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EVALUATION OF PHAGE ANTIBIOTIC SYNERGISM AGAINST *STAPHYLOCOCCUS AUREUS* ISOLATED FROM THE WOUNDS OF DIABETIC PATIENTS

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that can thrive under both aerobic and anaerobic conditions. It is spherical in shape and forms clusters resembling grapes. On agar media, it produces colonies with a distinctive golden or yellow color. *S. aureus* has the ability to tolerate high concentrations of salt. It yielded positive results for catalase, coagulase and mannitol fermentation tests. This pathogenic bacterium is the cause of infection in hospital and community settings. In immunosuppressed individuals, such as diabetics, foot ulcers are a common complication, affecting approximately 15% of diabetics. These ulcers create an environment that promotes microbial colonization and growth, of which *S. aureus* is an important colonizer. In addition, *S. aureus* has developed resistance to a variety of antibiotics. In this study, we collected 50 samples from different hospitals in Faisalabad. These samples were cultured on a specific agar medium to isolate and purify *S. aureus*. To confirm the presence of this bacterium, biochemical tests were performed. Phages specifically targeting *S. aureus* were isolated using the double agar overlay method. We assessed the synergistic effect of phage (10^6 PFU/ml) and antibiotic against *S. aureus* by measuring the optical density (OD) of bacterial cultures before and after treatment. Analyze the difference in OD values to assess the effect of the treatments. The main purpose of this study was to explore the potential of phages as an alternative therapy and to investigate their synergistic effects when combined with antibiotics in the treatment of *S. aureus*. Our findings suggest that the

combination of phages and antibiotics holds promise for the development of phage-based approaches to control bacterial infections.

Key Words: *S. aureus,* Catalase, Mannitol Salt Agar, Blood Agar, Bacteriophages, Antibiotics, Double Agar Overlay.

INTRODUCTION

Wound infection is most prevalent among the *Diabetes mellitus* patients due to their weak immune system. Oxygen deprivation and inflammatory state develop in diabetic wounds. A diabetic wound is the complicated consequence of neuropathy and antipathy causes the feet to go numb and the wound to remain untreated (Singh, Armstrong, & Lipsky, 2005). It is estimated that 25% of diabetic patients suffer from wound problems during their lifetime (Kavitha et al., 2014). In 2021, the prevalence was predicted to be higher in urban areas (12.1%) as compared to rural areas (8.3%) and high in lucrative countries (11.1%) as compared to impoverished countries (5.5%). Diabetes patients had a 4.75% colonization rate of Methicillin-resistant *Staphylococcus aureus* (MRSA) than non-diabetic patients (Sun et al., 2022).

Colonization and proliferation of microbes occur in open wounds that can spread to deeper tissues and cause severe infections (Richard, Lavigne, & Sotto, 2012). Microorganisms colonize diabetic foot ulcers either individually or by forming a community (Zubair, Malik, & Ahmad, 2011). The major colonizer of a diabetic foot ulcer is *S. aureus*. It is a cocci-shaped bacterium organized in a cluster form. Golden (*aureus* means golden) or yellow colonies formed on media and these organisms can tolerate up to 10% salt. Virulence factors enhance wound adherence, persistence, and infection. There is a wide variety of toxins and enzymes prompted by *S. aureus* such as hyaluronidases, nuclease, lipases, protease, collagenase, and hemolysins (Boulton, Vileikyte, Ragnarson-Tennvall, & Apelqvist, 2005).

MRSA has a Staphylococcal chromosomal cassette *mec* (*SCCmec*) region which contains the *mecA* gene. This gene is responsible for antibiotic resistance (Rasigade & Vandenesch, 2014). These pathogenic strains have been found resistant to cephalosporin, monobactams, carbapenem groups, and the beta-lactam groups, including the penicillinase-resistant penicillin (dicloxacillin, cloxacillin, nepheline, methicillin, and oxacillin) antibiotics. Quinolones are broad-spectrum antibiotics, used in the therapy of infections acquired from hospitals. In hospitals, a subclass of quinolones, fluoroquinolones, are usually used against MRSA. However, *S. aureus* also develops resistance against fluoroquinolones, and its application is not encouraged (Shahi & Kumar, 2016).

Many burn wound pathogens develop antibacterial resistance throughout the world, especially nosocomial samples. The enterococci, MRSA, and extended-spectrum β-lactamases that have resistance to vancomycin are evolving as a threat in patients because these can be transferred from one patient to another. Therefore, resistant organisms caused an explosive and extended outbreak of infections (Rathod & Kasturi, 2017). Recently, the rise of antibiotic-resistant superbugs has highlighted the need for innovative methods in the fight against infectious diseases. Large-specific infectious antibiotic-resistant organisms have become an important hazard to public health. The WHO advisory committee promoted new methods of treatment instead of antibiotic treatment (Pallavali, Degati, Lomada, Reddy, & Durbaka, 2017).

