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Characterization and Encapsulation of Bacteriophages Isolated from Sewage Investigating its Impact on Multiple Drug Resistant Organisms: A Comparative Study

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ABSTRACT

This study aimed at isolating and characterizing multidrug-resistant *Klebsiella pneumoniae* and *Staphylococcus aureus* from sewage samples, and to investigate the stability and release kinetics of encapsulated bacteriophages targeting these pathogens. Sewage samples were collected, processed and subjected to serial dilution and bacterial isolation on selective media. Isolates were identified via gram staining, biochemical tests and 16S rRNA sequencing. The Kirby-Bauer disc diffusion method was employed to determine the antibiotic resistance profiles of the bacterial isolates, while the plaque assay method was used to quantify phage activity.Antibiotic sensitivity assays confirmed multidrug resistance in both bacteria. Bacteriophages were isolated from sewage and encapsulated using chitosan-alginate beads, with and without alum as an adjuvant. Encapsulated and non-encapsulated phage were tested for stability and release under simulated intestinal and thermal conditions. Results indicated that encapsulation significantly improved phage stability and controlled release, particularly in simulated intestinal environments. Encapsulation with alum further enhanced release kinetics. Thermal stability assays at 37 and 80°C showed variable phage stability, with encapsulated phage exhibiting better resilience compared to non-encapsulated ones. Statistical analyses (t-tests and ANOVA) confirmed significant differences in release behaviours between different encapsulation conditions. These findings suggest that the encapsulation, especially with adjuvant, enhances phage stability and release, offers a promising strategy for phage therapy applications targeting gastrointestinal infections. Further research into optimizing encapsulation methods could improve the therapeutic efficacy of bacteriophage treatments.

Key words: Bacteriophages, encapsulation, chitosan-alginate, stability, release kinetics, antibiotic resistance, *Klebsiella pneumoniae, Staphylococcus aureus*

INTRODUCTION

The escalating challenge of emergence of antibiotic resistance, MDR strains present a significant public health threat globally, necessitating the exploration of alternative antimicrobial strategies (Fish *et al.,* 2016). Both *Klebsiella pneumoniae* and *Staphylococcus aureus*, notorious for their roles in nosocomial infections and their capacity to develop resistance to multiple antibiotics (Basak *et al.,* 2016), were chosen as target pathogens. The study aimed at isolating and identifying MDR strains of *K. pneumoniae* and *S. aureus* from sewage samples (Ben *et al.,* 2017), which serve as reservoirs for diverse and often resistant microbial populations (Kebriaei *et al.,* 2023). Conventional disinfectants are increasingly ineffective against pathogenic and MDR pathogen and MDR bacteria (Samar *et al.,* 2023). One promising approach is bacteriophage therapy, which utilizes viruses that specifically infect and lyse bacterial cells (Fauconnier, 2019). Bacteriophages, or phages, have been recognized for their potential to combat multidrug-resistant (MDR) bacterial infections. Studies have isolated bacteriophages from sewage samples, such as En5822 and EMCL318, showing high lytic activity and specificity against drug-resistant *Enterobacter cloacae* and *Escherichia coli* strains (Soumya *et al.*, 2018; Nair *et al.,* 2022). These phages exhibit notable antimicrobial properties, including biofilm reduction capabilities, short latent periods and high burst sizes, making them effective against drug-resistant bacteria (Nitasha *et al.,* 2021; Samar *et al.,* 2023). Additionally, the isolation of a *P.*

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aeruginosa bacteriophage, AM.P2, from wastewater in India, highlights its potential in inhibiting multidrug-resistant clinical isolates, suggesting its utility in phage therapy and environmental decontamination. However, phage therapy faces several obstacles, including maintaining phage viability, ensuring targeted delivery to infection sites and protecting phages from harsh environmental conditions within the gastrointestinal tract (Lin *et al.,* 2017). Encapsulation technology offers a viable solution to enhance the stability and efficacy of phage therapy. Encapsulation involves encasing phages in biocompatible materials, thereby shielding them from adverse conditions and facilitating controlled release (Abdelsattar *et al.,* 2019). This study investigated the encapsulation of bacteriophages in chitosan-alginate beads, a method noted for its simplicity, cost-effectiveness and potential for protecting phages during oral administration.

