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Evaluation of the Efficacy of Bacteriophages Targeting Multidrug-Resistant *Staphylococcus aureus*

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Abstract

To address diseases brought on by bacteria that are resistant to antibiotics, bacteriophages—viruses that particularly target and destroy bacteria—are utilized as an alternative to antibiotics. In this study, phages from hospital sewage were isolated and characterized in relation to the clinical strain of multidrug-resistant Staphylococcus aureus. The phages were isolated and identified using biochemical methods, including coagulase tests, catalase tests, microscopy, and mannitol fermentation on mannitol salt agar. When antibiotic resistance was evaluated for these isolated bacteria, it was shown that all isolates had no resistance to Vancomycin, but they were 100% resistant to Augmentin, 68% to Ciprofloxacin, 53% to Cefotaxime, 50% to Cefixime, 33% to Streptomycin, 30% to Chloramphenicol, and 13% to Oxacillin. Consequently, specific phages identified as PA, PB, and PC were isolated and characterized for further study. The PA phage showed a broad host range in the host range analysis, lysing eight of the twenty different strains of Staphylococcus while not infecting any other bacteria. While a notable drop in titer was noted at pH levels 5 and 9, this phage maintained stability at temperatures as high as 60 °C and showed peak activity at pH 7. Investigating the biological properties of these phages can provide valuable insights and contribute to the development of potential therapeutic agents against multidrug-resistant Staphylococcus aureus.

Keywords: *Bacteriophages, Antibiotic Resistance, MDR, Staphylococcus.*

Introduction

Staphylococcus aureus belongs to genus Staphylococcus and this genus contains about 40 species among which Staphylococcus considered as most pathogenic specie. S. aureus is gram positive facultative anaerobic, non-spore farming cocci shape having the diameter of about 0.7 to 1.3 µl, arranged in pairs and sometimes, form irregular grapes like cluster, having ability to tolerate high level salt concentrations, in humans mostly this bacterium present on the skin, in respiratory tract and act as a normal flora of body (Somerville and Proctor, 2009).

S. aureus related to gram positive, having ring or round shape belonging to the firmicutes phylum, and is a member of normal flora of the body, and usually present in the respiratory tract and on the skin. S. aureus is recognise due to its ability for development of resistance to almost all types of antibiotics, methicillin resistant S. aureus which is resistant to methicillin are commonly known as (MRSA) they are universal microorganism that are causative agent of group of the diseases range from skin, and soft tissues diseases to life terrifying problems some of them are sepsis bacteremia, and pneumonia. S. aureus is one of the main human related diseases that can cause infection in public community and nosocomial diseases which create demand to stop its spread and advancement in treatment of all infections which are caused by this specie. Some reports show that methicillin-resistant and susceptible S. aureus show resistance against antibiotics used (Saba *et al.*, 2017).

Material and Methods

This study was conducted at the University of Haripur's Microbiology lab, where sewage samples from hospitals in Haripur were collected for bacteriophage isolation. Samples were transported on sterile swabs and stored at 4°C. All laboratory items and media were sterilized at 121°C for 15 minutes, and bacterial isolates from local hospitals were cultured on nutrient and Mannitol Salt Agar (MSA) plates, which were then incubated overnight at 37°C.

Culturing on Mannitol Salt Agar

MSA was prepared in compliance with the manufacturer's guidelines. Samples were inoculated using sterile swab, incubated at 37°C and incubated for 24–48 hours.

Gram Staining and Microscopy

A bacterial colony was smeared on a sterile slide, heat-fixed, and subjected to a Gram staining procedure and observed under microscope.

Biochemical Testing

For the catalase test, a drop of 3% hydrogen peroxide was placed on a glass slide, and a bacterial culture was mixed in; bubble formation indicated a positive result. In the coagulase test, 1 mL of fresh plasma was mixed with several colonies of pure bacterial culture, incubated at 37°C for four hours, and the results were recorded.

Antimicrobial Susceptibility Profiling

The Kirby-Bauer disc diffusion method involved suspending bacterial colonies in normal saline and spreading the inoculum onto Mueller-Hinton Agar (MHA) to achieve confluent growth. Antibiotic discs were placed on the agar, which was incubated at 37°C for 18 to 24 hours. Clear zones of inhibition were then measured in millimeters (mm) to evaluate the sensitivity or resistance of the bacteria to the antibiotics, following CLSI guidelines (2015).