Bacteriophages have the power to kill the bacteria at the last stage of the infectious cycle. Phages were known for their auspicious ability to eradicate or decrease the liable organisms that cause disease in a definite atmosphere (Rastogi et al., 2018). Bacterial parasites can steal all the information needed to reproduce in a suitable host from its genome (Kasman & Porter, 2022). Bacteriophages have been divided into two groups (lytic and lysogenic) based on the replication cycle. Lytic bacteriophage takes over the machinery of the host, replicates within it, destroys the bacterial cell, and then releases the progeny. Lytic bacteriophages use the holing-lysin system to kill bacteria. Holin protein makes holes in the cytoplasmic membrane and lysin, also called amurin

disturbs the peptidoglycan synthesis. Lysogenic phages integrate their genome into the host genome (Seed, 2015).

Phage treatment is effective against MDR bacteria, so it is an appropriate method in place of antibiotic therapy. Bacteriophage sources are hospitals and sewage waste (Haq, Chaudhry, Akhtar, Andleeb, & Qadri, 2012). Phage applications are safe because they are environmentally friendly and cost-effective, and no human phage infection has been reported to date. Thus, bacteriophages have high specificity, high safety, and low dosage and antibiofilm activity advantages over antibiotics (Esmael et al., 2021).

Although phage treatment appeared as the most suitable process to combat infection, this strategy also has some problems. The major dilemma regarding phage therapy is the phage resistance (Rahmani, Zarrini, Sheikhzadeh, & Aghamohammadzadeh, 2015). For protection against attacking viruses, bacteria have developed plenty of safeguard mechanisms. Two of them are R-M and CRISPR systems. Restriction modification (R-M system) is a process in which invading phage DNA is attacked by specific restriction endonucleases before methylation of foreign DNA occurs. Methylation of DNA protects it from endonucleases. As methylation does not occur, endonucleases cut the foreign DNA at specific restriction sites while CRISPR is a very familiar process in phage resistance. In this process, CRISPR proteins are guided by RNA and cleave the invading phage DNA (Dupuis, Villion, Magadán, & Moineau, 2013).

In recent times, innovative methods have been suggested to conquer resistance by bacteria. Scientists have suggested that instead of using phages in place of antibiotics or using them both independently; a phage-antibiotic combination may be more effective. The phage-antibiotic combination has advantages such as reduced bacterial load, reduced resistance against phage or antibiotic, and stronger effective penetration into biofilms. Numerous investigational models, comprising plaque technique, liquid plankton, animal experiments, and biofilm assessments, have been performed to effectively evaluate the phage-antibiotic synergism. Remarkably, the alternate outcome of host advancement enlightens the synergistically antibacterial effect of phage and antibiotics (Diallo & Dublanchet, 2022). This research aims to isolate *S. aureus* from the wounds of diabetic patients and to isolate bacteriophages against *S. aureus* from sewage water sources. Furthermore, we evaluated the synergistic effect between antibiotics and phages for treating MDR *S. aureus*.

MATERIALS AND METHODS

Ethical Approval

The study was conducted after the ethical approval provided by the International Biosafety Committee (IBC) of the University of Agriculture Faisalabad. The study was approved under permit number 3296. The sampling in the study was done according to patient consent.

Sample collection and transportation

Fifty samples were collected from the wounds of diabetic patients from Allied Hospital Faisalabad, Pakistan. The sample collection was done from wounds through Amies transport swabs as previously described by (Church, Elsayed, Reid, Winston, & Lindsay, 2006). After sample collection, the swab was put into the tube and sealed properly to avoid contamination. Each sample was labeled and transported to the Bacteriophage Laboratory, Institute of Microbiology at the University of Agriculture, Faisalabad.

Isolation of bacteria

The collected samples were streaked on prepared sterile nutrient agar (Staph-110 agar, and Mannitol Salt agar) in a biosafety cabinet. Each plate was marked according to the sample number and incubated for 24 hours at 37℃. After 24 hours of incubation, the inoculum was picked from the plates and streaked on the blood agar for the evaluation of hemolytic properties (Tong, Davis, Eichenberger, Holland, & Fowler Jr, 2015).

Identification of bacteria

The morphological characterization of *S. aureus* was done using Gram staining and the morphology was observed under a light microscope (Gómez Inca & Véliz García, 2023). The biochemical characterization was performed for further identification of *S. aureus*. Catalase, Coagulase, Oxidase, Citrate utilization, Methyl Red, Voges-Proskauer, Indole, Urease, and Triple Sugar iron tests were performed (Vashist, Sharma, & Gupta, 2013).