The research further explored the encapsulation of isolated phages with and without an alum adjuvant. Alum, known for its immunostimulatory properties, was included to investigate whether it could enhance the therapeutic efficacy of encapsulated phages (Liu *et al.,* 2018). Filamentous phage vB-AbaI-TMU2 was isolated from sewage in Tehran and was found to be stable at pH 5 and 7, temperatures 25 and 37°C (Esmaeili-Fard-Barzegar *et al.,* 2022). By addressing the challenges associated with phage stability and delivery, this study seeked to pave the way for more reliable and effective phage-based treatments for bacterial infections, particularly those caused by MDR pathogens.

MATERIALS AND METHODS

Plastic bottles were utilized for the collection of sewage samples, ensuring a volume of 1 litre per sample from various depths. After thorough mixing, the samples were stored at 4°C. Each sample underwent serial dilution up to 10^{-9} using sterilized distilled water. For the isolation of *Klebsiella pneumoniae*, 500 µl from each dilution was spread onto MacConkey agar plates and then incubated at 37°C for 24 h. Pure *Klebsiella* spp. colonies were sub-cultured onto Eosin Methylene Blue (EMB) agar plates and nutrient agar slants. A total of 80 isolates were recovered from 25 samples, identified by their mucoid appearance and Gram staining. Similarly, for the isolation of *Staphylococcus aureus*, 500 µl from each dilution was spread onto Mannitol Salt Agar (MSA) plates and then incubated at 37°C for 24-48 h. A total of 50 isolates were recovered from 25 samples, identified by the appearance of yellow zones and Gram staining. Successful growth of microorganisms led to further sub-culturing of suspected *S. aureus* colonies onto blood agar plates and nutrient agar slants, followed by additional incubation at 37°C. The serial dilution method facilitated the individual colony isolation from sewage samples.

Gram staining was performed to differentiate Gram-positive and Gram-negative bacteria, and morphological characteristics were observed under a microscope. Biochemical tests, including Oxidase, Triple Sugar Iron, Simmons Citrate Agar, Indole and Urease tests, were conducted for further characterization of *K. pneumoniae*. Similarly, biochemical tests, including Catalase test, Coagulase test and Mannitol Salt Agar (MSA) fermentation, were conducted for further characterization of *S. aureus*.

The Kirby-Bauer disc diffusion method was employed following CLSI guidelines. Sterilized Muller Hinton Agar plates were inoculated with bacterial suspension and antimicrobial discs, followed by overnight incubation at 37°C. Inhibition zones were measured and seven antimicrobial discs were placed onto the agar surface for *K. pneumoniae*, while seven antimicrobial discs were placed for *S. aureus*. Multidrug resistance (MDR) was defined as non-susceptibility to at least one agent in three or more antimicrobial categories.

DNA extraction was carried out from the provided culture, exhibiting a single high-molecular-weight band on agarose gel. A fragment of the 16S rRNA gene was amplified, resulting in a 1500 bp PCR amplicon. After purification, the PCR products underwent forward and reverse sequencing. The consensus sequence was generated and compared using BLAST against the NCBI GenBank database. The top 10 sequences with the highest identity scores were aligned using Clustal W, and a phylogenetic tree was constructed using MEGA 7 based on the distance matrix (Zhao *et al.,* 2024).

Serial dilutions of the sewage sample were prepared, ranging from undiluted to 10^-6, using microfuge tubes. Each dilution underwent spot isolation on DNB bottom agar plates, where a mixture of processed sewage sample and *S. aureus*/*K. pneumoniae* suspension was incubated at 37°C for 24 h. Clear, well-defined plaques were observed, indicating areas where phage particles had lysed bacterial cells. Selected plaques were spot-isolated and phage particles were released from the agar using phage buffer. The isolated phage suspension was stored at appropriate temperatures for further characterization.