Enrichment of Bacteriophages

Sewage samples were centrifuged at 10,000 rpm for 10 minutes to remove sediments, and the supernatant (40 mL) was combined with 10 mL of 5X LB broth and 1 mL of log-phase bacterial culture, followed by incubation at 37°C with shaking at 120 rpm for 18 to 24 hours. After a second centrifugation, the supernatant was filtered through a 0.22 µm sterile syringe filter, stored at 4°C, and assessed for phage presence using a spot test (Twest and Kropinski 2009).

Purification of Phages

Phage serial dilutions were prepared by adding 100 µL of overnight bacterial culture to each dilution, followed by a 5-minute incubation at 37 °C. The mixture was spread onto LB agar plates, overlaid with 3–4 mL of semi-solid LB medium, and incubated for 24 hours at 37 °C, resulting in clear plaques. The plaques were isolated using a sterile micropipette tip, with the purification process repeated 2–3 times before storing the purified phages at 4 °C for short-term use and -20 °C for long-term storage (Bibi *et al.*, 2016).

Characterization of Isolated Phage

Host range determination:

The host range of the phage was evaluated using a spot test, where 100 µL of log-phase bacteria was added to Falcon tubes containing 3 mL of agar, followed by the addition of semi-solid medium before pouring onto LB agar plates. After allowing the agar to solidify, 10 µL of phage suspension was added at various positions, and the plates were incubated at 37 °C for 24 hours to observe plaque formation.

Phage titer determination

The double-layer agar method was utilized for phage titration by serially diluting a stock phage solution and mixing 100 µL of each dilution with a bacterial culture. After resting for 10 minutes, the mixture was combined with 3 mL of 0.7% top agar and poured onto nutrient agar plates. The plates were incubated at 37 °C for 24 hours to allow plaque formation, with a distinct single plaque selected for phage titer determination.

The double-layer agar technique was applied for the phenotypic characterization of phages, allowing for the observation of individual plaque size and shape (Khawaja *et al.*, 2016).

Physiological Characterization

Thermal stability test

The thermal stability of the isolated bacteriophages was tested at various temperatures by placing 1 mL of the phage solution in separate autoclaved Eppendorf tubes. These tubes were then incubated in a water bath set to different temperatures: 30ºC, 40ºC, 50ºC, and 60ºC.

pH stability test

The pH stability test was performed as described by Verma *et al.* (2009), where fresh nutrient broth was adjusted to various acidic or basic pH levels using 1M HCl or 1M NaOH. A 1 mL phage suspension was added to 9 mL of the adjusted medium and incubated at 37 °C for 3 hours, with a neutral pH (7) suspension serving as a control. After incubation, phage titre was assessed against *S. aureus* using the double-layer agar method.

Organic Solvent Sensitivity Test

The organic solvent sensitivity test evaluated phage stability in chloroform and ethanol by mixing 1 mL of phage solution with an equal volume of each solvent and incubating at room temperature for 1 hour in a shaking incubator. After incubation, the mixtures were centrifuged at 10,000 rpm for 10 minutes, and phage titer was determined using the double-layer agar assay.

Bacterial Growth Reduction Assay

The reduction assay assessed phage growth by culturing equal amounts of phages and bacterial cells in LB broth, alongside a control flask, all incubated at 37 °C with shaking. Samples were taken every two hours to measure optical density at 550 nm, allowing for evaluation of the phage's ability to inhibit bacterial growth (Chaudhry et al., 2014).

Phage stock preparation

Phage stocks were prepared by adding 100 µL of S. aureus overnight culture to 100 mL of nutrient broth with 1 mM $CaCl_2$ and $MgCl_2$, incubating at 37 °C with shaking. After 1 hour, 100 µL of purified phage was added and incubated overnight for lysate formation. The lysate was then centrifuged, filtered through a 0.22 µm filter, and stored at 4 °C (Bonilla et al., 2016).

Results

Culturing on Mannitol Salt Agar and Biochemical Identification

As illustrated in figure 1, *S. aureus* exhibits the beneficial effects of mannitol fermentation when its proliferation causes the colour of Mannitol Salt Agar to change from red to yellow. Gram staining shows S. aureus as a gram positive; the cocci form and organize themselves in clusters. A positive catalase test result is indicated by the appearance of bubbles on a glass slide. When the coagulase test produced positive findings, S. aureus was validated by the production of clots, as seen in fig. 2.

