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EVALUATION OF PHAGE ACTIVITY AGAINST ANTIBIOTIC-RESISTANT ENTEROCOCCUS SPECIES

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Abstract

Enterococcus faecalis is an opportunistic pathogen native to intestinal microbiota that causes severe infections including bacteremia, endocarditis, and neonatal sepsis. Multidrug-resistant (MDR) E. faecalis strains were isolated from clinical samples using Bile Esculin Azide agar, biochemical profiling, and microscopy. Antibiotic susceptibility testing confirmed resistance to tetracycline, ciprofloxacin, chloramphenicol, and vancomycin, with retained sensitivity to rifampin. This resistance demands an immediate therapeutic alternate and phages serve to be the suitable alternative. Lytic phage PA-814 was isolated from hospital sewage and evaluated as antibiotic alternatives. Phage PA-814 significantly suppressed bacterial growth for 16 hours compared to controls. Phage PA-814 exhibited narrow host range and optimal stability at 37°C and pH 7. DNA-based genome was confirmed via nuclease treatment and electrophoresis. These findings support phages as promising therapeutics against MDR E. faecalis infections.

1. Introduction

Enterococcus is a genus of facultative anaerobic, Gram-positive cocci that was formerly classified as group D streptococci. These bacteria are commensals of the gastrointestinal tracts of humans and animals and have a striking capability to survive in harsh environmental conditions. Even though Enterococcus species form part of the normal intestinal flora, certain species, most notably *Enterococcus faecalis* and *Enterococcus faecium* have emerged as significant opportunistic pathogens associated with nosocomial infections and multidrug resistance (MDR) (Gilmore et al., 2013). Enterococcal bacteremia is linked to extended hospitalization and significant mortality burden. Of the genus, *E. faecalis* and *E. faecium* are the most implicated species in the cause of bloodstream infection (Kalode & Patil, 2023). Though several species of *Enterococcus* have been identified, *E. faecalis* is by far the most common isolate, representing about 80–90% of nosocomial enterococcal infections, while *E. faecium* accounts for about 10–15% of the infection burden. The *Enterococcus* species have also been identified as common etiological agents of lower urinary tract infections such as prostatitis, cystitis, and epididymitis (Al Bshabshe et al., 2024).

The ability of *E. faecalis* and *E. faecium* to survive in a hostile, antimicrobial-rich environment is the main factor contributing to their success as hospital-associated pathogens. Both species have pathogenic potential and capacity to spread illness have been connected to a number of characteristics. They can attach to inert materials and the extracellular matrix to the host cells. including various medical devices, they can also avoid the immune system and they can build biofilms that increase their resistance to phagocytic attack and antibiotic death. *E. faecalis* exhibits greater virulence factors, which may account for its continued dominance in enterococcal infections. Numerous proteins have been identified as contributing to pathogenicity (García-Solache & Rice, 2019). For instance, a collagen-binding protein was found in high frequency in urinary isolates of *E. faecalis* (Codelia-Anjum et al., 2023). Virulence factors are a number of additional strategies that *Enterococcus* uses to boost its pathogenicity. Molecules known as virulence factors make bacteria more harmful and help them survive and colonize their host environment. Collagen binding protein, TcpF, gelatinase, enterococcal surface proteins, aggregation compounds, and pilin gene clusters (PGCs) are a few known virulence factors present in urine isolates of *Enterococcus* species. Bacterial aggregation is caused by aggregation substance (AS), a required adhesion on the surface that mediates adherence to host cells makes it easier for pheromone-responsive plasmids to conjugate with cells. For instance, a collagen-binding protein was found in high frequency in urinary isolates of E. faecalis (Codelia-Anjum et al., 2023). For instance, a collagen-binding protein was found in high frequency in E. faecalis (Codelia-Anjum et al., 2023). The use of phages in therapy is conceptually simple; there are side effects that may otherwise necessitate extremely complicated pharmacology. The ability of phages to proliferate and exert antibacterial action is the primary cause of this issue. Phages must reach bacterial targets in large enough quantities to surpass a minimum inhibitory concentration just like any antimicrobial medications. Successful phage penetration can be facilitated by a combination of the phages capacity to lyse bacterial biofilm (such as during bacterial biofilm removal) and its capacity to penetrate target bacteria (Chan et al., 2013)).