The Power Soil Kit extraction protocol involved storing Solution CD2 at 2-8°C and other reagents at room temperature. If Solution CD3 precipitated, it was heated at 60°C, and centrifugation was done at room temperature. The Power Bead Pro Tube was spun briefly, and Solution CD1 was added to the sample, followed by vortexing and centrifugation. Additional steps included adding Solution CD2 and CD3, vortexing and centrifugation to isolate DNA. The DNA was then loaded onto an MB Spin Column for purification. For library preparation using the NEB Ultra II FS DNA Library, 250 ng of genomic DNA underwent fragmentation and end prep. A thermal cycler was used for incubation, followed by adaptor ligation, addition of USER Enzyme, and purification. PCR enrichment was done using Q5 master mix and index primer, followed by cleanup. Library quality was assessed on a Tapestation, and quantification was done using Qubit. Sequencing was performed on Illumina-Novaseq 6000 using V1.5 Chemistry (Jakociune and Moodley, 2018).

Bacteriophages were mixed with 0.3% commercial honey and 0.25% gelatin for *Beads 1* and with 3% honey and 2.5% gelatin for *Beads 2*. *Beads 3* involved suspending bacteriophages in 50 mM Tris-HCl buffer (pH 7.4), while *Beads 4* utilized a mixture of 0.01% gelatin, 0.05% honey, 0.15 M NaCl and 10 mM $MgSO_4 \cdot 7H_2O$. Each matrix solution was combined with 1.5% sodium alginate, loaded into syringes, and extruded into a $CaCl_2$ solution to form spherical beads. After incubation, the beads were rinsed and immersed in a chitosan-acetate buffer solution for 30 min to coat them with a chitosan layer. Finally, the encapsulated bacteriophages were stored at 4°C until use (Kaewkod *et al.,* 2016).

The encapsulation process with alum as an adjuvant involved several steps. Firstly, alum was prepared by reacting aluminium pieces with potassium hydroxide to form potassium alum crystals, which were then harvested, washed and stored. The alum adjuvant solution was prepared by dissolving aluminium salt in sterile water or saline. Bacteriophages were suspended in a mixture containing 3% honey, 2.5% gelatin and 0.5% alum adjuvant solution. This mixture was combined with sodium alginate and extruded into a calcium chloride solution to form beads. After solidification, the beads were washed and coated with a chitosan layer by immersion in a chitosan-acetate buffer solution. Finally, the encapsulated bacteriophages were stored at 4°C until use, suitable for controlled release applications.

Encapsulated bacteriophages were placed in flasks with distilled water at 4°C. Samples were collected at various time points over 8 days and after 8 weeks. No phage release was observed during storage. The protocol for testing stability and release of non-encapsulated and encapsulated bacteriophages under simulated intestinal conditions involved submerging beads with encapsulated bacteriophages in artificial intestinal juice and incubating them at 37°C for 6 h. Released phages were quantified using plaque assays, providing insights into encapsulated bacteriophage stability and release in simulated intestinal conditions.

The protocol for thermal stability assays and bead morphology examination included exposing encapsulated and non-encapsulated phages to 37°C and 80°C, with samples taken at intervals for plaque assays to assess bacteriophage stability under various conditions.

RESULTS AND DISCUSSION

Klebsiella pneumoniae typically lacked indole production but exhibited positive results in the Citrate Test. These biochemical tests aided preliminary identification, but confirmatory tests were necessary for accurate identification. The isolate showed high similarity with *K. pneumoniae* based on nucleotide homology and phylogenetic analysis. The genome DNA sequence appeared in GenBank under the Accession Number NR_117686.1.

Staphylococcus aureus typically exhibited positive results in the Catalase and Coagulase tests, aiding in its identification. *S. aureus* colonies were identified by their characteristic golden-yellow colour on Blood Agar plates. These biochemical tests served as preliminary identification methods, with further confirmatory tests possibly needed for accurate identification and characterization. The isolate showed high similarity with *S. aureus* based on nucleotide homology and phylogenetic analysis. The genome DNA sequence appeared in GenBank under the Accession Number NR_037007.2.

The *K. pneumoniae* isolated from sewage sample was found to be multi drug resistant organism as it was found resistant to all the seven antimicrobial discs obtained from Oxoid (England), including Ceftazidime (30 µg), Cephotaxime (30 µg), Ertapenem (10 µg), Imipenem (10 µg), Clindamycin (2 µg), Tobramycin (10 µg) and Colistin (10 µg).

The *S. aureus* isolated from sewage sample was found to be multi drug-resistant organism as it was found resistant to five antimicrobial drugs Co-Trimoxazole (25 µg), Ceftriaxione (30 µg), Amikacin (30 µg), Piperacillin (10 µg) and Norfloxacin (10 µg).