Antibiotic Susceptibility Test

The antimicrobial profile of *S. aureus* isolates was determined using the Kirby-Bauer disc diffusion assay, following the protocol described by (Kebede, Kemal, Alemayehu, & Habte Mariam, 2016). A variety of antibiotics were used for the susceptibility test, including Oxacillin (OX) 5µg, Vancomycin (VA) 5µg, Gentamycin (Sun et al.) 10µg, Erythromycin (E) 30µg, Cefoxitin (FOX) 30µg, Ampicillin (Wang, Tkhilaishvili, & Trampuz) 10µg, Ciprofloxacin (CIP) 5µg, Sulfamethoxazole (SMX) 30µg, Tetracycline (TE) 30µg, Chloramphenicol (C) 30µg, Amikacin (AK) 30µg, Ceftazidime (CAZ) 30µg, Streptomycin (STR) 10µg and Penicillin G (PG) 30µg. To get turbidity equal to 0.5 (1.5×10^8 CFU/mL) according to McFarland standards, the *S. aureus* colonies were inoculated into peptone water and incubated at 37 ℃. A sterile cotton swab was dipped into the prepared inoculum. Excess fluid from the swab was removed by pressing it around the edges of the tube. The bacteria were streaked evenly over the MH agar. The above-mentioned antibiotics were then applied to the surface of the agar, and the plates were incubated for 24 hours at 37 ℃. After 24 hours, the zone of inhibition was measured to determine antibiotic sensitivity according to the guidelines provided by CLSI.

Screening of MRSA Strains

The oxacillin agar test has been used for the screening of MRSA strains. The reason is that methicillin is no longer easily available because of widespread resistance. An Oxacillin agar screen test was performed for the detection of MRSA according to the protocol described by (Perez, Dias, & d'Azevedo, 2008). Muller Hilton agar weighed 38g and was mixed in 1000mL of water in a conical flask. 4% NaCl and 6µg/mL oxacillin were added into the media. Then mix the solution properly. The media was autoclaved at standard temperature and pressure. After autoclaving, the media was kept still to cool down. When the media reached to temperature of 45-50℃, it was poured into sterile petri plates and allowed to solidify. To get turbidity equal to 0.5 (1.5×10^8 CFU/mL) according to McFarland standards, the *S. aureus* colonies were inoculated into peptone water and incubated at 37 ℃. A sterile cotton swab was dipped into the prepared inoculum. Excess fluid from the swab was removed by pressing it around the edges of the tube. The bacteria were streaked evenly over the MH agar. The plates were incubated overnight. The result was interpreted after 24 hours.

Bacteriophage Isolation

Total 30 samples of sewage water were collected from different sewage systems of Faisalabad, Pakistan. The sewage water samples were collected in 40ml falcon tubes. The samples were transported to the Bacteriophage Laboratory, Institute of Microbiology at the University of Agriculture, Faisalabad for phage isolation against *S. aureus*. A 0.22µm syringe filter was used to filter the supernatant after the samples had first been centrifuged at 6000 rpm for 12 minutes. Subsequently, 900µL of broth was mixed with 300µL of bacterial culture and 800µL filtrate in a 2mL Eppendorf tube. The incubation was done in a shaking incubator at 37 ℃ for 24 hours (Sangha, Kumar, Agrawal, Deka, & Verma, 2014). Repeated centrifugation and filtration were carried out to obtain enriched bacteriophage lysate and processed phage lysate to determine the presence of phages.

Spot Assay

The spot test was designed to examine for the presence of the bacteriophage in the lysate. Following incubation, the presence of the bacteriophage was indicated by the formation of clear zones on the agar (Jensen et al., 2015). Staph-110 agar plates were prepared by pouring 10mL of media into each plate and allowing it to solidify. Then, a 100µL sample of a 48-hour-old bacterial culture was poured onto the Staph-110 agar plates and evenly spread with a spreader. The plates were left on the desk to harden. Subsequently, 5µL of the bacteriophage lysate was carefully spotted onto the agar using a micropipette and allowed to be absorbed into the agar layer. The plates were then incubated for 48 hours under anaerobic conditions. A clear zone was observed after incubation