2. Methodology

Sample Collection and Isolation

This study was planned and carried out at the University of Haripur's Microbiology Research Laboratory. Based on sampling convenience, sewage samples for phage isolation were gathered from a number of hospitals in the Haripur district, including Yahya Hospital and DHQ Hospital Haripur. Samples were carried to the lab in falcon tubes containing 50 milliliters of sewage water and sterile swabs.

Collection and maintenance of *Enterococcus* clinical isolates

The Pakistan Institute of Medical Sciences Islamabad supplied all of the bacterial isolates utilized in this study. Fresh Bile Esculin Azide Agar (BEA) was streaked with bacterial samples and overnight incubated at 37 °C. Before every experiment, freshly subcultured bacterial samples were used with log phase bacterial cultures.

Culturing and identification of bacterial isolates

Primary culturing on Bile Esculin Azide Agar

Bile Esculin Azide Agar (BEA) was prepared according to instructions of the manufacturer. To prepare 1000ml of BEA, 44.5gram of BEA powder was dissolved in 1000ml of distilled water. Appropriate quantity of BEA powder was measured and added to clean conical flask. It was heated on hot plate until it became a clear solution. Flask was autoclaved at 121°C under 15 psi pressure for 15 minutes. After sterilization, media was poured into sterilized glass Petri-dishes in biosafety cabinet and left to solidify media and samples of *Enterococcus spp* streak on plates and check results after 24 hours and pure culture is formed by re-streaking.

Sub-culturing and pure culture preparation

To keep the cultures of *Enterococcus species* fresh and viable for additional analysis, sub-culturing was done. The quadrant streak method was used to create pure cultures by selecting well-isolated colonies from primary plates and re-streaking them on sterile bile esculin azide agar. Every transfer was carried out aseptically with sterile inoculating loop. The inoculation plates were incubated for 24 hours at 37°C. Pure colonies were chosen by selecting single colonies with the same morphology.

Biochemical identification of bacterial strain

Enterococcus faecalis was identified, and Gram-positive traits were distinguished using biochemical assays including Simmon's citrate, catalase, oxidase, and Gram staining. The bacterial strains were kept at -20°C in LB broth with 30% glycerol to preserve their viability.

Antimicrobial Susceptibility Testing of *Enterococci* species

The Kirby–Bauer technique was used to test the antibiotic susceptibility of *Enterococci* species grown on bile esculin (Alhamadani, 2025). For testing, making 1000ml MH agar, 38g of Muller Hinton Agar was taken and mixed with 1000ml distilled water. The autoclaved MHA was used for the purpose of sensitivity.

Sample collection for phage Isolation

Wastewater samples were collected from various hospitals located in Haripur, Khyber Pakhtunkhwa, Pakistan. A total of ten samples were obtained and promptly transported to the Department of Microbiology at the University of Haripur for further microbiological analysis and processing.

Initial confirmation of phages

Isolation of phages against *Enterococcus* species were carried out from wastewater collected from different hospitals of Haripur. Phages were isolated following an already established method (Adnan et al., 2020). Briefly, 40-ml sewage sample and 1ml of log phase bacterial culture was added to 10ml of 5X NB broths and incubated for 18–24 hours at 37°C and 120 rpm in a shaking incubator. Following that, silt, germs, and other debris were eliminated by centrifuging for 15 minutes at 8,000 rpm. The supernatant was collected in a fresh, clean falcon tube after filtering with a sterile (0.22 micrometer pore size) syringe filter.

Enrichment of Phages

For phage enrichment from sewage samples, a modified protocol by (Nasr-Eldin et al., 2021) was used. After collection, all sediments, other large particles, and pathogens were eliminated from the sewage by centrifuging it at 1000 rpm for 10 minutes. Log phase bacterial culture (1 ml) was introduced after supernatant (40 ml) and 5X NB broth (10 ml each) had been combined. The mixture was then shaken at 200 rpm for 18 to 24 hours at a temperature of 37 °C. The supernatant was filtered through a 0.22 micrometer syringe filter. Afterwards, 100µl bacteria and 3ml soft agar (0.7%) was mixed and overlaid on nutrient agar plate and allowed to dry. Following that, 10µl filtrate was further spotted on the agar plate and incubated at

37°C for 24 hours. The results of spot assay were recorded on next day.

