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BIOPROSPECTING AND EVALUATION OF LYTIC BACTERIOPHAGES AGAINST EXTENSIVELY DRUG-RESISTANT SALMONELLA ENTERICA SEROVAR TYPHI FROM DIVERSE BIOLOGICAL MATRICES

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Abstract

Extensively drug-resistant (XDR) Salmonella enterica serovar Typhi has pose public health challenge globally and predominantly in LMIC countries. Pakistan serves as a country with the highest burden of typhoid and Karachi is known as an epicenter. Lytic Phages are the natural killer of bacteria and can be exploited for the development of countermeasures. This study aimed to explore various biological matrices for the isolation of lytic phages against XDR Salmonella enterica serovar Typhi. It is also intended to evaluate the isolated phages on the basis of their plaque size, reproducibility in the laboratory environment, phage titers and stability during storage in a controlled environment. A total of 45 environmental samples were collected, comprising of 13 sewage, 14 drainage water, 8 poultry waste, 4 marine samples and 6 tap water were collected. Sequential enrichment, centrifugation, membrane filtration, and the double-layer agar overlay plaque assay were applied. The bacterial host (ST-1) used in this study was identified and characterized as XDR Salmonella enterica ssp. enterica through the MALDI-TOF MS, Vitek 2 compact system and serological test. Most of the sewage samples, indeed hospital-associated sewage yielded the phages with significant titer in a reproducible manner. We also found phages in poultry waste, drainage, and marine water, whereas no phages were isolated from tap water. Our findings provide ecological and microbiological insight into various biological matrices for specific lytic phages. In conclusion, hospital-associated sewage at Karachi, Pakistan is a good resource of phages. Our results indirectly verify the prevalence of XDR S. typhi in the community.

Keywords: *Biological matrices, Double-layer agar overlay plaque assay, Extensively drug-resistant, Lytic phages, Salmonella enterica serovar Typhi, Typhoid*

INTRODUCTION

Phages are viruses and one of the most abundant entities in environmental niches on earth. It is estimated that 10^7 to 10^8 phages are present per milliliter of ocean water. However, it depends on the varying properties and depth of the ocean. Phages are specific for each bacterial species and strain as well. Phages are present wherever their host bacteria exist. It is also projected that the phage-to-bacteria ratio is about 10 times more phages than there are bacteria in the environment. We can estimate the richness of the environment with bacteria indirectly by the number of specific phages or vice versa. For example, a single gram of soil can harbor approximately 10^{10} bacterial cells representing tens of thousands of diverse species. Phages are of two types depending on their life cycle, i.e., lytic and lysogenic phages. Lytic phages are natural predators of bacteria. They multiply inside the bacterial cells, resulting in the lysis or killing of bacterial cells. They are very sensitive and robust; they can tolerate a wide range of temperature and pH. Relating to the translation of phage R&D into commercialization or real-time use, it is highly economical to produce phages in a controlled laboratory environment. Foregoing in view, the phages have emerged as the inevitable need for the advancement of diagnostics and countermeasures (1-2).

In addition, antimicrobial resistance (AMR) constitutes a critical global public health threat. According to the World Health Organization (WHO), AMR was directly responsible for approximately 1.14 million deaths, with an additional 4.71 million deaths associated with AMR bacterial infections worldwide in 2021. It is estimated that deaths due to AMR will reach 10 million people by 2025. It is predicted that

AMR could surpass cancer as the leading cause of death by 2050 (3-4). In Pakistan, AMR is recognized as the third leading cause of mortality, following cardiovascular disease and maternal and neonatal disorders. Bacterial infections resistant to third- and fourth-generation antibiotics are estimated to cause 0.3 million deaths annually, while AMR broadly contributes to approximately 0.7 million deaths each year. Pakistan ranks as the third largest consumer of antibiotics globally, after China and India, with total antibiotic consumption valued at PKR 126 billion in 2023 (5).

Salmonella enterica serovar Typhi causes a systemic infection, typhoid fever. It remains one of the major public health challenges in endemic regions due to insufficient sanitation and inadequate access to hygienic food and water. Pakistan serves as a country with the highest burden of typhoid fever; particularly, Karachi holds its position as an epicenter due to its population density, mobility, and diverse healthcare setup. During the last decade, the clinical management of typhoid fever has been altered due to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) phenotypes of *Salmonella enterica* serovar Typhi. The world witnessed the first XDR *Salmonella enterica* serovar Typhi outbreak, which occurred in Hyderabad and Karachi during 2016–2018. This emergence rapidly sped through transboundary movements. Clonal extension of a single dominant ancestry carrying plasmid-mediated resistance was proved by genomic analyses of the emerging strains. This is further verified by the subsequent rapid dissemination across countries and continents (6-7).