Bacteriophage Purification

The purification of bacteriophages was done by using the double agar overlay method (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009; Luong, Salabarria, Edwards, & Roach, 2020). The procedure entailed making 250mL of soft agar with nutrient agar. In a 250mL conical flask, media powder (1.4g) was suspended, and deionized water was added to ensure proper measurement. For bacteriophage adsorption, $CaCl_2$ and $MgSO_4$ were added to the suspension. The flask was wrapped in aluminum foil and sterilized at 121 ℃ under 15 lbs pressure for 15 minutes. After that, the soft agar was immersed in a 45°C water bath. After that, $\sim 1\times10^7$ cfu/mL of bacterial culture was mixed with $\sim 1\times10^4$ pfu/mL of phage lysate. The mixtures were gently mixed and incubated at 37°C for 60 minutes to allow the bacteria to absorb the phage. This mixture was gently mixed with molten soft agarose (at 45°C). The mixture was quickly poured and evenly distributed onto pre-warmed plates. The plates were left undisturbed to cool and solidify for 15 minutes. The plates were incubated at 37℃ for 24 hours. The plates were examined for plaque formation.

Bacteriophage Storage

S.M. buffer is extensively used as a storage buffer**.** The single isolated plaque was picked up using the tips of a pipette. Then poured into Eppendorf tubes containing 100µL S.M buffer and 10µL chloroform was added to the final suspension. S.M. buffer containing the plaque was stored at 4℃ for long-term usage (Luong et al., 2020).

Minimum Inhibitory Concentration of Antibiotics

The micro broth dilution method was used for the determination of the minimum inhibitory concentration (MIC) of Chloramphenicol and Gentamicin (Owuama, 2017). In a 96-well microtiter plate, 100µL broth was added. 100µL of 10% Chloramphenicol was added to 1st well and mixed properly. After mixing, 100µL of the solution was transferred into the 2^{nd} well and the two-fold dilution procedure until the 11^{th} well (Positive control). Positive control consisted of chloramphenicol and broth. Negative control (12^{th} well) was consisted of bacteria and broth. The bacterial inoculum of 5×10^5 dilutions from the overnight culture was added. The same procedure was followed for gentamicin in 2^{nd} row. Then incubated at 37℃ for 24 hours. The optical density was measured at 600nm using a spectrophotometer after incubation. The Clinical and Laboratory Standard Institute guidelines were followed for the result interpretation.

Phage Antibiotic Synergism

The broth microdilution method was used for the evaluation of phage antibiotic synergism (Simon, Pier, Krüttgen, & Horz, 2021). For this purpose, a 96-well micro-titration plate was used. To determine phage antibiotic synergism, Gentamicin and Chloramphenicol were studied by using 1/4 the concentration of the MIC. Three controls were used, including antibiotic only, broth only, and phage only. Bacteria were adjusted to 6×10^6 CFU/mL and 10µL of bacteria was added to each well. Phages were adjusted to 10^6PFU/mL and 50µL was added. Then incubated at 37 ℃ for 24 hours. The optical density was measured at 600nm using a spectrophotometer after incubation

Statistical Analysis:
The test was repeated three times for statistical analysis. The statistical analysis for the effectiveness of phage antibiotic synergism was determined by applying one-way ANOVA. The significant difference was determined at $p \leq 0.05$.

RESULTS

Prevalence of *Staphylococcus aureus*

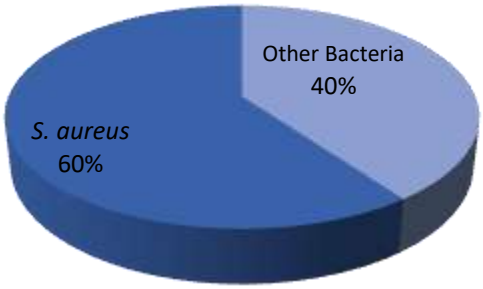
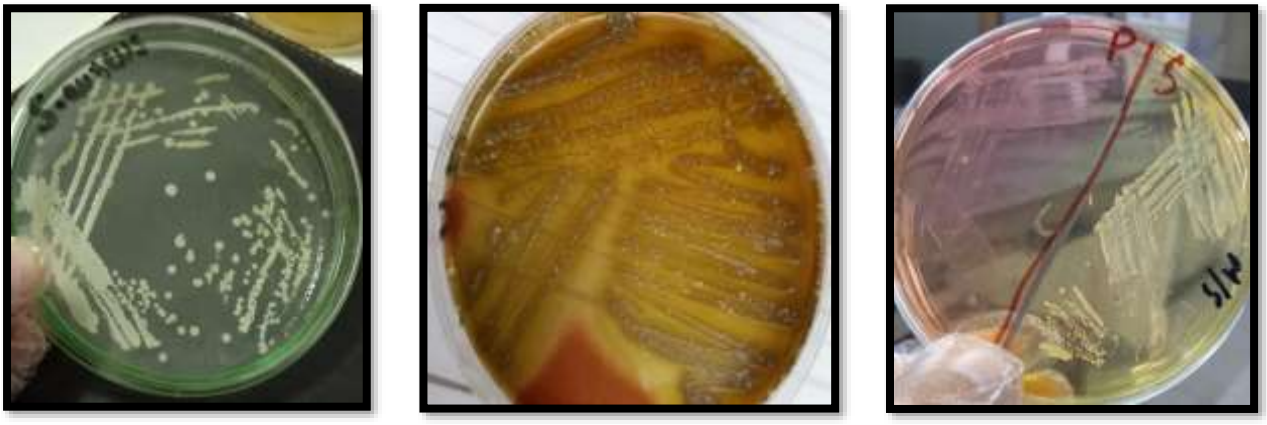


