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Harnessing Bacteriophages to Overcome Tuberculosis: Challenges and Advancements.

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Abstract

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (Mtb) remains a significant global health challenge, leading to a substantial number of deaths each year. The emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), presents a serious threat to TB control efforts. Phage therapy uses phages like mycobacteriophages, which infect species including *M. tuberculosis,* as a potential treatment for TB. Some mycobacteriophages, such as D29, TM4, and DS6A, have shown promise in preclinical studies by effectively targeting drug-resistant TB strains. The role of the lytic enzyme system involving LysA and LysB genes dedicatedly targets the specific Mycobacterium species' mycolic acid and peptidoglycan layer in due course of action. Besides this, the review also suggests the possibility of an efficient delivery system in the mammalian cell microenvironment. The new strategies also involve the CRISPR-Cas system as a molecular tool to facilitate the generation of mycobacteriophage variants with desired functional attributes. However, phage therapy faces shortcomings such as identifying specific phage-bacterial strains, regulatory compliances, and addressing potential immune responses. Although, the optimal delivery to the infection site, determining the ideal dosage, and addressing concerns about bacterial resistance are under critical consideration. Ongoing efforts focused on overcoming these limitations and translating promising preclinical results into clinical applications.

Keywords: Multidrug-resistant Antibiotics, *Mycobacterium tuberculosis, Mycobacterium smegmatis,* Mycobacteriophages, CRISPR-Cas, Phage delivery system

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Introduction

Tuberculosis is one of the oldest infectious diseases known to mankind, caused by the deadly pathogen *Mycobacterium tuberculosis* or less likely by closely related organisms *Mycobacterium africanum*, *Mycobacterium bovis* or *Mycobacterium caprae*(Pai et al., 2016). TB accounts for being the second leading fatal infectious disease following coronavirus disease post-2019 and globally ranks 13th as the leading cause of death. (*Global Tuberculosis Report 2022*, ; *Global Tuberculosis Report 2023*,). Tuberculosis disease occurs worldwide, infecting all countries and age groups. Across the globe, a quarter of the population is estimated to be affected with tuberculosis, despite 90 years of vaccination and 60 years of chemotherapy. (Holmes et al., 2017). According to the World Health Organization (WHO) in 2022, it was estimated that over 10.6 million people were infected with TB and 1.6 million deaths worldwide.

Tuberculosis is an airborne infection that is primarily transmitted through the respiratory route and mostly affects the lungs in humans. However, it also has many manifestations in other bodily tissues, affecting the central nervous system, the brain, and even the bones (Mbuh et al., 2019). The infectious agent i.e. *Mycobacterium tuberculosis* is transmitted from infectious to non-infectious healthy individuals through nasal secretion during sneezing or coughing (Al-Humadi et al., 2017). After the bacterium entry via inhalation, *M. tuberculosis* invades the dominant cell target, the pulmonary alveolar macrophage cell (Ravesloot-Chávez et al., 2021). Once the bacteria are internalized it restrains all the resistance mechanisms by the host and ensures its survival in the microenvironment (Zhai et al., 2019). Irrespective of the pathway, the bacterium now gets access to the lung parenchyma which stimulates the recruitment of immune cells by the host to the infection site. This creates a cell mass forming granuloma, within which the bacterium persists and proliferates. Gradually, the microbial load increases and if the granuloma fails to hold the infection, it eventually migrates to infect other parts of the body including the brain and bloodstream. Thus, granuloma becomes the central point for further establishment of pathogenicity and interaction with the host immune system (Cronan, 2022). The primary infection can be characterized by coughing sometimes with blood mixed in sputum, weakness, weight loss, fever, and night sweats. However, exposure to *M. tuberculosis* can result in two fates: elimination or persistence. The pathogen can be eliminated either through the cascade events of the innate or adaptive immune system of an individual. However, in some instances during the late phase of infection, the pathogen may remain in a latent state and be asymptomatic (Khabibullina et al., 2022; Zaidi et al., 2023). Latent tuberculosis infection (LTBI) is when someone is infected with the pathogen but does not have active TB. It is defined as the condition of evident immune response by the antigens of *M. tuberculosis*, without showing any clinical symptoms of active tuberculosis. Unlike active tuberculosis, LTBI is not contagious (Brett et al., 2020). About 23% of the world's population is estimated to have latent tuberculosis infection (LTBI) (O'Connell et al., 2022), within which approximately 5-15% reactivation to active symptomatic tuberculosis disease may occur and is governed by host, bacteria, and environmental factors (Kiazyk & Ball, 2017). The epidemiological data suggests that TB is considered to be the sixth cause of death among children specifically aged between one to fifty-nine months. Children serve as a major reservoir for active tuberculosis and worldwide about 67 million children are carriers of latent tuberculosis infection (Maphalle et al., 2022).