Purification of Phages

Phage were purified as described (Mazzocco et al., 2009), by picking clear phage plaques with the help of sterile tips, resuspend in phage buffer, 1 ml phage buffer with pH 7.2. The phage buffer having phage plaque was further centrifuged for 10 min, at 8000 rpm and passed through 0.22 micrometer syringe filters. The process was repeated again following filtration through the plaque assay technique. It was repeated 5 to 6 times to obtain pure plaques on plate and pure plaques were stored at 4 °C for further use.

Table 1: ***SM Buffer or phage buffer preparation (pH at 7 to 8)***

INGRADIENTS	QUANTITY
NaCl	2.9gm
$MgSO4·7H2O$	1gm
1M Tris Cl	25ml
Distilled water	upto500ml

Phage-Titer Determination

Using (Adams, 1959) double-layer agar method, phage titration was carried out and 100µl of each successive dilution of the phage stock solution were added to several sterile test tubes. Each test tube received 100 µl of the bacterial culture before being left at room temperature for 10 minutes. Following this, 3 ml of 0.7% top agar were added. Mixed thoroughly and poured onto sterile nutritional agar plates. Top agar was Left to dry for 10 to 15 minutes and plates were incubated for 24 hours at 37°C. A plate with a definite clear plaque was then picked for phage titer investigation.

3. Characterization of Phages

Thermal Stability Assay

To assess the stability of isolated phages at each of the following temperatures: 20°C, 30°C, 37°C, 45°C, 55°C and 65°C phage aliquots from this experiment were incubated for 1 hour at each of these temperatures. After an hour, these phages aliquots were brought to room temperature followed by double layer agar assay and titer of the phages was calculated as pfu/ml.

pH Stability Assay

Phage efficiency on pH with a wide range was examined using the method employed by (Verma et al., 2009)with just minor modifications. Briefly, different pH values used for pH determination were pH 2, 4, 6, 7, 8, 10 and 11. Phage suspension with a variable pH was added to 9 ml of tryptic soy broth (TSB), with incubation at 37°C for 24 hours. Plaque assay technique was used to evaluate the phages viability and effectiveness.

Host Range Determination

For determining phage host range, 6 isolated strains of *Enterococcus* species were selected for activity of phage against them. Spot assay was used to determine the host range. The plates were observed for plaques formation on next day, after overnight incubation.

Phage Genome Extraction

Phage lysate (100 L) was treated with DNase and RNase to extract bacterial DNA/RNA before incubation for four hours at 37°C. After adding Proteinase K, EDTA, and SDS and the mixture was incubated for an hour at 55°C after 20 minutes at 65°C (to denature DNase and RNase). After the incubation period, PCA (phenol chloroform isoamyl alcohol) was added in an equivalent volume, and the mixture was centrifuged for 10 minutes at 13,000 rpm. The resultant supernatant was then combined with sodium acetate (50 L for a 1ml combination) and 95% cold ethanol. After ice placement for five minutes, the mixture was centrifuged at 13,000 rpm for ten minutes. The resulting pellet was then treated with 70% ethanol and spined for 5 minutes after the supernatant was collected. Blotting paper and TAE buffer were used to recover the pellet after the supernatant was discarded.

Electrophoretic characterization of Phage genome

The isolated genome was placed in two different reaction tubes, treated with DNase 1 and the other with RNase A. Before visualization in 1% agarose gel with 1% TAE buffer, both reaction solutions were incubated for 4 hours at 37°C (Khawaja et al., 2016). If a band occurred in a combination that had been subjected to DNase treatment, the genome is likely RNA; if it appeared in a mixture that had undergone RNase treatment, the genome is likely DNA.

4. Results

Isolation and Identification of *Enterococcus Faecalis*

Microscopic examination of *E. faecalis* revealed small round shaped, gram positive cocci, usually appearing as gray or grayish white colonies on BAE. Figure 1 illustrates the growth characteristics of the isolate. The oxidase test yielded a negative result, as no color change was observed. Similarly, the catalase test was negative, indicated by the absence of bubble formation (Figure 2). In the Simmons citrate test, no color change was detected, confirming a negative reaction.