Fig.1 Prevalence of *Staphylococcus aureus*

Total 50 samples were taken from the wounds of diabetic patients. Out of these 50 samples, 30 (60%) were confirmed positive for *S. aureus* based on colony morphology and biochemical characterization while the rest of the 40% were other bacteria (Fig. 1).

Morphological Characterization

On Staph-110 *S. aureus* produced cream color colonies as shown in (Fig.2a). Whereas on blood agar, *S. aureus* showed a β-hemolytic pattern (Fig. 2b). Mannitol Salt agar is a differential and selective media for *S. aureus*, turns the color of the media from pink to yellow. After 24 hours of incubation at 37℃, golden-yellow colonies, and color change was observed on Mannitol Salt agar (Fig. 2c).



(a) (b) (c)

Fig.2 *S. aureus* growth on different types of media

Microscopic Characterization

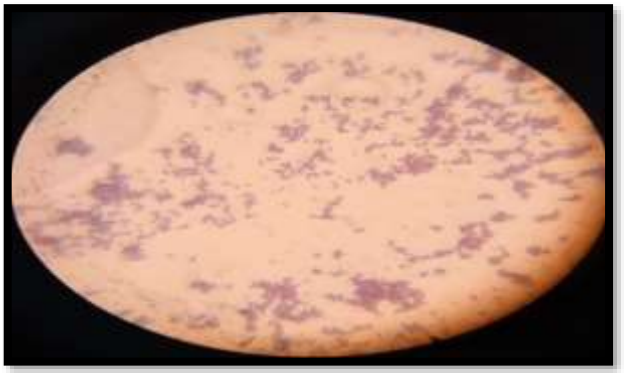


Figure 3. Microscopic examination of *S. aureus*

Under the microscope, purple color and cocci-shaped bacteria arranged in clusters were observed (Fig. 3).

Biochemical Characterization

Various biochemical tests were performed for the confirmation of S. aureus. The complete list of different tests was enlisted in (Table. 1) along with the confirmatory result.

Biochemical tests	**Results**
Catalase test	Positive
Coagulase	Positive
Oxidase	Negative
Citrate test	Positive
Methyl red test	Positive
Triple sugar iron test	Positive
Voges Proskauer test	Positive
Indole test	Negative
Urease	Positive

Antibiotic susceptibility test

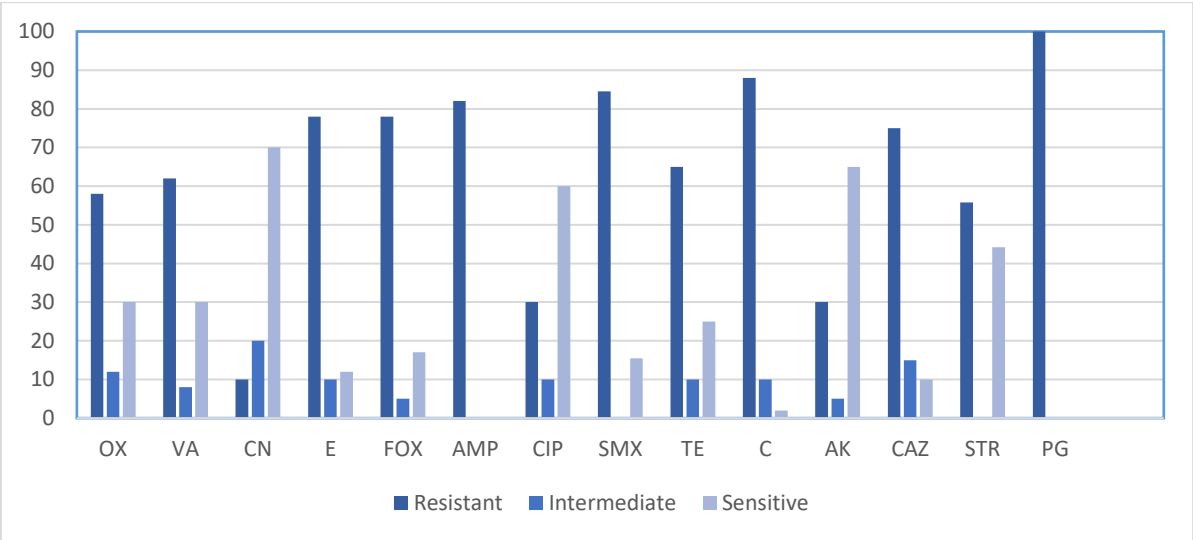


Figure 4: Antibiotic Sensitivity Patterns of *S. aureus* isolated from the wounds of diabetic patients

