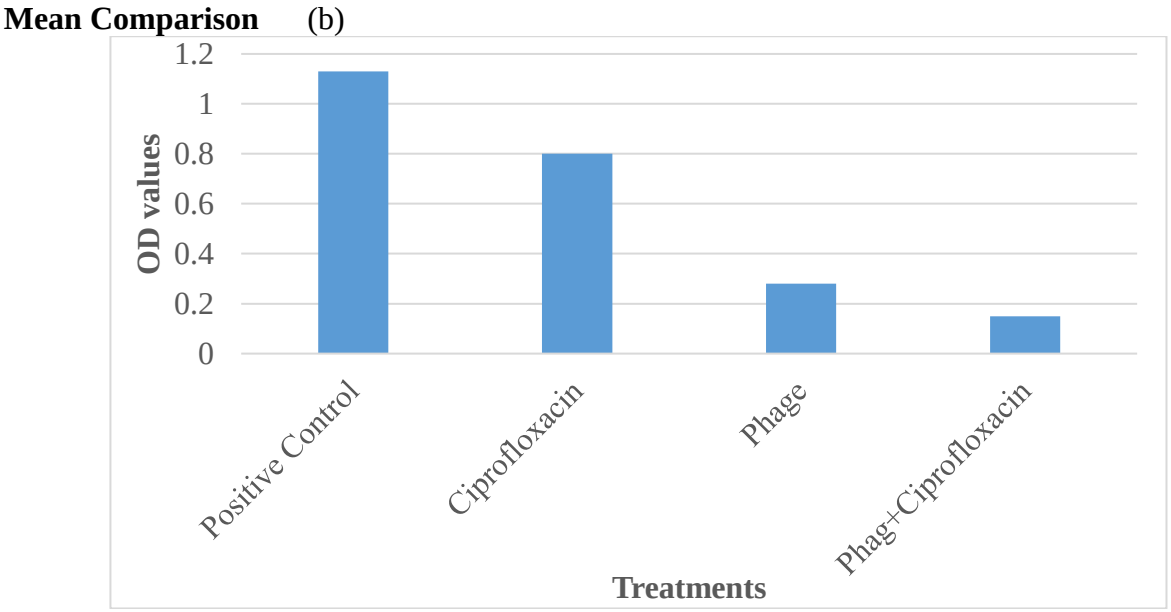

Fig.7b Graphical presentation of mean comparison of OD values of different treatments. The results showed that treating bacterial cultures with a combination of both phage and antibiotic (right panel) had greater inhibitory effect than treating bacteria alone with antibiotic (left panel) or phage (middle panel)

Statistical analysis based on comparison of mean values of optical densities of control well versus treatment well. Antibiotics (Meropenem and Ciprofloxacin), phage alone and their combination has revealed an overall significant ($p \leq 0.03$) difference in optical density values relationship among treatmentgroups. *P.aeroginosa* in microtitration plate was treated with antibiotics, Meropenem and Ciprofloxacin separately and optical density was observed under spectrophotometer at 600nm and showed higher values of OD 0.62 (Meropenem) and 0.80 (Ciprofloxacin) respectively. While treatment of phage alone showed lesser value of OD (0.28) against bacteria than antibotics. Meropenem and phage showed a high synergistic effect with each other after combined treatment and showed a high reduction in OD value (0.08). Also combined treatment of Ciprofloxacin and phage also showed synergistic effect with each other and OD value recorded was (0.15) which means bacterial load reduced even more after combined treatment of phage and antibiotics. The mean values of all treatment groups vary significantly from each other depicting the strong importance of this experiment with maximal results in synergistic therapy using antibiotic and phage combination (Fig.7).

Discussion

Diabetes mellitus is a major health issue of the whole world. According to a projection, 80 million of the population will be affected by diabetes. Contribution of Asia to diabetes is > 60% of world's population out of which India and China contribute the largest part (Shaw *et al.*, 2010).

P. aeruginosa is a motile, Gram-negative, oxygen utilizing bacillus, non- spore forming. Biofilm produces mono-flagellated bacterium which has pearlescent appearance and grape like odor. Its aptitude to grow at 42°C differentiates it from other Pseudomonas species. *Pseudomonas aeruginosa* is the most widespread reason of hospital-acquired infections. Due to biofilm production, delay in abrasion curing and confrontation to antimicrobial treatment is becoming a major problem by imposing a great burden on the public health sector.Chronic wound infections, including diabetic foot, leg, pressure, surgical site, and trauma ulcers, can result from bacterial biofilm formation (Rahim *et al.*, 2017).