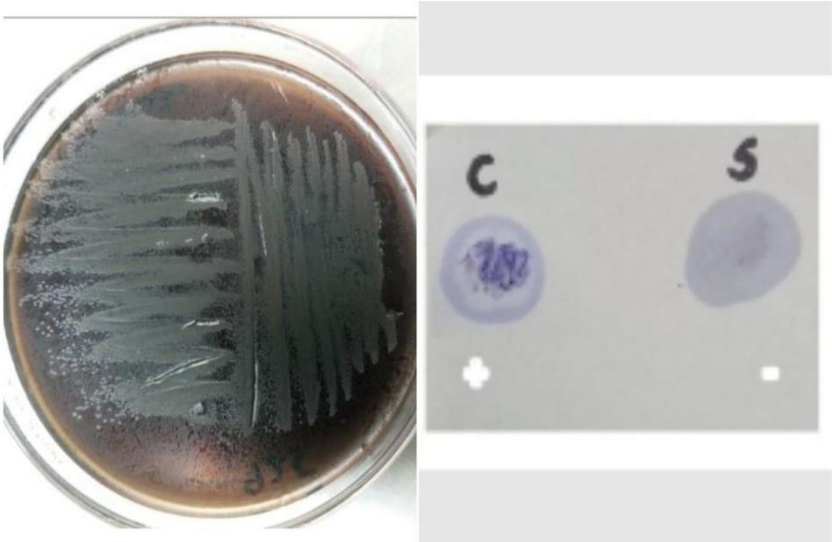

Figure 1: Growth and oxidase test result of Enterococcus faecalis

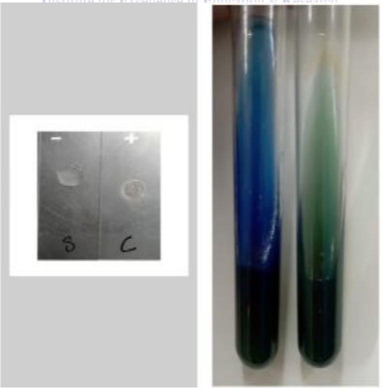

Figure 2: Catalase and simmon citrate test result of Enterococcus faecalis

Antibacterial susceptibility test of *E. faecalis*

Enterococcus faecalis showed resistance against Tetracyclin, Ciprofloxacin, Chloramphenicol, Vancomycin and was sensitive to Rifampin as shown in Figure 3.

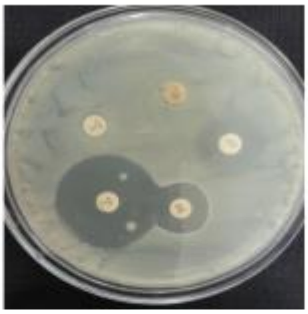

Figure 3: Antibiotic resistant test of Enterococcus faecalis

Table 2: *Inhibition zones (mm) formed by Enterococcus Faecalis against different antibiotics*

Antibiotics	EF-814	EF-925
Tetracyclin (TE 30)	0	0.8
RD 5	24	27
Ciprofloxacin (CIP 5)	0	0
Chloramphenicol (C 30)	14	22
Vancomycin (VA 30)	13	14