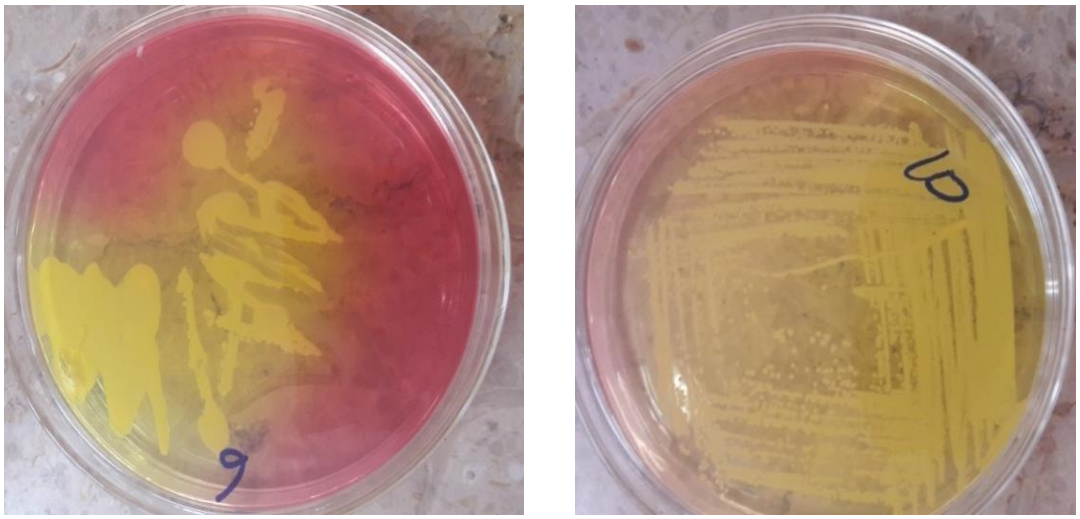

Fig1: Showing the MSA fermentation by *S. aureus*

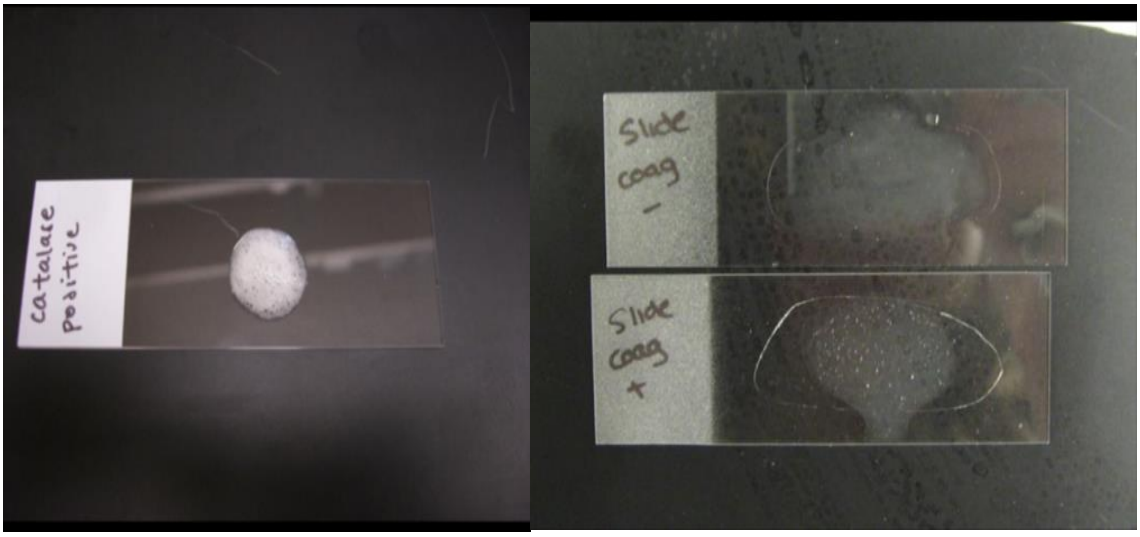

Fig 2: Catalase and coagulase test showing positive results

Antimicrobial Susceptibility Test

These isolated strains were tested for antibiotic resistance; all isolates showed 100% resistance to Augmentin, 68% to Ciprofloxacin, 53% to Cefotaxime, 50% to Cefixime, 33% to Streptomycin, 30% to Chloramphenicol, 13% to Oxacillin, and no resistance to Vancomycin.