The significant difference was observed in the p value between *K. pneumoniae* and *S. aureus,* non-encapsulated bacteriophages (Table 1) and encapsulated bacteriophages with adjuvant (Table 3). The lack of significant difference between encapsulated bacteriophages *K. pneumoniae* and *S. aureus* indicated that encapsulation may have normalized the release of bacteriophages (Table 2).

The significant overall difference between the groups in the ANOVA test and t test indicated that there were significant variations in the release of bacteriophages under different conditions (non-encapsulated, encapsulated and encapsulated with adjuvant; Tables 4, 5, 6 and 7). This suggested that both encapsulation and adjuvant played roles in influencing the release of bacteriophages under simulated intestinal conditions.

At 37°C, both encapsulated and non-encapsulated *K. pneumoniae* and *S. aureus* phages exhibited varying degrees of stability and release kinetics, with differences observed between encapsulated and non-encapsulated formulations (Tables 4, 5, 6 and 7). At 80°C, encapsulated *K. pneumoniae* phage appeared to have slightly higher stability compared to encapsulated *S. aureus* phage, while non-encapsulated *S. aureus* phage showed faster degradation compared to non-encapsulated *K. pneumoniae* phage (Tables 8, 9, 10 and 11). The presence of adjuvant enhanced the release kinetics of both encapsulated *K. pneumoniae* and *S. aureus* phages under thermal conditions, suggesting a potential strategy to improve the stability and release profiles of

Table 1. Stability and release of non-encapsulated bacteriophages under simulated intestinal conditions

	1 h	2 h	3 h	4 h	5 h	6 h	p value
Non-encapsulated *K. pneumoniae* phage in simulated intestinal juice (1 x 10^4 pfu/ml)	13	10	7	5	3	2	
Non-encapsulated *S. aureus* phage in simulated intestinal juice (1 x 10^4 pfu/ml)	3.8	5.6	2.4	0.7	0	0	$0.00030897e^{-12}$

Table 2. Stability and release of encapsulated bacteriophages under simulated intestinal conditions

	1 h	2 h	3 h	4 h	5 h	6 h	p value
Encapsulated *K. pneumoniae* phage in simulated intestinal juice (1 x 10^6 pfu/ml)	3	5	7	12	17	20	
Encapsulated *S. aureus* phage in simulated intestinal juice (1 x 10^6 pfu/ml)	2.3	5	9	16	17.5	19	$0.966212e^{-10}$

Table 3. Stability and release of encapsulated bacteriophages along with adjuvant under simulated intestinal conditions

	1 h	2 h	3 h	4 h	5 h	6 h	p value
Encapsulated *K. pneumoniae* phage along with adjuvant in simulated intestinal juice (1 x 10^6 pfu/ml)	0.3	0.9	10	76	120	160	
Encapsulated *S. aureus* phage along with adjuvant in simulated intestinal juice (1 x 10^6 pfu/ml)	0.25	0.78	3.6	12.5	69	150	$4.0209170e^{-10}$

Table 4. Stability and release of encapsulated bacteriophage release under thermal conditions (37^{0}C)

	0 sec	30 sec	180 sec
Encapsulated *K. pneumoniae* phage (1 x 10^3 pfu/ml)	8.9	6.7	5.3
Encapsulated *S. aureus* phage (1 x 10^3 pfu/ml)	2	2.5	4

Table 5. Stability and release of non-encapsulated bacteriophage release under thermal conditions (37^{0}C)

	0 sec	30 sec	180 sec
Non-encapsulated *K. pneumoniae* phage (1 x 10^2 pfu/ml)	4.6	2	0
Non-encapsulated *S. aureus* phage (1 x 10^2 pfu/ml)	2.3	1.5	0

Table 6. Stability and release of encapsulated bacteriophage along with adjuvant release under thermal conditions (37^{0}C)

	0 sec	30 sec	180 sec
Encapsulated *K. pneumoniae* phage along with adjuvant (1 x 10^3 pfu/ml)	5.6	9.8	13
Encapsulated *S. aureus* phage along with adjuvant (1 x 10^3 pfu/ml)	2.5	8	11