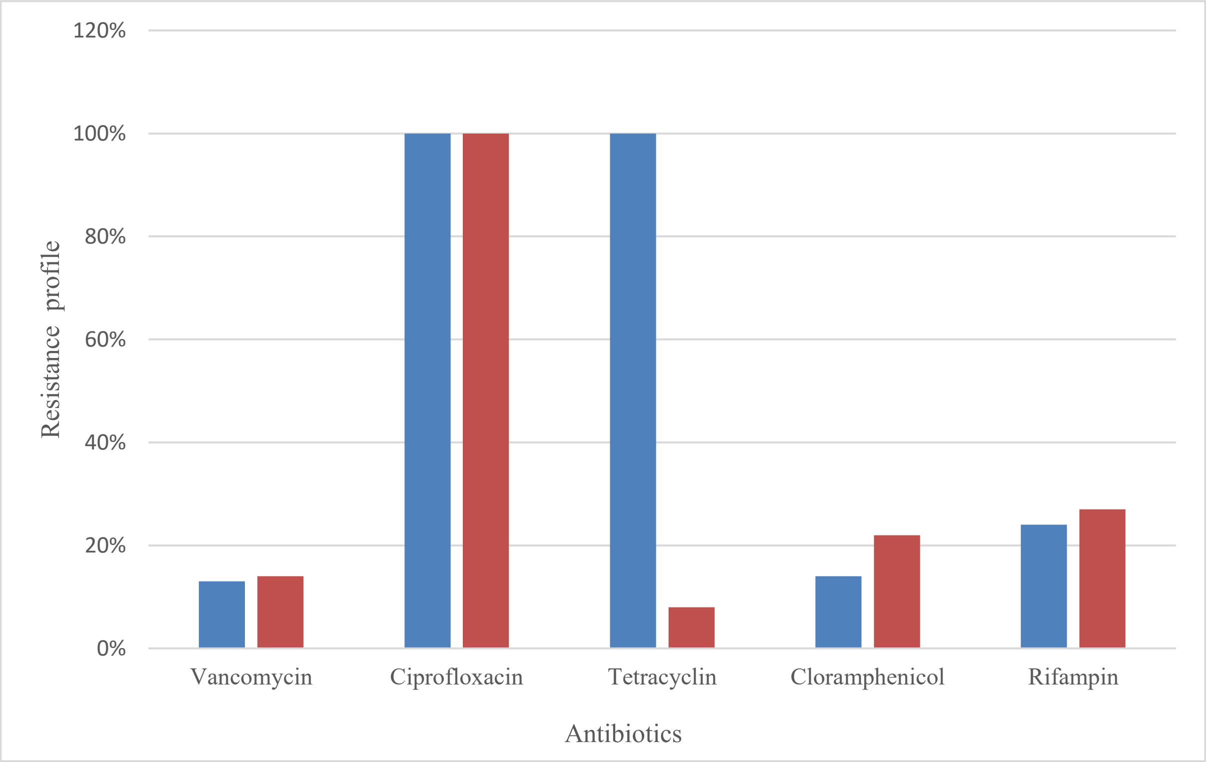

Figure 4: Antibiotic resistance profile of Enterococcus faecalis

Table 3: *CLSI antibiotics breakpoint*

ANTIBIOTICS	RESISTANT ≤	INTERMEDIATE	SENSITIVE ≥
Tetracyclin 30	≤ 14	15-18	≥ 19
Rifampin 5	≤ 16	17-19	≥ 20
Ciprofloxacin 5	≤ 15	16-20	≥ 21
Chloramphenicol 30	≤ 12	13-17	≥ 18
Vancomycin 30	≤ 14	15-16	≥ 17

ISOLATION AND CHARACTERIZATION OF PHAGES

ISOLATION OF PHAGE

Phage PA814 was isolated from sewage water showing positive spot test. It was further evaluated by double layer agar (DLA) assay where phage 814 produced clear lytic plaques with 2-3mm diameter. Phage 814 was further purified 8 times by picking same size plaque from each DLA assay plate (Figure 5).

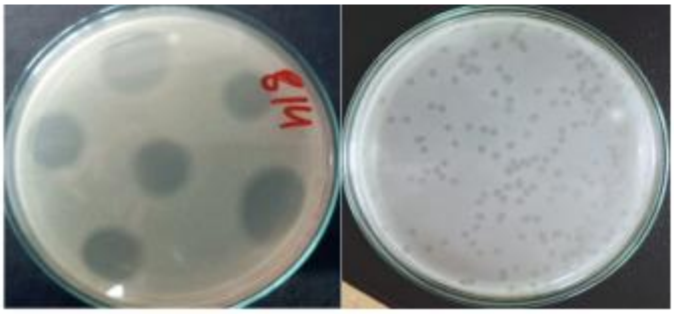

Figure 5: Phage PA814 spot and DLA assay against E. faecalis

CHARACTERIZATION OF PHAGES

Host Range of phage

The Spot assay method was utilized to observe the host range of phage PA-814 6 distinct bacterial strains where Phage PA-814 showed lytic activity against three bacterial strains.

Table 5: Host Range of Phage PA-814 against Different Strains of *E. faecalis* (+) = Lytic, (-) = Non- lytic or Resistant.

Bacterial Strain	Phage PA814
925 *Enterococcus faecalis*	+
814 *Enterococcus faecalis*	+
867 *Enterococcus faecalis*	+
967 *Enterococcus faecalis*	-
510 *Enterococcus faecalis*	-
862 *Enterococcus faecalis*	-

pH Stability of Phage:

Phage PA814 was incubated for pH stability for 1 hour on different pH (2, 4, 6, 7, 8, 10 and 11). Phage PA814 was more stable at pH 7, while increase and decrease of pH cause reduction in the PFU of phage PA814. Phage PA814 showed better stability at pH 6, 7 and 8 (Figure 7).