The overall TB incidence and mortality rate have been adversely affected by COVID-19 pandemic challenges, and social and political conflicts in the recent past, which brought down the health community to address the global threat of TB. The circumstances of global aging, inadequate diagnosis, high latent infection pool, and major drug resistance pose significant problems in lowering the global TB burden (Villar-Hernández et al., 2023). Antibiotic resistance (AMR) is one of the major global health challenges which the

world is facing at present. The widespread occurrence of superbugs across the environmental niche is responsible for an increase in the mortality and morbidity rate, making it difficult to combat pathogenic infections. (B. Aslam et al., 2018). Studies conducted back in 2019 showed that bacterial antibiotic resistance was linked to 4.95 million deaths, with bacterial AMR accounting for 1.27 million of those deaths (*Global Antimicrobial Resistance and Use Surveillance System (GLASS)*,). Low or negligible susceptibility towards antibiotics among certain bacterial groups has evolved in due course of time as a result of spontaneous mutation. Alongside this, the efficient transfer of drug-resistance genes among different bacterial species favored the phenomenon of multidrug resistance (MDR) (Urban-Chmiel et al., 2022). In the case of TB, the genesis of mutated strains of *M. tuberculosis* that are resistant to almost all of the major anti-tuberculosis drugs is a serious public health concern. Back in the year 2019, reports analyzed 180000 global deaths due to antimicrobial resistance against rifampicin, which is the first line of defense drug for tuberculosis treatment (Dean et al., 2022).

It is of utmost importance to withstand the antimicrobial resistance emergency and drastically change the treatment algorithm for the well-being of mankind. Lately, the dependency of the whole world on antibiotics has accelerated bacterial resistance. Antibiotics are not only nonspecific for target bacteria but even impede the beneficial resident microorganisms, disrupting the normal microbiome of an individual (Levy et al., 2017). Nowadays, treatment with antibiotics against those pathogens poses a multitude of resistance mechanisms for a wide range of drugs is a critical question to deal with. Determining and developing novel compounds with target-specific action is an extremely slow and expensive process. The Centre for Disease Control (CDC) reported and published an estimated annual death of 10 million people by multidrug resistance (MDR) by the year 2050 (Church & McKillip, 2021). Otherwise, recent studies' methodologies and phages can be taken into consideration to fight against the same with effective proven results. The rapid decline in the proficiency of antibiotics has paved the way for the resuscitation of phage therapy in the present-day scenario. The answer to the ongoing challenges can be traced back to the past where phage could be used to constrain bacterial growth. The therapeutic usage of bacteriophage can be a potent and multidimensional strategy that may alleviate the problem. Phages are the most abundant species in the biosphere constituting 10^{31} phage particles i.e., tenfold more than the bacterial population (Shkoporov et al., 2022). This leads to the theoretical possibility of having at least one distinct phage particle against each bacterial species.