According to the WHO and Centers for Disease Control and Prevention (CDC) defined categorization, multidrug-resistant (MDR) *Salmonella enterica* serovar Typhi strains are resistant to the first-line antibiotics ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. Extensively drug-resistant (XDR) *Salmonella enterica* serovar Typhi strains exhibit additional resistance to fluoroquinolones and third-generation cephalosporins, thereby severely limiting available therapeutic options. In 2024, WHO called on scientists, academia, biopharmaceutical companies, and health policymakers for joint efforts for safe and effective therapeutics and biocontrol products as alternatives to antibiotics. In contrast to broad-spectrum antibiotics, phages are very selective for their hosts while negligibly disturbing the surrounding microbiota, making them attractive candidates for precision antimicrobial therapy (8-9).

Therefore, the aim of the present study is to selectively enrich the samples collected from different biological matrices and isolate phages against XDR *Salmonella enterica* serovar Typhi. It is also intended to investigate their distribution, recovery, and storage-based stability.

MATERIALS AND METHODS

STUDY DESIGN

A comparative cross-sectional research study was conducted for the isolation of phages against XDR *Salmonella enterica* serovar Typhi (bacterial host) from various biological matrices. The study was approved by the Institutional Review Board. Good laboratory practices and ethical considerations were taken into account. The research was conducted at Microbiology Department of Jinnah University for Women, Nazimabad, Karachi, Pakistan and Centers of Excellence in Science & Applied Technologies, Islamabad, Pakistan

ACQUISITION OF BACTERIAL HOST STRAIN

The host strain used in this study was blood isolate of *S. typhi* acquired from a diagnostic laboratory located in Karachi in 2024. The strain was named as ST-1 and transported to the lab in a cold chain. *S. typhi* ATCC® 13076™ was used as quality control strain.

PRIMARY PROCESSING AND STORAGE OF BACTERIAL HOST

According to biosafety protocols, the bacterial strains were handled in a BSL-2 lab. Primarily, ST-1 was subcultured on 5% blood agar to check the purity. Long-term preservation was achieved by preparing bacterial stocks in Tryptic Soy Broth (TSB) supplemented with 15% glycerol and keeping them at -80°C with proper cataloguing. Working stocks of bacterial culture were maintained on MacConkey agar (10).

IDENTIFICATION AND CHARACTERIZATION OF BACTERIAL HOST

The host strain was grown overnight on Tryptic Soy Agar (TSA) and identified through protein mass fingerprinting using the VITEK® MS MALDI-TOF MS system (bioMérieux, France; database version 3.2.0) (11). The quality control strain of *Escherichia coli* ATCC 8739 was used with test isolates simultaneously for calibration and validation as per user guideline. For biochemical characterization, the VITEK® 2 compact system (bioMérieux, France; database version 9.02) and GN identification cards (bioMérieux, France) were used. Automatically, the report was generated from the 9.02v version of the database after 10 h of incubation (12). The serotypes of all isolates were confirmed with a commercial antiserum kit (Cat No: BD Difco™ Becton, Dickinson and Company (BD), US) following the Kauffmann–White classification scheme for Salmonella O and H antisera (13). All systems were operated in accordance with the manufacturer's guidelines. Further characterized on the basis of antimicrobial susceptibility testing through the VITEK 2 system using AST-GN81 cards (bioMérieux, France). Results were interpreted as susceptible, intermediate, or resistant based on established clinical breakpoints of the Clinical & Laboratory Standards Institute (CLSI), M100. The WHO / CDC definition of extensively drug-resistant (XDR) *Salmonella enterica* serovar Typhi strains was applied to confirm ST-1 as XDR (14-15).

SAMPLE COLLECTION FOR PHAGE RECOVERY

The samples from different biological matrices, including poultry waste, sewage, drainage water, marine, and tap water, were aseptically collected and transported to the laboratory within 6 hours. The 500 mL sterile polypropylene screw-capped containers were used for the collection of all types of water samples. Poultry wastes were collected from a poultry farm in 15 mL conical tubes. Sewage samples were collected from municipal channels and hospital-associated sewage outlets. Drainage samples are from house hold sources. Marine water samples were collected from surface at coastal regions. Tap water samples were collected from different residential water supplies. All the samples were collected from Karachi.