In current research, 25 patients were selected for sampling from the General ward of Allied Hospital, Faisalabad from the patients related to diabetic wounds. Samples were processed by direct swabbing to nutrient broth, on which green turbidity was produced. Then, all the specimens were sub-cultured on the MacConkey's agar and Cetrimide agar on which colorless colonies were produced as *P. aeruginosa* is a non-lactose fermenter and green colored, pigmented colonies were produced respectively. The10 samples were found positive for *P. aeruginosa* by growing on cetrimide agar and microscopy at 100X. The positive samples were then preceded by Gram's staining and biochemical identification in which catalase, oxidase and citrate test which all were positive for *P. aeruginosa*.

The extensive use of antibiotics is a major cause of developing resistance in *P. aeruginosa.* This is seen due to the over administration, prolonged use of antibiotics, self-medication and random prescription of improper drugs(Shahi and Kumar, 2016). Inherent resistance mechanism makes this microorganism less agreeable to manage by drug cycling. A combination of resistance mechanisms, which includes multi-drug efflux pumps, C lactamase and aminoglycoside modifying enzymes and target size modifications are responsible for the MDR in *P. aeruginosa*, resulting in weakening ability of *P. aeruginosa* against conventional antibiotic treatments and an increasing interest towards lytic phage therapy (Tenover, 2006).So, it is needed to develop new curative and prophylactic measures to control of bacterial infections in diabetic wound patients. In this study, antibiotic sensitivity of different antibiotics were checked against *P. aeroginosa* isolates. The isolates of *P. aeruginosa* were found susceptible only to Meropenem.

Phage therapy was one of the best possible therapeutic treatment and given a major concern during the late 1980s(Brüssow, 2005). In the middle of 20 century, bacteriophages specific to Pseudomonas were isolated. *P. aeruginosa* is solitary reason of hospital acquired infections and given great concern for bacteriophage utilization and genome sequencing projects related to the organism (Abedon *et al.*, 2011). Bacteriophages are the viruses that only infect bacteria but cannot infect eukaryotic cells. The particular characteristics which distinguish phage therapy from antibiotic therapy are: only target to specific bacteria without effecting to normal microflora of the body, proliferate particularly to infection site and they can adjust to resistant bacteria(Debarbieux *et al.*, 2010).

Hospital sewage samples were used for isolation of bacteriophages in this research work. Bacteriophages obtained in this research were lytic. The formation of clear region of lysis against host bacteria using particular bacteriophage lysate indicated that all the isolated bacteriophages were lytic.

The combined use of phage with a certain antibiotic has shown increased antimicrobial effects over the single use of phage or antibiotics (Chhibber *et al.*, 2013). The combined use of phage
with sublethal concentrations of antibiotics has been shown to synergistically increase antibacterial effects so called phage–antibiotic synergy (PAS) and has been shown effective against bacteria including *Klebsiella pneumoniae*, *Staphylococcus aureus*, *P. aeruginosa*, *Escherichia coli*, and *Burkholderiacepacia* complex(Knezevic *et al.*, 2013). In addition, the combined use of different antimicrobial agents such as phage and antibiotics is expected to decrease the chances of resistant bacteria emerging(Ventola, 2015; Khan *et al*., 2017).

In the present study, Phage antibiotic synergism was screened against *P. aeruginosa* using microtitration method. Phages were used in combination withMeropenem and Ciprofloxacin. Phages showed synergistic effect with both antibiotics resulting in more killing of bacteria. The highest amount of killing was observed with Meropenem in combination.

Conclusion

Phage-antibiotic synergism offers a possible opportunity for developing treatment strategies for infections caused by *P. aeruginosa* in the future using corresponding antibiotics and phages which meet the prerequisites for therapeutic application. Since emergence of resistant strains has already been reported therefore, combination of two agents will always help in decreasing the development of resistant mutants. Hence, co-therapy using phage and antibiotics (Meropenem and ciprofloxacin) can take care of the critical problem of resistance in modern medicine. PAS therapy therefore presents

a new window and reinforces the view that it can act as an alternative treatment option for MDR *P. aeruginosa* infections in diabetic patients.

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