Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/avkwy072

ISOLATION OF BACTERIOPHAGES AND THEIR EFFECT AGAINST MULTI DRUG RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Background: Antibiotic resistance poses a significant public health threat, prompting scientists to explore safer alternatives like phages for controlling bacteria.

Objectives: The study aimed to isolate indigenous bacteriophages and explore their antibacterial properties against multidrug-resistant *Staphylococcus aureus*.

Methodology: Ear samples were used for *S. aureus* isolation, cultured on Mannitol Salt Agar, and identified through Gram staining and biochemical tests. MDRSA strains were confirmed using antibiotic disc diffusion and phage antibacterial activity determined. The study isolated phages from sewage water samples using a 0.22µm filter and MDRSA, and their antibacterial activity was assessed using the spot test method.

Results: Out of 44 samples, 45.45% (20) showed yellow-colored colonies on MSA, indicating gram-positive cocci in bunches, catalase, and coagulase test-positive. The antibiotic sensitivity results showed that 100% of the 20 *S. aureus* isolated strains were resistant to penicillin, ampicillin, cefotaxime, oxacillin, and amoxiclav drugs, while 100% were sensitive to gentamycin, amikacin, and Clindamycin. 90% to cefoxitin, 75% to ciprofloxacin, 65% to erythromycin, 30% to tetracycline, 20% to streptomycin, vancomycin, and norfloxacin, 15% to chloramphenicol, and 10% to kanamycin. Bacteriophages were isolated from sewage water and confirmed based on plaques. In the spot test, bacteriophages plaques were observed on spotted places.

Conclusion: In this study, the indigenous bacteriophages were successfully isolated, and they exhibited good antibacterial activity against MDRSA.

Keywords: Bacteriophages; Sewage; Multi-drug resistance; *Staphylococcus aureus*; Antibacterial activity.

1 INTRODUCTION

S. aureus is normal flora and opportunistic pathogen. It has created major Health problems by producing wide variety of infections like diseases of the skin, including pimples, impetigo, etc. It might cause boils (furuncles), folliculitis, carbuncles, scalded skin syndrome and abscesses, lung

infections or pneumonia, brain infections or meningitis, bone infection or osteomyelitis, bacteremia, and septicemia, generalized life-threatening blood infections or Toxic Shock Syndrome, mastitis and moreover *S. aureus* have ability to make biofilms on tissues and medical equipment's that have ability to resist antimicrobials. it is difficult to eradicate biofilm-embedded bacteria [1].
Many strains of *S. aureus* have capability to resistant multi drugs MDR, and As methicillin resistant (MRSA) strains have caused major outbreaks in universal, MDRs are increasing [2] Vancomycin resistant (VRSA) are being increasing in the globe [3]. In Pakistan number of MRSA is also increasing, It was reported as 5% in 1989 and since then has increased up to 51%[4]. The nonstop appearance of resistant *S. aureus*, particularly MRSA, renders treatment difficult, [5] while the medicine choice after (MRSA) is vancomycin, but now (VRSA) are spreading [3] Rising number antibiotic resistance created great problem to scientists to attention on new antimicrobial agents such as phytochemicals, silver nanoparticles, therapeutic enzymes, pigments and bacteriophages[6].

Virulent bacteriophages have genes that code for enzymes like: Endolysins, depolymerase (EPS) and Phospholipases/Esterase. Endolysins: Lysin A and Lysin B that help the phages to releasing of phages particles from the host bacteria by hydrolyzing the component of the bacterial cell wall called peptidoglycan [7].

Bacteriophage may be used to dissolve biofilms, to diagnose and treat various forms of infectious diseases Efficient therapies are currently in place for Staphylococcus along with MRSA, E. *coli*, Salmonella [8].

The growth of pathogenic bacterial resistance is a major concern for public health; if all currently available antimicrobial medicines fail to suppress or eradicate these resistant infections, they will constitute a major problem. Due to the above-mentioned problem of rising antibiotic resistance among bacteria, the use of bacteriophages in medicine and the food industry may be appropriate for killing infectious germs. Therefore, in this study, bacteriophages were isolated from sewage water samples, and bactericidal activity was tested against their host multidrug-resistant *S. aureus* isolated from ear canal samples.