An antibiotic sensitivity test was performed on all *Staphylococcus aureus* strains. Different antibiotics were tested on them. The zone of inhibition was measured according to CLSI guidelines and categorized into sensitive, intermediate, and resistant (Fig. 4).

Screening of Methicillin-resistant staphylococcus *aureus* (MRSA)

Among the total of 30 S. aureus isolates, screening was conducted to determine the presence of MRSA. The Oxacillin test was employed to methicillin resistant. The growth observed over oxacillin agar after 24 hours, the strain was classified as resistant to Methicillin. Conversely, if no growth was observed within the required time frame, the strain was considered methicillin sensitive. Out of all 30 isolates, 17 isolates (56.67%) were found to be Methicillin-resistant over the methicillin agar test as shown in (Fig. 5).

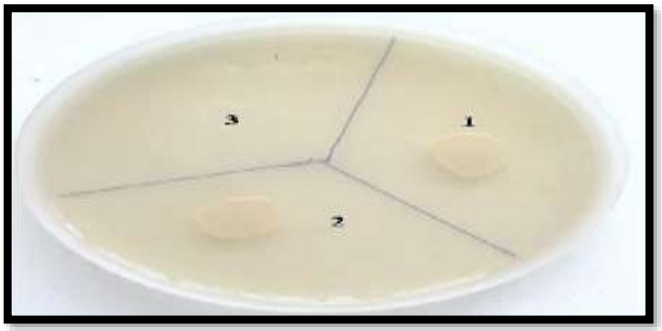


Fig 5. Oxacillin agar method showed that strain no 1 and 2 are sensitive to oxacillin and 3 resistant to oxacillin

Minimum Inhibitory Concentration of Antibiotics

Clinical and Laboratory Standard Institute guidelines were followed for the minimum inhibitory concentration determination of chloramphenicol and gentamicin. The isolates were susceptible to chloramphenicol and gentamicin with MIC ≥33 μg/mL and 0.5 μg/mL respectively**.**

Spot Test

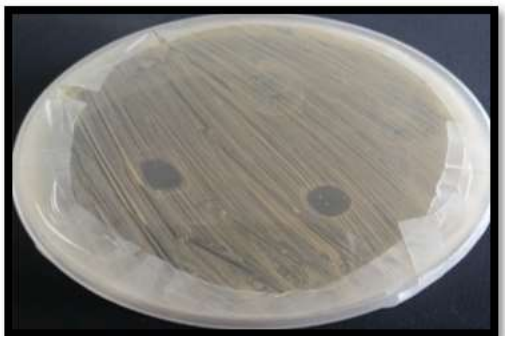


Fig6. Clear circular zones indicate the presence of phages in sewage sample

The spot test was used as an initial test for the presence of phage and is sometimes referred to as a rapid test. The phage lysate is directly poured onto the agar plate, which has the bacterial lawn. A clear zone resulting from the lysis of host bacterial cells after incubation indicated the presence of phages (Fig. 6).

Bacteriophage Isolation

Figure7. Small plaques formation

The isolation of lytic bacteriophages against *S. aureus* was performed using the double agar overlay. The process involved incubating bacterial suspension with phage lysate for 20 minutes. To facilitate the absorption of the bacteriophage, the suspension, along with molten soft agar, was poured onto nutrient agar plates. After 24 hours of incubation, the plates were examined, and clear plaques were observed (Fig. 7).