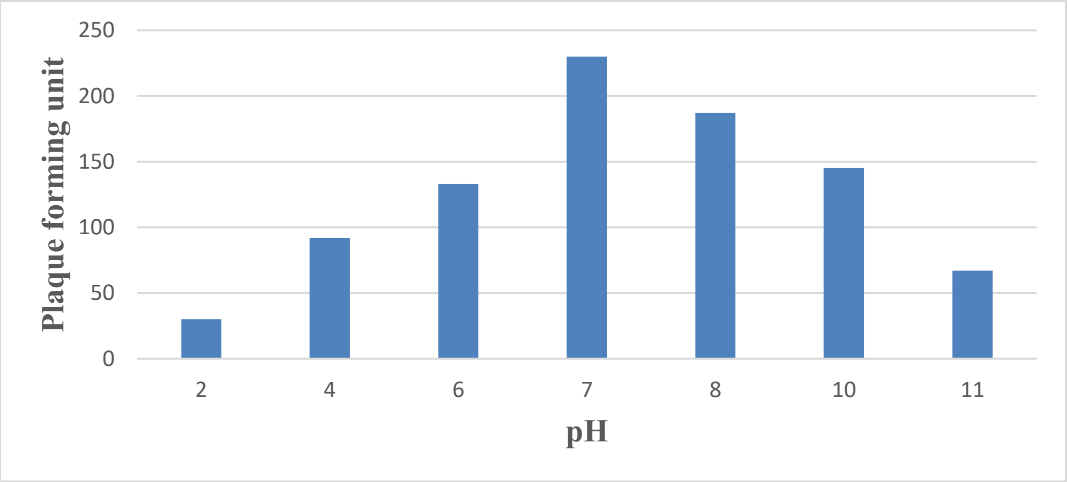

Figure 7: pH stability test of Phage PA814 on various pH

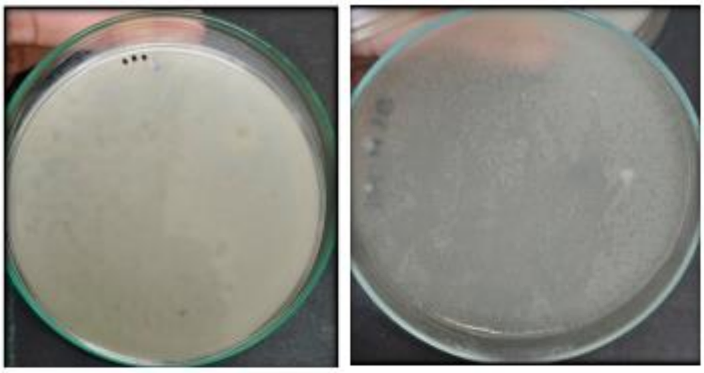

Figure 8: pH stability test of Phage PA814 on various pH

Thermal stability of Phage

Phages have ability to survive according to host temperature. Isolated phages were checked for one hour at different temperatures at 20°C, 30°C ,37°C ,45°C ,55°C ,65°C and pfu/ml was calculated. Phage-814 showed significant growth at range of 30°C to 50°C and easily survived but at 65°C, pfu/ml was decrease as shown in Figure 9.