2 MATERIALS AND METHODS

2.1.1 Sample Collection for *S. aureus* isolation

For bacterial isolation total 44 external ear canal samples were collected from patients using sterile cotton swabs from Kausar Hospital Khairpur. Swabs were inserted into external canal and rotated three times, **counterclockwise** and clockwise. Samples were transported to postgraduate laboratory institute of microbiology Shah Abdul Latif University Khairpur.

2.1.2 Bacterial Isolation and identification

The specimens were inoculated on MSA and incubated aerobically for 24 h at 37°C. The bacteria were identified on the basis of colony morphology, Gram's stain and diverse biochemical tests like catalase and coagulase tests [9].

2.1.3 Antibiotic Susceptibility Testing

The Kirby–Bauer disc diffusion method was used to assess antibiotic susceptibility on Muller Hinton Agar (MHA) using standard methods as specified by CLSI recommendations. Penicillin, ampicillin, oxacillin, cefotaxime, gentamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, norfloxacin, amikacin, amoxiclav (ceftazidime/clavulanic acid), imipenem, ciprofloxacin, kanamycin, Cefoxitin, clindamycin and vancomycin were among the antibiotics examined. The results were classified as "sensitive," "resistant," and "intermediate sensitive" according to the CLSI zone size interpretative chart [10]. Bacteria were preserved in a broth containing 16 percent glycerol.

2.2 Sample Collection, Isolation, and Evaluation of Antibacterial activity of Bacteriophages against *S. aureus*

2.2.1 Sample Collection for Isolation of Bacteriophages

Sewage samples were collected for phages isolation from Village Bugro and layari Khairpur using sterile syringes. Samples were transferred to postgraduate laboratory institute of microbiology, Shah Abdul Latif University Khairpur. Samples were labelled according to location of sampling Bugro and Layari Khairpur.

2.2.2 Isolation of Bacteriophages

Phages were isolated from sewage using the standard dual-layer agar overlay method. In detail Sewage water samples were centrifuged at 12,000 g for 10 minutes and passed through 0.22µm membrane filter. The filtrate was propagated with MDR *S. aureus* overnight in the nutrient broth at 37 °C for 24h. After incubation bacterial residues were removed from broth culture, by centrifugation at 12,000 g for 10 minutes. The supernatants were collected and passed through a 0.22µm filter. Using sterile pipette 100µL of MDR *S. aureus* and 100µL filtrate were aliquoted and mixed with 5 mL unsolidified nutrient agar containing 1mL Calcium chloride, and after that , the mixture were poured on top of the solidified nutrient agar plates containing 1mL Calcium chloride [11].

2.2.3 Purification of Bacteriophages

The tip of a pipette were gently touched in the center of a single plaque of each newly extracted for phage purification and then phage material on the tip were transferred into 100µL of phage buffer (Tris-HCl pH 7.5, MgSO4, NaCl and $CaCl_2$) [12].

2.2.4 Evaluation of phages antibacterial activity using spot test against MDR *S. aureus*

Using sterile pipette 0.5µL MDR *S. aureus* were mixed with 4.5mL of unsolidified top nutrient agar, containing 1 mL Calcium chloride, and poured on the top of solidified nutrient agar plates containing 1mL Calcium chloride. The spot test was carried out 10µL of Coliphages were pipetted on specific areas in the plates and then allowed to dry completely. Plates were incubated at 37°C for 24h, after incubation plates were checked for lysis zones [13].

3 Results and Discussion

3.1.1 Bacterial Isolation and Identification Results

For bacterial isolation using sterile cotton swabs, 44 external ear canal samples were collected from the Kausar Hospital Khairpur. All samples were inoculated onto MSA plates and incubated at 37°C for 24 h. After incubation, the plates were inspected for colonial features. A total of 20 (45.45%) out of the 44 samples tested positive on mannitol salt agar, with yellow colonies visualized on those plates. Figure: 1 shows the growth of bacteria with yellow in color. The mannitol negative, on the other hand, appeared red or did not show change in color.