Phage Antibiotic Synergism

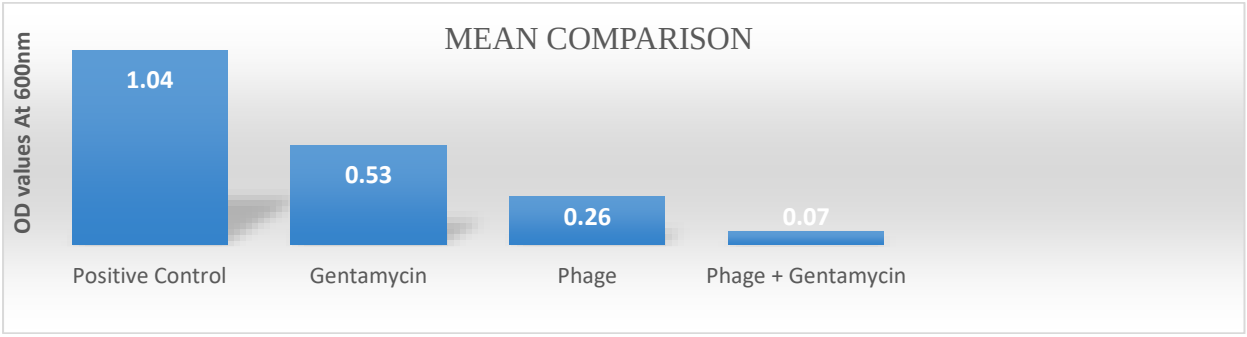


Fig8. Mean comparison of OD values of different treatments (Gentamycin, Phage, and both in combination) given to the S. aureus

The microtiter plate method was employed to evaluate the activity of antibiotics and phages against MRSA. Specifically, the activity of gentamycin, chloramphenicol, and phages was assessed individually and compared to their activity when used in combination. To measure the difference in optical density (OD), a spectrophotometer was utilized. A statistical analysis of mean values revealed a highly significant relationship ($p<0.01$) between three different treatment groups. Staphylococcus aureus was treated with the antibiotic gentamicin in the microtiter plate experiment, and the optical density (OD) was measured at 600nm with a spectrophotometer. The gentamicin treatment alone resulted in an OD decrease of up to 0.53. Similarly, phage treatment alone resulted in an OD reduction of up to 0.26nm against the bacteria. When gentamicin and phages were combined, however, a synergistic effect was observed, resulting in a greater decrease in OD of up to 0.07. This suggests that the combination of gentamicin and phages effectively reduced the bacterial load. The mean values of all treatment groups differed significantly from one another, emphasizing the significance of this experiment, particularly the synergistic therapy using the antibiotic and phages (Fig. 8).

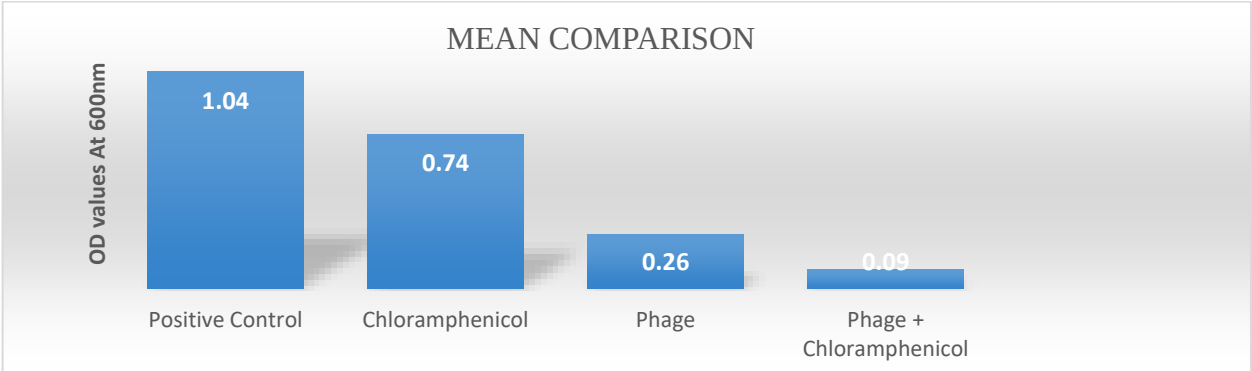


Fig9: Mean comparison of OD values of different treatments (Chloramphenicol Phage and both in combination) given to the S. aureus

Similarly, after treatment with the antibiotic chloramphenicol, the optical density of Staphylococcus aureus at 600nm was measured using a spectrophotometer. The chloramphenicol treatment alone resulted in an OD decrease of up to 0.74. The use of phages alone resulted in an OD reduction of up to 0.26 against the bacteria. When chloramphenicol and phages were combined, however, a synergistic effect was observed, resulting in a greater decrease in OD of up to 0.09. This shows that the combination of chloramphenicol and phages effectively reduced the bacterial load. The mean values of all treatment groups differed significantly from one another, highlighting the significance of this experiment and the maximum results obtained with synergistic therapy using the combination (Fig. 9)