Table 7. Significance of release of bacteriophages under different conditions (non-encapsulated, encapsulated and encapsulated with adjuvant)

	p value
Encapsulated vs non-encapsulated *K. pneumoniae*	0.0021
Encapsulated vs non-encapsulated *S. aureus*	0.2485
Encapsulated with adjuvant vs. encapsulated *K. pneumoniae*	0.0015
Encapsulated with adjuvant vs. encapsulated *S. aureus*	0.0192
ANOVA	$1.2127e^{-07}$
Encapsulated *K. pneumoniae* and *S. aureus* phage at 37°C	$0.00213e^{-14}$
Encapsulated *K. pneumoniae* and *S. aureus* phage with adjuvant at 37°C	$0.019176e^{-12}$

Table 8. Stability and release of encapsulated bacteriophage release under thermal conditions (80^{0}C)

	0 sec	30 sec	180 sec
Encapsulated *K. pneumoniae* phage (1 x 10^3 pfu/ml)	5.6	0.8	0.3
Encapsulated *S. aureus* phage (1 x 10^3 pfu/ml)	2.3	0.4	0.1

Table 9. Stability and release of non-encapsulated bacteriophage release under thermal conditions (80^{0}C)

	0 sec	30 sec	180 sec
Non-encapsulated *K. pneumoniae* phage (1 x 10^2 pfu/ml)	3.7	1.5	0
Non-encapsulated *S. aureus* phage (1 x 10^2 pfu/ml)	15	2	0

Table 10. Stability and release of encapsulated bacteriophage along with adjuvant release under thermal conditions (80^{0}C)

	0 sec	30 sec	180 sec
Encapsulated *K. pneumoniae* phage along with adjuvant (1 x 10^3 pfu/ml)	5.7	1.1	0.9
Encapsulated *S. aureus* phage along with adjuvant (1 x 10^3 pfu/ml)	2.3	0.9	2.5

Table 11. Comparison of the p-values obtained from the t- tests and ANOVA test for the release of encapsulated bacteriophages under thermal conditions at 80°C

	p value
Encapsulated vs. non-encapsulated *K. pneumoniae*	0.1014
Encapsulated vs. non-encapsulated *S. aureus*	0.398
Encapsulated with adjuvant vs. encapsulated *K. pneumoniae*	0.4213
Encapsulated with adjuvant vs. encapsulated *S. aureus*	0.1800
ANOVA	0.0001
Encapsulated *K. pneumoniae* and *S. aureus* phage at 80°C	0.1014
Encapsulated *K. pneumoniae* and *S. aureus* phage with adjuvant at 80°C	0.1800

bacteriophage formulations comparable to previous reports of phage encapsulation (Colom *et al.,* 2017).

Recent studies demonstrated the potential of encapsulated phages in treating *Clostridium difficile* infections by showing efficacy in reducing bacterial colonization and increasing survival in animal models (Vinner *et al.,* 2017, 2020). However, challenges such as maintaining phage viability and ensuring targeted release in the colon need to be exploited. Studies included many techniques such as encapsulation in liposomes enhanced phage stability for medical applications (Salvo, 2020), microencapsulation of bacteriphages in pH-responsive solid dosage form (Gurinder *et al.*, 2019) to test stability in acidic pH. Bead encapsulation offered a simple and cost-effective method for delivering phages orally, making it highly suitable for on-farm applications aimed at controlling intestinal colonization by zoonotic and pathogenic bacteria. These encapsulation strategies alongwith use of adjuvant aimed at protecting phages from the acidic stomach environment and ensured their release at the infection site in the intestines, improving the effectiveness of phage therapy in gastrointestinal applications (Abdelsattar *et al.,* 2019).

CONCLUSION

The findings suggested that encapsulation and adjuvant addition influenced bacteriophage release behaviour under simulated intestinal conditions, with potential implications for applications such as phage therapy and biocontrol. Further research exploring additional parameters and conditions is warranted to comprehensively understand bacteriophage release dynamics, optimize formulation strategies and to validate the therapeutic potential of encapsulated bacteriophages in clinical settings. Moreover, exploring additional factors such as storage conditions, adjuvant selection and delivery mechanisms will be crucial for advancing the field of bacteriophage therapy.

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