Gram staining and Biochemical assays such as catalase and coagulase were used for further characterization of the organisms. Under a microscope, spherical, purple-colored bacteria were observed organized in clusters in Gram staining (Figure 2). Bubbles were noticed in the tube when bacterial colonies were transferred into a test tube containing hydrogen peroxide indicating positive catalase test (figure 3). Plasma was coagulated on a slide indicated positive coagulase test (figure 4).

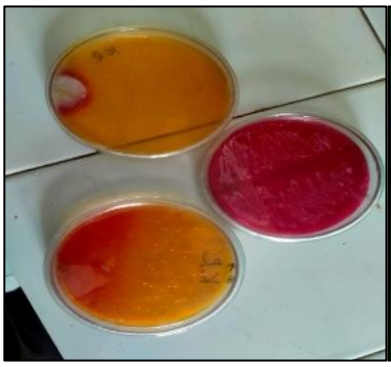

Figure 1. *S. aureus* on MSA (A, B) positive and C Negative

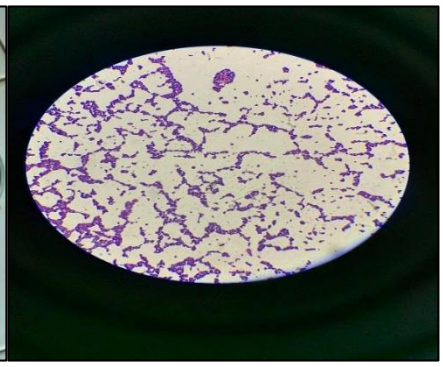

Figure 2. Gram staining of *S. aureus*

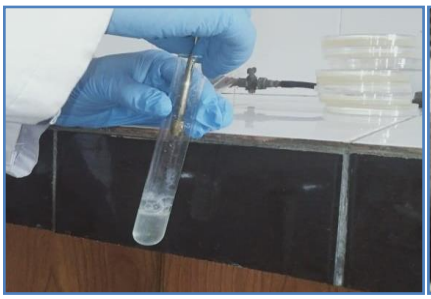

Figure 3. Catalase Test

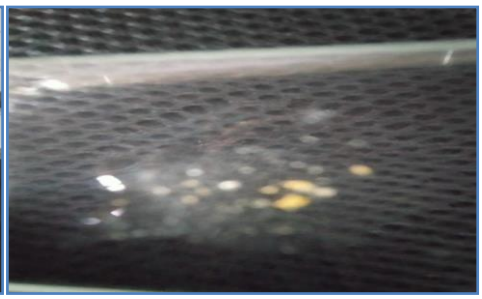

Figure 4. Coagulase Test

3.1.2 Antibiotic Susceptibility Test

The Kirby Baur technique was used for determination of the antibiotic resistance profile of the isolated *S. aureus* strains. Zones of antibiotic susceptibility testing are depicted in Figure 5 and table 3. The results showed that 100% of the 20 *S. aureus* isolated strains were resistant to penicillin, ampicillin, cefotaxime, oxacillin, and amoxi clavulanate drugs, while 100% were sensitive to gentamycin, amikacin, and clindamycin. 90% of all *S. aureus* isolates were resistant to cefoxitin, 75% to ciprofloxacin, 65% to erythromycin, 30% to tetracycline, 20% to streptomycin, vancomycin, and norfloxacin medicines, 15% to chloramphenicol, and 10% to kanamycin. showed in (table 4 and figure 7).