DISCUSSION

Wound healing may be influenced by mechanical tension at wound site. Vitamin D insufficiency has recently been suggested as a potential cause for foot ulcers infection. Limited growth hormone production, raised markers of inflammation, elevated concentrations of enzymes, and decreased cell multiplication are major factors that impede healing process in diabetic foot infection (DFI) (Tiwari et al., 2013). MRSA is the most common bacteria that cause post-operative wound infections and other soft tissue infections as reported in (Chang et al., 2019; Sun et al., 2022). The resistance in the pathogenic bacteria is perceived as a major threat to general health and effecting the population around the world. MDR pathogens not only raised in hospital settings but also emerged in general environment and community. Bacteria adapt and evolve by giving response to the attack of antibiotics. This is the consequence of survival of fittest. Bacteria show resilience in genetic makeup that trigger response to antibiotic. This is done by doing mutation, by acquiring foreign genetic elements and by expressing resistance genes (Munita & Arias, 2016). The treatment of *S. aureus* is becoming more difficult day by day due to antibiotic resistant according to (Chambers & DeLeo, 2009; García et al., 2010; Nobrega, Costa, Kluskens, & Azeredo, 2015; Plipat, 2012). There is a need to find alternative therapies to overcome the resistance. Previous reports have shown the importance of phage therapy (Garneau et al., 2010; Rasool, Yousaf, Siddique, Saqalein, & Khurshid, 2016; Sangha et al., 2014). The major dilemma regarding phage therapy is phage resistance. For protection from invading viruses, bacteria have developed plenty of safeguard mechanisms. Two of them are the RM system and the CRISPR system (Kim et al., 2018). To deal with this problem, another innovative strategy that is being studied is the PAS (Phage antibiotic synergism) (Kaur, Harjai, & Chhibber, 2012; Simon et al., 2021; Wang et al., 2020). Many researchers discussed phage antibiotic synergism. Kaur *et al* studied the lytic bacteriophage and linezolid co-therapy against methicillin-resistant *S. aureus* (MRSA) from diabetic foot infections. In this study, they took diabetic BALB/c mice and established *S. aureus* ATCC 43300 hind paw infection in mice. This infection was treated with lytic bacteriophage, MR-10 alone and in combination with linezolid. Then investigated the whole process of wound healing. The results showed that combination therapy proved more effective during the entire process of healing (lesion

score, foot myeloperoxidase activity, bacterial load, and histopathological analysis) as compared to a single administration of phage and antibiotics. Co-therapy reduced the emergence of resistant mutants. The combination of antibiotics with phage causes plaque size enlargement, amplification of phage accelerates and increases the burst size. Then phage enzymes degrade the cell wall of bacteria and antibiotics diffuse into the cell (Jo, Kim, Ding, & Ahn, 2016). Ara *et al* studied the phage-antibiotic synergistic effect in *S. aureus.* They used this combination to reduce antibiotic resistance in *S. aureus*. In this study, treated the *S. aureus* with phages, ciprofloxacin, and phage-antibiotic combination. Then observed the results after incubation. According to the results, *S. aureus* numbers were significantly reduced by 4.62, 3.47, and 5.75 logs CFU/ml, respectively. The phage-antibiotic treatment effectively controlled the growth of *S. aureus* (Jo et al., 2016). Li *et al* characterized the Henu2 bacteriophage and evaluated the synergistic action between antibiotics and phage. This research revealed that Henu2 phage alone had just a minor inhibitory effect on *S. aureus* growth. However, combined treatment of phage and antibiotic efficiently inhibited the *S. aureus* growth, with a reduction of more than three logs in less than 24 hours in lab. These findings suggest that Henu2 phage can be used in conjunction with antibiotics to boost phage Henu2 production and hence improved bacterial killing efficiency (Li et al., 2021).

In our study, Statistical analysis based on a comparison of mean values has revealed an overall highly significant ($p<0.01$) relationship among three different treatment groups. *S. aureus* in the microtitration plate is treated with antibiotics, phage, and in combination. After the measurement of optical density at 600nm, it was concluded that 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. The combined use of antibiotics and phages reduced the bacterial load and decreased the optical density. We concluded from our study that antibiotics and phages enhance each other's efficacy, and their combined potential capability to kill microbial entities is greater than their one as reported in (Jo et al., 2016; Kaur et al., 2012; Li et al., 2021).

CONCLUSION

This study presents a novel approach for combating antimicrobial-resistant pathogenic bacteria through the combined use of antibiotics and bacteriophages. Bacteriophages were isolated from sewage water and their effectiveness in combination with antibiotics was assessed using a titration method. The findings revealed a significant reduction in bacterial growth when antibiotics were used in combination with phages. These results highlight the potential of combining phages with antibiotics as a promising strategy for controlling bacterial infections and suggest the applicability of phage-based approaches in addressing the challenge of AMR.

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