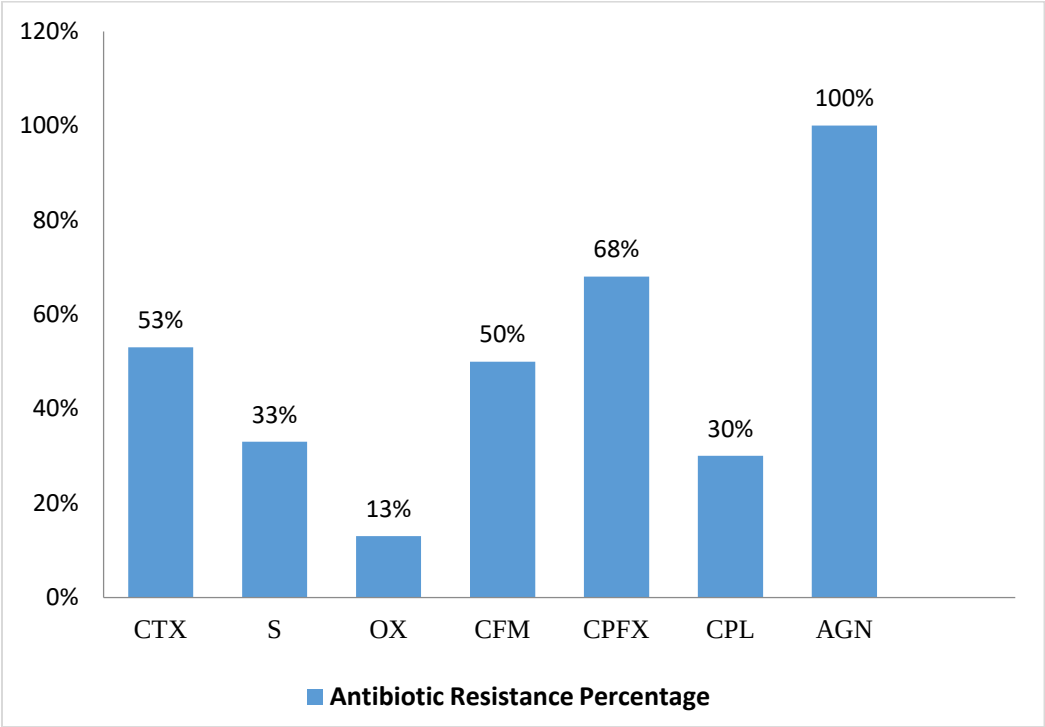

Fig 3: A bar graph displaying *Staphylococcus aureus's* resistance trend.

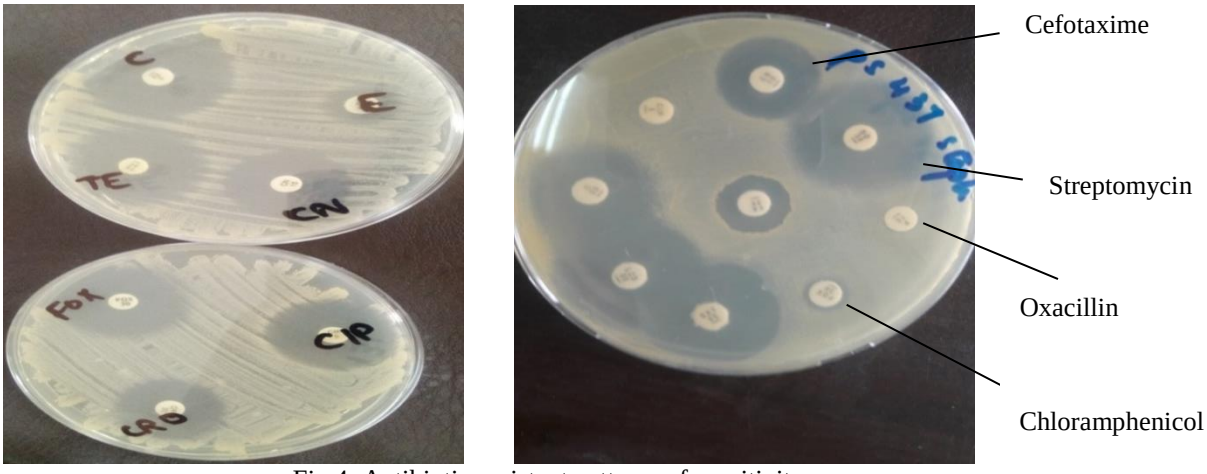

Fig 4: Antibiotic-resistant patterns of sensitivity.