PROCESSING OF SAMPLES FOR PHAGE ISOLATION

Poultry samples were homogenized by mixing 10-15g of sample with 25mL of sterile sodium chloride/magnesium sulfate (SM) buffer. The mixtures were vortexed for 15 sec and subjected to incubation at 35-37°C for 1 h of mild shaking to facilitate the release of phages into the buffer. Other samples, including sewage, drainage water, and marine water samples, were directly subjected to incubation with shaking under the same condition. However, the concentration was 2-3 % in medium for marine sample. Later, all the samples other than tap water were then centrifuged at 8,000x g for 10 min at 4°C to remove debris. The membrane filtration technique was implemented for each sample using sequential filtration through 0.45 µm and 0.22 µm cellulose acetate sterile syringe filters. Separate filters were used for each sample. In case of choking of filters, more than one filter was used to obtain clarified supernatant, which contains total viral particles. These were stored at 4°C until further processing (16-17).

ENRICHMENT WITH HOST BACTERIA FOR PHAGE SELECTION

The ST 1 were grown overnight in a Tryptic Soy Broth. An amount of 100µL of grown culture and 10 mL of filtered supernatant (total viral particles) were added to 10 mL of TSB. This suspension was incubated at 37°C for 18-24 h under agitation (150 rpm). Later, the enriched sample was centrifuged at 8,000x g for 10 min at 4°C. The supernatant was decanted into a sterile tube without disturbing the debris pellet, filtered by using a 0.2µm membrane filter (cellulose acetate), and the filtrate was then collected in a sterile tube. This tube was expected to contain host-specific phages and was stored at 4°C until further processing (18-20).

ISOLATION AND PURIFICATION OF PHAGES

The specific phages were isolated using a double layer agar overlay plaque assay. Briefly, 100µL enriched sample and 100µL overnight culture of ST-1 were mixed in 3mL of 0.6% (*w/v*) top TSB supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ maintained at 48–50°C and poured onto the TSA. Later,

the plate was incubated at 37°C for 16–20 h. Phage isolation was demonstrated by the presence of distinct plaques, which were then selected on the basis of consistent size and shape. The selected plaques were transferred to their respective labeled tubes containing 1mL SM buffer and left for 24 h. Three passages were carried out using the same method to purify and rule out the reproducibility of phages in a laboratory environment. To get the amplified phages, a volume of 5 mL SM buffer was added onto the plate once it had confluent lysis, and the plate was left to stand at room temperature for 1–2 h. The top agar layer was then collected with buffer using cell scrapers aseptically. The suspension was then centrifuged at 8,000 x g for 10 min at 4°C. The supernatant was filtered through 0.2µm cellulose acetate filters. Purified phage stocks were stored at 4°C for further analysis (21-23).

DETERMINATION OF PHAGE TITER

The enumeration of phages in terms of plaque-forming units, i.e., PFU/mL, was carried out using dilutions of phage suspension through a double layer agar overlay plaque assay. The dilutions were mixed with host bacteria in a dilute, molten agar or agarose matrix (the "top agar" or "overlay"), which was distributed evenly to solidify on a standard agar plate (the "bottom agar" or "underlay"). After overnight incubation, plaques were counted and PFU/mL was determined. The assay was run in triplicate (24-26).

PHAGE STORAGE STABILITY

Phages were stored at -75 to -80°C for one year. The stability in terms of viability and phage titer was determined at 3rd month, 6th month and 1 year of storage.

STATISTICAL ANALYSIS

Graphic statistical analysis was used to investigate and summarize the distribution pattern of XDR *Salmonella enterica* serovar Typhi phages.

RESULTS

IDENTIFICATION AND CHARACTERIZATION OF BACTERIAL HOST

The ST-1 was identified as *Salmonella enterica ssp. enterica* through the MALDI-TOF MS system with a 99.9% confidence level (Fig. 1a). Additionally, it was biochemically characterized as *Salmonella enterica* serovar Typhi with 99% probability through the Vitek 2 compact system. The MALDI-TOF MS and Vitek 2 compact systems are the high-throughput identification and characterization platforms recognized for rapid and accurate results. The serological latex particulate antigen-antibody assay verified successfully the serotype of ST-1. After testing the host susceptibility for various classes of antibiotics on the basis of minimum inhibitory concentration (MIC), the ST-1 was declared an XDR strain in accordance with the WHO / CDC criteria for the categorization of MDR and XDR strains of *Salmonella enterica* serovar Typhi.