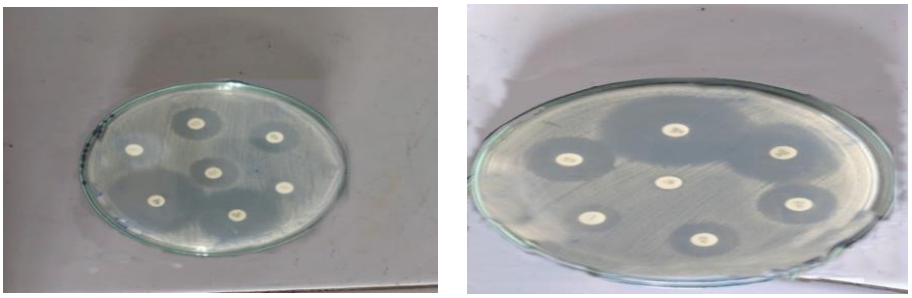

Figure 5. Antibiotics Susceptibility Test

Table 1. Average and standard deviation of zones of inhibition by antibiotics against MDR *S. aureus* isolates

Antibiotic	Zone of inhibition (mm) Average ± S.D
Penicillin	26.53333±0.503322
Gentamycin	13.33333 ± 1.527525
Streptomycin	18.666667 ± 0.5773503
Ampicillin	27.33333± 0.763763
Tetracycline	24.16667± 0.763763
Erythromycin	20.33333±1.527525
Amikacin	15 ± 1

Chloramphenicol	18.33333±1.527525
Ciprofloxacin	20.66667 ± 1.527525
Cefotaxime	27 ± 1
Kanamycin	18 ± 1
Oxacillin	22 ± 1
Norfloxacin	15.66667± 1.414214
Clindamycin	18 ± 1
Amoxi clavulanate	28.66667 ± 0.57735
Cefoxitin	21 ± 1.732051
Vancomycin	16.5 ± 1.5

Table 2. Antibiotics Susceptibility Test percentages of *S. aureus* Isolates

ANTIBIOTICS	SENSITIVE NO = 20 %	RESISTANT NO = 20%
Penicillin	(0%)	(100%)
Gentamycin	(100%)	(0%)
Streptomycin	(80%)	(20%)
Ampicillin	(0%)	(100%)
Tetracycline	(70%)	(30%)
Erythromycin	(35%)	(65%)
Amikacin	(100%)	(0%)
Chloramphenicol	(85%)	(15%)
Ciprofloxacin	(25%)	(75%)
Cefotaxime	(0%)	(100%)
Kanamycin	(90%)	(10%)
Oxacillin	(0%)	(100%)
Norfloxacin	(80%)	(20%)
Clindamycin	(100%)	(0%)
Amoxi clavulanate	(0%)	(100%)
Cefoxitin	10%)	(90%)
Vancomycin	(80%)	(20%)