Becteriophages Isolation and Purification

Phages were isolated using the double layer agar test method against strains U1, U3, U6, and U7 of sewage samples. By repeating the DLA and spot test procedures, Phage A was selected and purified.

Spot test

Among four enriched water samples, only one produced a clear lytic zone, with individual plaques observed through the double-layer agar method. The plaque with the largest, clearest zone was selected for further study. Host range was assessed by spot testing on LB plates, where zones of lysis after 24 hours indicated bacterial susceptibility.

Fig. 5: Results of a positive spot assay.

Plaque morphology

The isolated phage produced distinct circular plaques on the agar plate, with each plaque displaying a clear 3 mm centre surrounded by an 8 mm opaque outer ring when tested against the host bacteria.

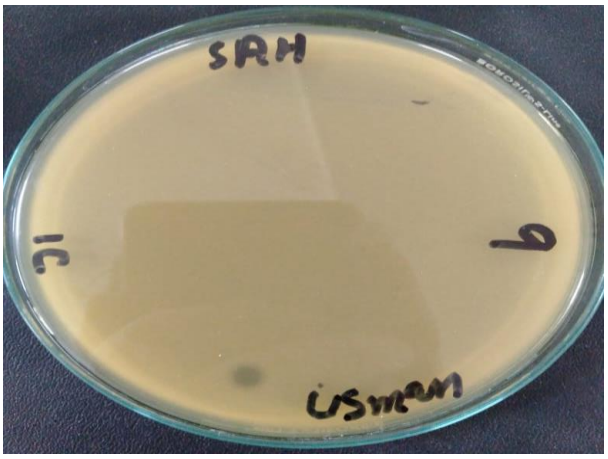

Figure 6: A double-layer test plate shows the formation of an individual PA bacteriophage plaque.

Morphological Characterization of Phages

Determination of phage titre

The phage titre was determined using the double-layer agar (DLA) method, resulting in a titre of 10^8 pfu/mL. The propagation and subsequent enumeration were repeated, consistently yielding phage titres above 10^8 pfu/mL. Morphologically, the PA and PC plaques were circular, while the PB plaques appeared as small dots. The sizes of the PA and PC plaques ranged from 3 to 5 mm, whereas the PB plaques measured 1 to 2 mm (see figure).