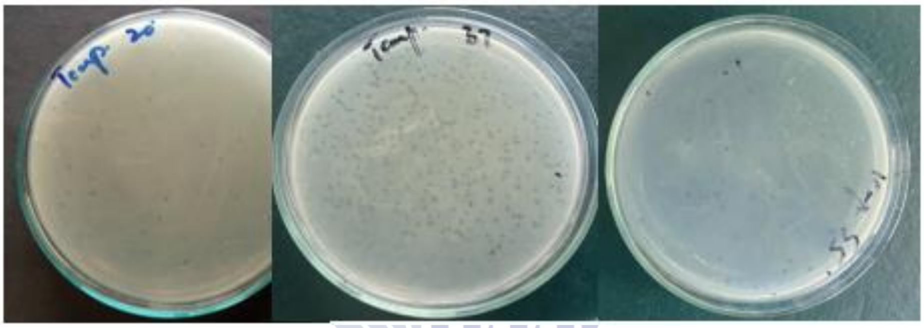

Figure 9: Thermal stability test of Phage PA-814 on various temperature

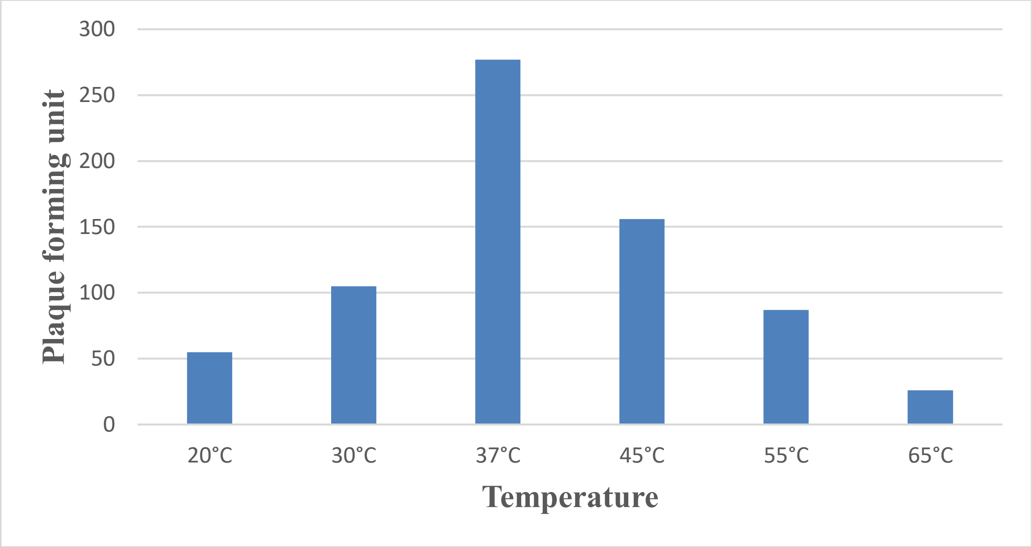

Figure 10: Plaque assay result at different temperatures (20 °C, 30 °C, 37°C, 45°C, 55°C, 65°C)

Gel Electrophoretic of phage genome

Genomic characterization, which reveals the characteristics of the genome, is a crucial criterion for classifying phages. On gel electrophoresis, the mixture treated with RNases had a visible band, whereas the mixture treated with DNases showed no band, indicating that the extracted genome of the separated phage was DNA as shown in Figure 11. The findings showed that the phage's genome was larger than 10 kb. A high concentration and purity of retrieved phage DNA are shown by the band in lane 2 appearing quite clearly in the gel picture.

Figure 11: Lane 1 DNA ladder 10kb, Lane 2, completely digested genome with DNase and Lane 3 Genome treated with RNase (undigested)

Discussion

Phage therapy has drawn interest lately for the treatment of bacterial infections, such as infection caused by MDR *E. faecalis*. The rise in antibiotic resistance and the ability of phages to infect and eradicate bacteria has increased interest in phage therapy. Phages have proved to be an effective, safe, and natural way to stop and manage MDR pathogens (Gebara et al., 2019).

Drummer Frederick Twort (Fildes, 1951) made the first independent discovery of phages called "bacterial eaters" in 1915. The growing antibiotic resistance of bacterial strains led to severe medical and social issues. Phages were previously utilized to treat a variety of human bacterial infections.

The rise of MDR bacterial pathogens has emerged as a serious global health concern, limiting the effectiveness of conventional antibiotics and increasing morbidity and mortality rates, especially in hospital settings (Arshad et al., 2024; Miller et al., 2014). Among these, *Enterococcus faecalis* has become a notorious pathogen due to its intrinsic and acquired resistance mechanisms that render many standard antibiotics ineffective (Fiore et al., 2019).

In our study, we specifically focused on the evaluation of phage as a potential alternative or complementary tool to manage infections caused by antibiotic-resistant *E. faecalis*. Our antibiotic susceptibility profiling revealed resistance to tetracycline, ciprofloxacin, and vancomycin among the tested *E. faecalis* isolates. This pattern aligns with recent surveillance reports highlighting the increasing occurrence of vancomycin-resistant enterococci (VRE) in clinical and environmental samples (Arias & Murray, 2012); Miller et al., 2014).