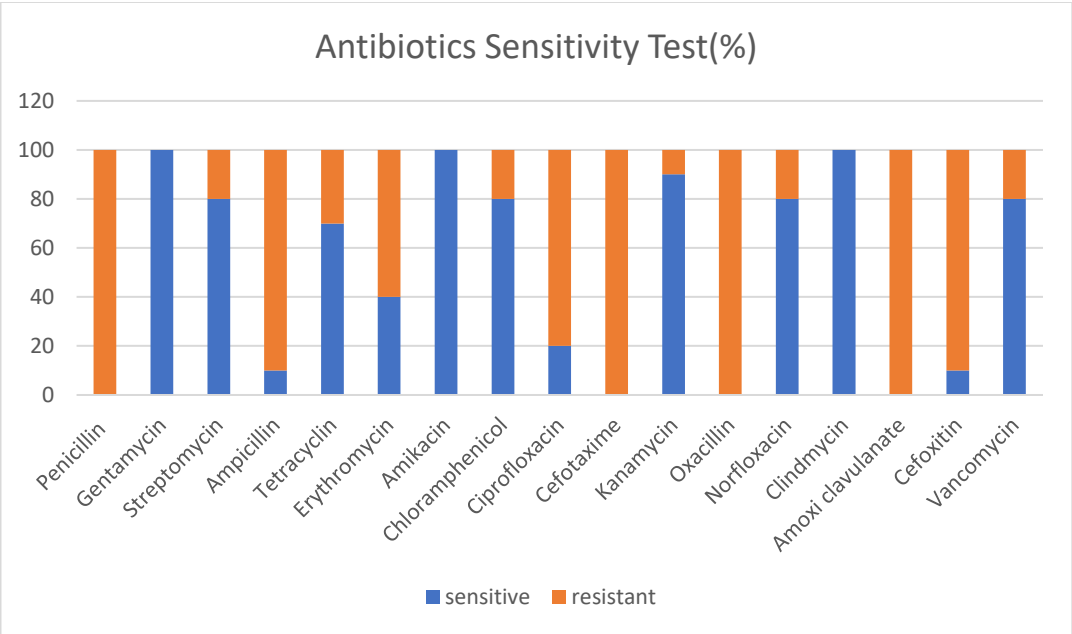

Figure 6. Displays Antibiotics Sensitivity Pattern of 20 *S. aureus* Strains Isolates

3.2 Bacteriophages Isolation

Bacteriophages were isolated using double layer agar technique on nutrient agar plates. Figure 7 depicts plaques of bacteriophages labelled with Layari and Bugro, while no plaque appears in the control plate, in which only MDR *S. aureus* was poured. Based on plaques, Bacteriophages were purified and confirmed. Plates labelled Bugro and Layari indicating the visible plaque produced by Bacteriophages. In the control plate, no plaque was seen.

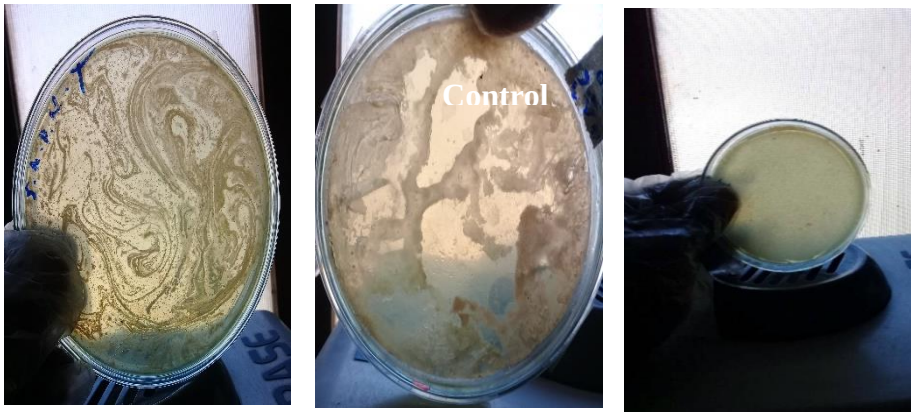

Figure 7. Plaques of Bacteriophages During Isolation Step

3.2.1 Antibacterial activity of phages using spot test against MDR *S. aureus*

Plates were also labelled according to sample, such as Layari and Bugro, for spot test. In spot test, using sterile pipette 10µL of pure phages were spotted on MDR *S. aureus* at selective segments, whereas control plates were spotted with sterile distilled water. Plates were checked after incubation. Bacterial cell lysis zones (plaques) were observed in plates, while dense bacterial growth was observed in control plates showed in figure 8.