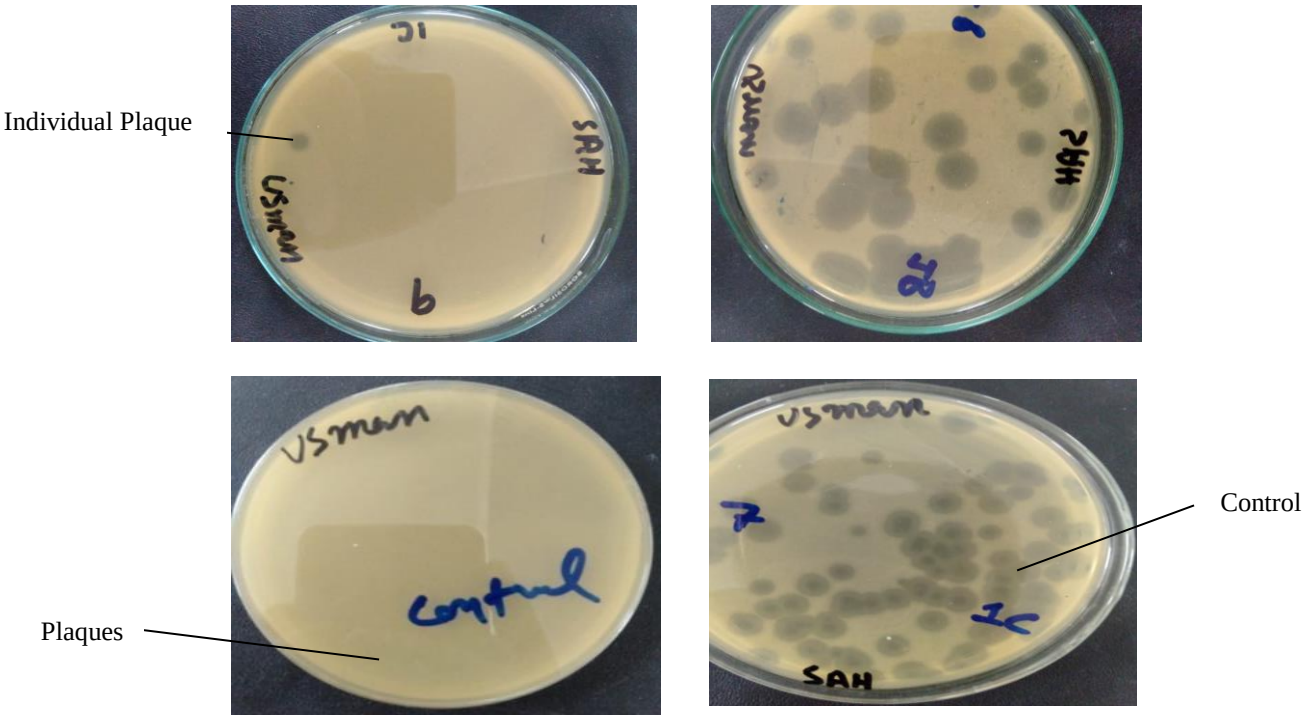

Fig 7: Morphology of isolated phages A and B and C and Control.

Plaque Augmentation

Plaques morphology was observed by the growth of bacteriophages on plates by double-layer agar assay their size and structure can be observed.

Physiologically Characterization of Bacteriophages

pH Stability of Bacteriophages:

Isolated phage PA show stability at different pH show optimum growth at pH 7 while decreases on 5 and 9.

Thermal Stability of Bacteriophages

Isolated phages were tested for thermal stability at 4, 25, 37, and 45 °C for one hour, showing substantial growth across this range. However, a marked decrease in plaque-forming units was observed at 60 °C, indicating reduced viability at higher temperatures.

Reduction Assay

Phage A showed persistent efficacy over a 24-hour period, as shown in Figure 8, with reduced bacterial growth at MOI:1 after 6 hours and at MOI:100 after 10 hours.At MOI:10, Phage B was only effective for six hours, as Figure 10 illustrates. Phage A and C cocktails were also investigated to provide a deeper understanding, as illustrated in fig 11.

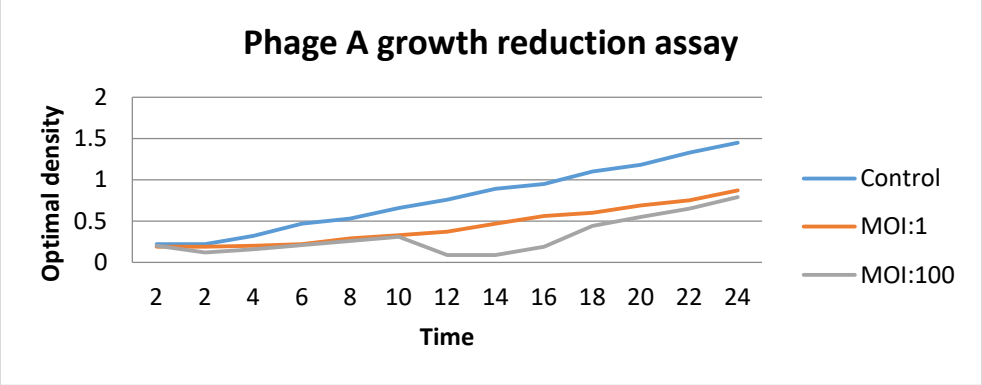

Fig 8: Growth control ability at MOI 1 and 100.

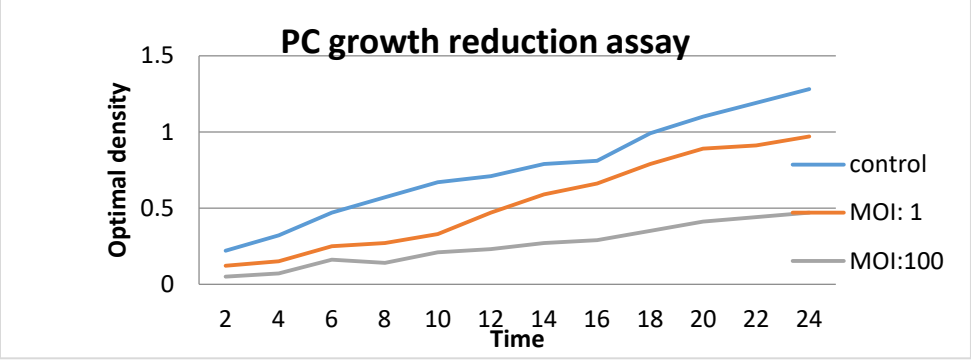

Fig 9: Phage C growth Control ability at MOI 10 and 100.