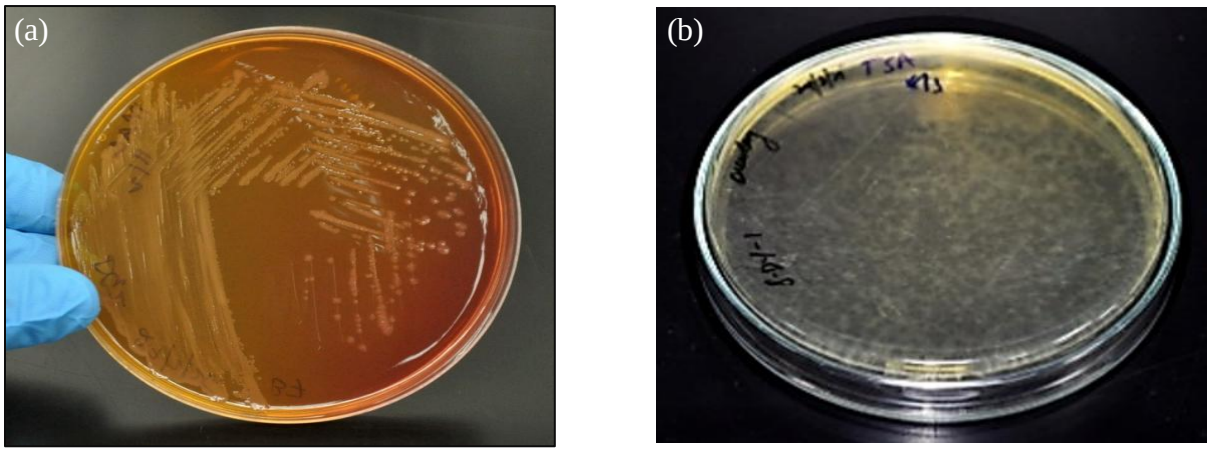

Fig. 1 (a). *Salmonella enterica* serovar Typhi (ST-1) on MacConkey agar; **(b).** Double-layer agar overlay plaque assay

It exhibited resistance to ampicillin, trimethoprim-sulfamethoxazole, fluoroquinolones and third-generation cephalosporins. Results were based on established clinical breakpoints of the Clinical & Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing, M100. The reason for the selection of the XDR strain as a bacterial host was to isolate potential lytic phages having potential against the antimicrobial-resistant strain of typhoid. Currently the world and especially low- or middle-income countries have been facing XDR typhoid as a major public health challenge. The situation leads to treatment failure, limited therapeutic options, prolonged hospitalization, high mortality, an import burden of antibiotics and other socio-economic losses (27).

ISOLATION OF PHAGES FROM DIFFERENT ENVIRONMENTAL MATRICES

A total of 45 samples from various biological matrices were successfully collected, enriched and processed specifically for the isolation of ST-1 phages (Fig. 2). Clear plaque formation was considered positive for the isolation of lytic phages. In our study, most of the lytic phages were isolated from sewage samples reflecting the abundance of XDR *S. typhi* phages. Precisely, hospital-associated sewage was dominating as compared to household sewage and drainage water. We did not find phages from tap water. However, the phages were also isolated from a few poultry samples. Only one sample of each marine water and drainage water was also positive for phages. Indirectly, our findings demonstrated the prevalence of XDR *S. typhi* in hospitalized patients (Table II, Fig. 3).

Fig. 2. Biological Matrices. **(a)** poultry waste; **(b)** marine surface water; **(c-d)** Sewage; (e)drainage water

S. *typhi* is transmitted through the oral-fecal route, and phages are obligate parasites of specific bacteria. Since the distributions are strongly prejudiced by the microbial burden of bacterial host XDR *S. typhi,* facilitating increased bacteriophage-host interactions and phage replication dynamics. Other studies also reported high recovery of phages from sewage samples. It is also reported that municipal sewage harbors a high density of pathogenic and commensal bacteria. Similarly, samples from hospital sewage systems were found to have lytic phages having therapeutic potential. This outcome is associated with the persistent bacterial contamination, facilitated bacteriophage-host interactions, rapid phage amplification, adaptation and evolution of phages in accordance with the prevalent clinical strains. Hence, these studies and facts also verified our findings. One of the limitations of our study is the unequal sample sizes from different sources (28-30).