Interestingly, our isolates retained susceptibility to rifampin, suggesting a potential therapeutic window. While rifampin monotherapy is not recommended due to rapid resistance development, it remains valuable in combination regimens against resistant enterococcal infections (Skinner et al., 2017). The presence of rifampin sensitivity in our isolates could be clinically significant for designing combination treatments, especially in resource-limited settings.

To address the rising resistance, phage therapy has re-emerged as an innovative biological control approach due to phages' specificity, self-replicating nature, and ability to destroy bacterial hosts. In our study, more than 20 wastewater samples were screened for phage isolation. This sampling strategy proved fruitful, yielding five lytic phages specific to *E. faecalis* where PA-814 was further characterized based on the host range.

Wastewater and sewage are well-documented sources for phage hunting due to their high bacterial load and diverse phage populations (Matos, 2024).

Following isolation, we examined the thermal and pH stability of the recovered phages to evaluate their practical applicability. The phages demonstrated significant lytic activity at temperatures 30°C, 37°C, and 45°C, indicating their robustness under physiological and slightly elevated temperature conditions. This observation aligns with (Bao et al., 2020), who reported that phages infecting *Enterococcus* species maintained infectivity across a moderate temperature range, a desirable feature for therapeutic applications.

Similarly, the pH stability assay showed that the isolated phages remained viable and retained lytic ability in the pH range 6, 7, and 8. This finding aligns with (Chaturongakul & Ounjai, 2014) who described that most therapeutic phages targeting Gram-positive bacteria demonstrate optimal activity in mildly acidic to neutral environments. The ability to withstand a broad pH range enhances the potential use of these phages in various infection sites, including the gastrointestinal tract, where pH can fluctuate.

A notable observation in this study was that the isolated phages displayed a narrow host range, efficiently lysing only specific *E. faecalis* strains. *Enterococcus* phages typically have narrow host ranges due to their dependence on specific bacterial surface receptors for attachment (Kortright et al., 2019). While a narrow host range reduces the risk of off-target effects on beneficial microbiota, it may limit the effectiveness of monophage preparations in diverse clinical settings where multiple resistant strains circulate.

To address this, many researchers recommend the phage cocktails development, which combines many complementary phages host range to enhance spectrum and reduce the emergence of phage-resistant mutants (Abedon et al., 2011)). Future work based on these findings could focus on formulating cocktails, optimizing phage dosing strategies, and exploring synergistic effects with antibiotics, to maximize therapeutic efficacy.

It is also important to consider potential limitations. Although in vitro results showed promising lytic activity and stability under varied conditions, further characterization, such as whole-genome sequencing of the isolated phages, is essential to confirm the absence of undesirable genes, including toxin or lysogenic conversion genes (Young et al., 2022). Moreover, preclinical in vivo studies should be carried out to evaluate safety, immunogenicity, and pharmacokinetics before clinical use can be recommended.

In conclusion, this study highlights that phages isolated from wastewater environments possess significant lytic potential against multidrug-resistant *Enterococcus faecalis*. These phages demonstrated robust stability over a practical range of temperatures and pH, which is critical for real-world application. However, the narrow host range indicates the need for phage cocktail development and further molecular characterization. Our results contribute to the growing evidence that phage therapy could be an effective and sustainable solution to tackle antibiotic resistance in *Enterococcus* species, complementing existing treatment options and paving the way for future clinical applications.

Conclusion

The phage against *Enterococcus faecalis* was isolated from wastewater samples as a result of this study, and it was subsequently further described by identifying several essential features for its stability. The current investigation concluded that the isolated phage exhibited lytic activity against the isolated *E. faecalis*. According to the current investigation, the phage exhibited a limited host range, meaning that it exhibited lytic activity against distinct *E. faecalis* strains but not against other taxa. Phage lytic activity was seen at temperatures ranging from 30 to 65°C, with the best efficacy occurring at 37°C.

Due to these properties, this phage is very appealing for possible application in both the elimination of *E. faecalis* and the treatment of illnesses associated with it. The whole phage or its lytic protein may be used to treat infectious illnesses. It is commonly known that phage treatment can be used in place of antibiotics. Emerging technologies such as bioengineered chimaeras indicate phage-derived lytic proteins as a new class of antimicrobials. Treatment using phages is often considered an alternative to antibiotics.

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