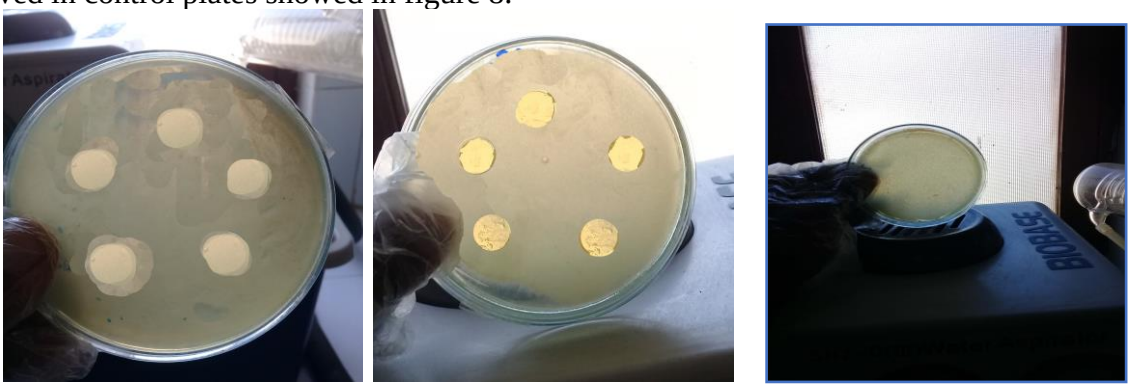

Figure 8. Plaques of Bacteriophages against MDR *S. aureus* Spot Test

Lysis Zones or Plaques were observed at spotted areas of bacteriophages against MDR *S. aureus*. And plate spotted with sterile distilled water displaying no zone or lysis plaque. This confirmed the antibacterial activity of bacteriophages against MDR *S. aureus*.

4 Discussion

Antibiotic resistance is becoming more common these days because of antibiotic overuse and misuse. In essence, the problem stems from the recent spread of resistance inside the community. MDR *S. aureus* is a serious problem, and managing the difficulties connected with this bacterium daily is getting increasingly challenging. Bacteriophages are a potential antibiotic replacement in the treatment of bacterial infections, and the purpose of this research is to isolate indigenous bacteriophages from sewage water sources and test their antibacterial effectiveness against MDR *S. aureus*.

In this study for MDR *S. aureus* isolation a total of 44 samples were collected. from 44 samples, 45.45% were positive for *S. aureus* and 54.54% were negative. The fact that 54.54% of samples were negative could be attributed to a variety of factors.

Differences in the prevalence of *S. aureus* strains between countries and hospitals may be explained in part by differences in the quality and size of samples, the use of different microbiological methods (from sampling technique to culture media) and different interpretation guidelines. Moreover, different levels of commitment to infection control measures may contribute to these differences *S. aureus* is responsible for a variety of human and animal ailments, including osteomyelitis, endocarditis, skin abscesses, and others [14]. Antibiotic misuse has resulted in the emergence of MRSA. MRSA strains are on the rise, highlighting the need for future therapeutic breakthroughs[15]. The results showed that 100% of the 20 *S. aureus* isolated strains were resistant to penicillin, ampicillin, cefotaxime, oxacillin, and amoxi clavulanate drugs, while 100% were sensitive to gentamycin, amikacin, and clindamycin. 90% of all *S. aureus* isolates were resistant to cefoxitin, 75% to ciprofloxacin, 65% to erythromycin, 30% to tetracycline, 20% to streptomycin, vancomycin, and norfloxacin medicines, 15% to chloramphenicol, and 10% to kanamycin. *S. aureus* strains that were multidrug resistant (MDR) were analysed. The multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan drug-resistant (PDR) bacteria have been thoroughly described, according to a guideline produced by the European Centre for Disease Control (ECDC) and the Center for Disease Control and Prevention (CDC), Atlanta. MDR was defined as acquired nonsusceptibility to at least one antimicrobial agent in three or more antimicrobial categories; Figure 7 illustrates a graphical representation of *S. aureus*' antibiotic susceptibility profile. The b-lactam antibiotics aminoglycosides, quinolones, macrolides, tetracycline, chloramphenicol, and vancomycin was all resistant to these *S. aureus* strains.

Bacteria may be resistant due to structural modification by enzymatic action that causes antibiotic inactivation; access to target was prevented due to altering the outer membrane permeability; antibiotic target site was altered, and efflux pumps may be involved, which pumps out the antibiotic and target enzyme bypass or overproduction was prevented; antibiotic target site was altered, and efflux pumps may be involved, which pumps out the antibiotic and target enzyme bypass or overproduction was prevented; antibiotic target site was altered, and efflux pumps may be involved, which pumps out the antibiotics [16]. Antibiotic-resistant bacteria treatment is a therapeutic issue. The susceptibility pattern can be used to predict future treatment problems.