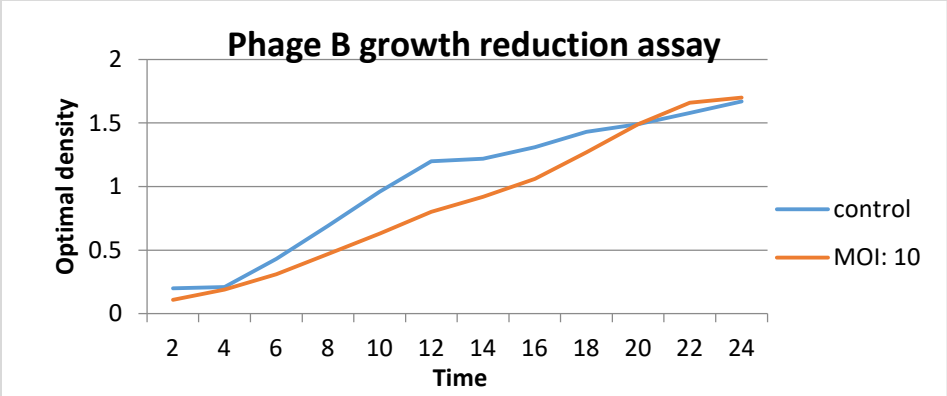

Fig 10: Phage B Growth Reduction Assay at MOI 10.

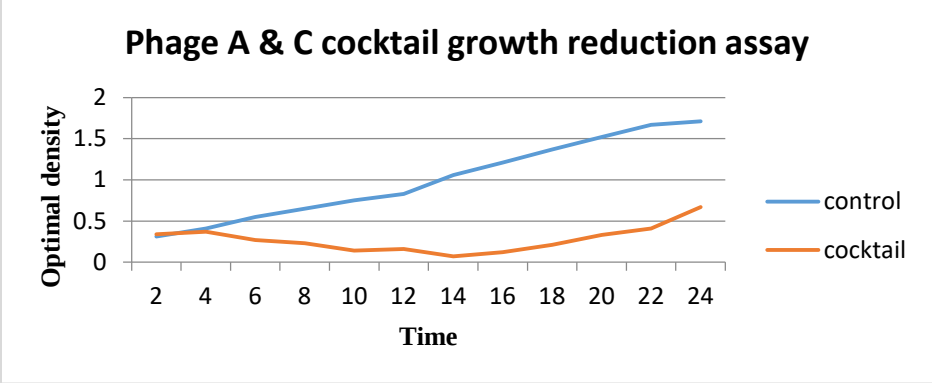

Fig11: Phage A and Phage C Cocktail growth reduction assay at MOI 1.

Discussion

The therapeutic application of bacteriophages has garnered considerable attention as an alternative to traditional antibiotics for managing antibiotic-resistant infections, first demonstrated successfully in 1919 (Elbreki *et al.,* 2014). The need for alternative treatments, especially against challenging pathogens like Staphylococcus aureus, has become increasingly crucial (Masoumi *et al.,* 2020). In a recent study, sewage samples collected from hospitals were screened for *S. aureus*-specific phages; only one of four samples yielded infective phages in spot testing, even after enrichment. This limited recovery may be attributed to low host bacterial density at the collection sites, as bacteriophages are obligate parasites whose growth depends on host presence (Uchiyama et al., 2007). Another consideration is that sediment in sewage may hold a higher concentration of phages, as they tend to attach and cluster at the bottom, requiring agitation for their release (De Flora *et al.,* 1975; Drake *et al.,* 1998). Only clear plaques, indicating complete lysis of S. aureus, were selected for further study; however, variability in plaque size suggested the presence of multiple phage types within the samples (Irving et al., 1990). Thermal and pH stability tests showed that phage viability was optimal between 0-5°C and remained stable across pH 4-9, though stability decreased below pH 4 or above pH 10 (Civerolo *et al.,* 1990; Krasowska *et al.,* 2015). Organic solvent resistance was also assessed, indicating that phage viability was retained in the presence of 5% chloroform, making it useful for phage storage in chloroform-aqueous solutions due to chloroform's antibacterial properties (Cotton and Lockingen, 1963). However, phages showed sensitivity to ethanol (Verma *et al.,* 2009). These findings emphasize the complex stability parameters required for phage therapy applications and reinforce the potential of bacteriophages as viable therapeutic agents against resistant pathogens.