Table I. Isolation of XDR *S, typhi* Phages from different biological matrices

Biological matrices	Total collected (n)	Sample positive (n)	Isolation rate (%)
Poultry waste	8	2	25
Drainage water	14	1	7.14
Hospital-associated sewage	6	6	100
House hold sewage	7	2	28.57
Marine water	4	1	25
Tap water	6	0	0
Total	45	12	26.66

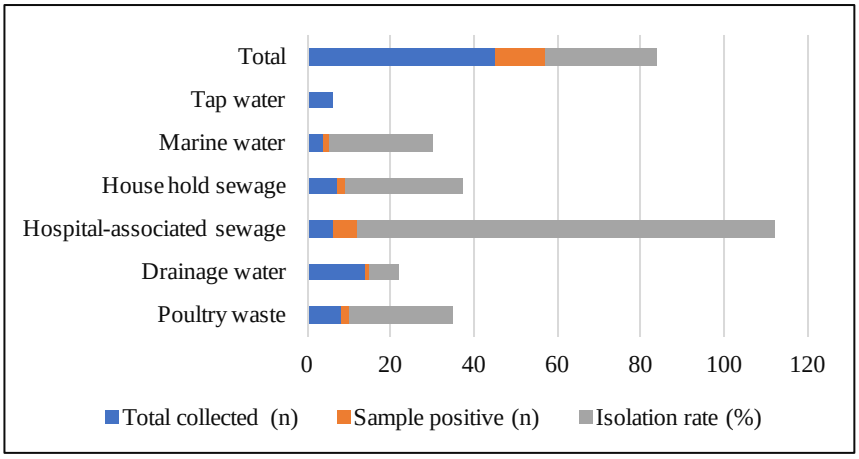

Fig. 3. Isolation frequency of XDR *S. typhi* phages

EVALUATION OF PHAGES

A double agar overlay method was successfully utilized for the evaluation of isolated phages (Fig. 1 b). A comparative evaluation of isolated lytic phages was carried out on the basis of plaque size, reproducibility in the laboratory environment, and phage titers and stability during storage in a controlled environment. Sewage-derived phages predominantly produced large (3-4 mm diameter) and circular plaques. However, phages isolated from marine and drainage water exhibited relatively irregular and small plaques (1-2 mm diameter). Varying sizes of plaques are associated with phage replication dynamics and interaction with bacterial hosts in the given lab environment. Another study demonstrated that phages with a short latent period and high burst size produce large plaques because they rapidly infect and lyse surrounding cells. Smaller plaques are usually associated with slower replication, lower burst size, or restricted diffusion through the agar medium. Additionally, agar density, bacterial growth phase, and receptor availability constitute probable factors associated with the lab environment (28, 30).

The phages from sewage and poultry samples were reproduced effectively through 3 consecutive passages in the laboratory environment as compared to the drainage and marine samples. The phages isolated from the sewage sample gave the highest titer in terms of PFU/mL as well (Table II). Mirmiran *et al.* (2025) reported twelve *Salmonella*-specific phages that were isolated from diverse environmental sources across China. Livestock manure in Shanxi Province (Northwest China), poultry wastewater in Suizhou, Hubei Province (Central China), slaughterhouse effluent in Guangdong Province (South China), and from hospital sewage in Zhejiang Province (Eastern China). This geographic and ecological diversity underscores the broad natural distribution of *Salmonella* phages (31).

Other studies showed effectiveness of phages against MDR *Salmonella* spp. However, inadequate data on safety of phage use, phage stability, and lack of regulatory framework remain major obstacles in the commercial application of phages (32).

Subsequently, the phage stocks were stored in a controlled laboratory environment and subjected to periodic stability testing to check the viability and phage titer determination. In our findings, no or very negligible reduction in phage titer was observed in sewage-derived phages. The phage titers of other phages were decreased 10^3-fold.

Table II. Mean of XDR *S. typhi* phage titers isolated from different matrices

Biological matrices	Mean phage titer (PFU/mL)
Poultry waste	$1x10^9$
Drainage water	$1.1x10^9$
Hospital-associated sewage	$1x10^9$
House hold sewage	$1.2x10^6$
Marine water	$1x10^6$

CONCLUSION

In conclusion, the findings offer ecological and microbiological insight into environmental reservoirs of lytic phages against extensively drug-resistant (XDR) *Salmonella enterica* serovar Typhi. Although we also found phages in poultry waste, drainage, and marine water, the majority of phages were isolated from sewage samples, predominantly hospital-associated sewage at Karachi, Pakistan. Hence, our results indirectly verify the prevalence of XDR *S. typhi* in the community. More studies related to detailed characterization of phages and animal modeling can be conducted by using such lytic phages to develop safe, efficient, and economical prophylactic and therapeutic products.

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Conflict of interest:

Authors declared no conflict of interest.

Authors' contribution:

DG Research work and manuscript writing; SK Resources and co-supervision of research; SGN Supervision and critical analysis.

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