To examine indigenous bacteriophages that are effective against MDR *S. aureus*, sewage water samples were collected from numerous areas in and around Khairpur. 90% of the samples tested positive for bacteriophages in our investigation, which is consistent with previous studies showing that bacteriophages may be found in sewage water [17,18].

The plaques developed by phages come in a variety of sizes and shapes. Clearing zones or plaque formation was observed on agar plates after 24 h of incubation. In the Nutrient Agar plates, these plaque forms suggest bacterial lyses or the presence of bacteriophage[18].

For the first time in clinical medicine, Felix d'Herelle described phage treatment [19]. Many experts believe that bacteriophages can aid in the fight against bacteria that are resistant to antibiotics, such as *S. aureus*. [17,20]. It is estimated that there are 10^{31} phage particles on earth, ten-fold more than bacterial population estimates, making phages the most abundant biological entities in the biosphere [21]. Most phages are found in the aquatic environment. In compared to saltwater and soil, sewage water is expected to have a phage count of 10^{8}-10^{10} [22].

In vitro, phages have been shown to be especially effective against a range of *staphylococcal* strains. Paul *et al*. Bacteriophage was discovered to be capable of killing >99.9% of *S. aureus* cells at a dosage of 2.5 g/mL. Bacteriophages killed 99.9% of a panel of 3000 *S. aureus* isolates, including MRSA (methicillin-resistant *S. aureus*), MSSA (methicillin-sensitive *S. aureus*), and mupirocin-resistant strains (at a dosage of 10 g/mL) [23]. MRSA and MSSA 48-hour biofilms might be reduced by up to 95.5 percent with 12.5 g/mL doses [24]. P128 Bacteriophages has also been shown to be effective in vitro in combination with standard-of-care anti-*staphylococcal* antibiotics such as daptomycin,

vancomycin, linezolid, gentamicin, ciprofloxacin[25], oxacillin [26], and cephazolin for treatment of sensitive and resistant *staphylococcal* isolates [26].

Staphylophages (30 mg/mL) were efficacious against 28 MSSA and 8 MRSA bacteria in an in vitro lysis experiment (OD reductions of 58.6 percent and 54.1%, respectively, compared to untreated controls) [27]. *Staphylococcus* epidermidis, S. hominis, *Staphylococcus* haemolyticus, and *Staphylococcus* lugdunensis strains, on the other hand, were only little harmed (1–15% reduction) [27]. This study emphasizes the importance of phage therapy as a potential treatment option [20].

Phage therapy has a wide range of potential applications in human medicine and dentistry, as well as veterinary science and agriculture, and it can be an effective alternative therapy, especially for multidrug-resistant bacteria [28,29]. There are several phages on the earth that are capable of eliminating a wide range of bacterial populations [28].

Phage treatment is less expensive and has less adverse effects on eukaryotic cells than traditional antibiotics. On bacterial cell membranes, phages have complementary receptors [30].

5 Conclusion

Bacteriophages were isolated from sewage wastewater and confirmed on the basis plaques formed by bacteriophages. and the MDR *S. aureus* that served as its host was isolated from external ear canal samples. Spot tests were used for to examine the antibacterial activity. The phages in this investigation have a high level of bacterial lysis activity against MDR *S. aureus*. However, these isolated phages must be further characterized before being employed in commercial lysate preparations, while their therapeutic potential against a larger spectrum of bacterial strains can be studied. In future research may be carried out in animal models and, eventually, clinical trials, which may give a powerful alternative treatment against the threats of multi drug resistant diseases.

Conflict of Interest

All contributing authors declare no conflicts of interest.

Author Contributions

SL and AAM helped develop the topic, design, supervise, correct, and approve the text. IK, ZAS, SFK, and SK helped in the analysis and writing the first draft, did the experimental analysis. MS and SA contributed to manuscript writing and IK wrote the final manuscript.

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