Conclusion

The findings highlight the stability and host specificity of phage, suggesting its promise as a therapeutic agent under optimal pH and temperature conditions, advancing alternative treatment strategies for combating antimicrobial-resistant pathogens.

References

Abedon, S. T. (2012). Bacterial "immunity" against bacteriophages. *Bacteriophage, 2*(1), 50-54.

Abedon, S. T. (2014). Phage therapy: Eco-physiological pharmacology. **Scientifica*, 2014*, Article 581639.

Abedon, S. T., García, P., Mullany, P., & Aminov, R. (2017). Phage therapy: Past, present and future. *Frontiers in Microbiology*, 8, 981.

Abuladze, T., Li, M., Menetrez, M. Y., Dean, T., Senecal, A., & Sulakvelidze, A. (2008). Bacteriophages reduce contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by Escherichia coli O157. *Applied and Environmental Microbiology*, 74(20), 6230-6238.

Ackermann, H. W., Tremblay, D., & Moineau, S. (2004). Long-term bacteriophage preservation.

Adams, M.H., 1959. Bacteriophages. Interscience Publishers.

Adriaenssens, E., & Brister, J. R. (2017). How to name and classify your phage: An informal guide. *Viruses*, 9(4), 70.

Appelbaum, P. C. (2006). MRSA—the tip of the iceberg. *Clinical Microbiology and Infection*, 12, 3-10.

Babushkina, E. A., Belokopytova, L. V., Grachev, A. M., Meko, D. M., & Vaganov, E. A. (2017). Variation of the hydrological regime of Bele-Shira closed basin in Southern Siberia and its reflection in the radial growth of Larix sibirica. *Regional Environmental Change*, 17(6), 1725-1737.

Bhardwaj, N., Bhardwaj, S. K., Mehta, J., Kim, K. H., & Deep, A. (2017). MOF–bacteriophage biosensor for highly sensitive and specific detection of Staphylococcus aureus. ACS Applied Materials & Interfaces, 9(39), 33589-33598.

Bruttin, A., & Brüssow, H. (2005). Human volunteers receiving Escherichia coli phage T4 orally: A safety test of phage therapy. **Antimicrobial Agents and Chemotherapy*, 49*(7), 2874-2878.

Calendar, R. (Ed.). (2012). The Bacteriophages: Volume 1. Springer Science & Business Media.

Cech, N. B., Junio, H. A., Ackermann, L. W., Kavanaugh, J. S., & Horswill, A. R. (2012). Quorum quenching and antimicrobial activity of goldenseal (Hydrastis canadensis) against methicillin-resistant Staphylococcus aureus (MRSA). *Planta Medica*, 78(14), 1556.

Centers for Disease Control and Prevention (CDC). (2001). Methicillin-resistant Staphylococcus aureus skin or soft tissue infections in a state prison--Mississippi, 2000. MMWR. Morbidity and Mortality Weekly Report, 50*(42), 919-922.Clark, J.R. and March, J.B., 2006. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends in biotechnology*, 24(5), pp.212-218.

Daum, R. S., Ito, T., Hiramatsu, K., Hussain, F., Mongkolrattanothai, K., Jamklang, M., & Boyle-Vavra, S. (2002). A novel methicillin-resistance cassette in community-acquired methicillin-resistant Staphylococcus aureus isolates of diverse genetic backgrounds. *The Journal of Infectious Diseases*, 186(9), 1344-1347.

David, M. D., Kearns, A. M., Gossain, S., Ganner, M., & Holmes, A. (2006). Community-associated meticillin-resistant Staphylococcus aureus: Nosocomial transmission in a neonatal unit. *Journal of Hospital Infection*, 64(3), 244-250.

DeLeo, F. R., & Chambers, H. F. (2009). Reemergence of antibiotic-resistant Staphylococcus aureus in the genomics era. *The Journal of Clinical Investigation*, 119(9), 2464-2474.

Duckworth, D. H. (1976). Who discovered bacteriophage? *Bacteriological Reviews*, 40(4), 793.

Evans, P. N., Boyd, J. A., Leu, A. O., Woodcroft, B. J., Parks, D. H., Hugenholtz, P., & Tyson, G. W. (2019). An evolving view of methane metabolism in the Archaea. *Nature Reviews Microbiology*, 17(4), 219-232.