The phage therapy practice over antibiotics can be drafted based on phage properties. Bacteriophages are host-cell-specific and predominately kill an individual species or subspecies of bacteria (Stone et al., 2019). Phages do not aimlessly kill bacteria, unlike conventional antibiotics. Thereupon, the capability to combat bacterial infections has led phages to be a potential “magic bullet”. It potentially degrades the distinct bacteria without influencing other entities, contributing towards narrow spectrum action (Nikolich & Filippov, 2020). These rule out the probability of secondary infections and pathogen resistance. The entire process for isolation and selection of bacteriophage for application with technological advancement is comparatively more affordable than an antibiotic leading to a magnificent saving and this makes it approachable to a wider population (Romero-Calle et al., 2019). As bacteria evolved, infections became more complex and difficult to treat. Thus, with better resources and sophisticated technology phage therapy can be reinstated. Though phage therapy has proven to be effective (R. M. Dedrick et al., 2019; Żaczek et al., 2020), scientists and pharmaceutical companies are apprehensive about using it as a replacement for conventional antibiotics (Brives & Pourraz, 2020). Certain countries have considered phage therapy to treat various diseases. Despite the large-scale production of antibiotics, the Soviet Nations still retained the traditional therapeutic approach of phages and tried to implement them with new enhanced technologies. Among the current deadly diseases, tuberculosis is endemic, especially in

developing countries. Malnutrition and poor living standards increase bacterial persistence (Sinha et al., 2019). *Mycobacterium tuberculosis* continues to prevail at present in a much more evolved and menacing form. Hence with the rise in antibiotic resistance, it is essential to find an alternative to curb this problem.

2. *Mycobacterium tuberculosis*: The pathogen

Mycobacterium tuberculosis is a highly pathogenic organism that belongs to the family of Mycobacteriaceae and the genus Mycobacterium. Robert Koch made the initial discovery of this causal agent of tuberculosis in 1882 (Barberis et al., 2017). It is a highly aerobic, non-sporulating, and non-motile bacillus with a generation time of 18-24 hours under optimal conditions (Verma et al., 2022). The bacterium cell envelope contributes to the virulence of the bacterium. *M. tuberculosis* uses its complex cell surface structures which are rich in lipids, to connect with host cell surface receptors (Dulberger et al., 2020). Fundamentally, the peculiar cell wall structure holds impermeability to targeted drugs and other compounds. The specialized bacterial ESAT-6 (Early Secretory Antigenic Target) secretion system called the ESX-1 is a major virulence factor in establishing successful infection. This system is also called type VII secretion which secretes immunogens, namely ESAT-6 and CFP-10 responsible for cytokine release in the host cell (Leem et al., 2018). In addition, a protein system called dormancy survival regulon (Dos) controls the expression of over 50 genes that are responsible for the pathogen's ability to survive in low-oxygen conditions, which contributes to its ability to cause disease. *Mycobacterium tuberculosis* has a genome size of around 4 megabases, and there is significant diversity in its genotypes, with some strains having evolved into global threats to human health (Koleske et al., n.d.). The widespread presence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and total drug-resistant (TDR) strains of *Mycobacterium tuberculosis* means that they do not respond to the current treatment regimen. The resistance that *M. tuberculosis* develops is usually due to spontaneous genetic mutations that affect the target of the drugs, activate mutations, or modify efflux pumps as a result of deletions, insertions, or inversions (Table 1). The *M. tuberculosis* genome contains over 4000 proteins responsible for cellular metabolic pathways, within which over 200 proteins solely participate in fatty acid metabolism. Clinical considerable drugs target these proteins for functional inactivation (Nimmo et al., 2022; Yan et al., 2022). The antituberculosis drugs used are highlighted in compliance with how *M. tuberculosis* escapes its mechanism.

Table 1 briefly outlines the antituberculosis drugs used in compliance with how *M. tuberculosis* escapes its mechanism by mentioning its target sites, mutation types, and genes involved. For example, Rifampicin resistance occurs when mutations in the rpoB gene prevent the drug from binding to RNA polymerase (Kumar & Jena, 2014). Isoniazid resistance involves mutations in the inhA gene and katG gene, hindering drug activation (Tseng et al., 2015). Pyrazinamide resistance is linked to mutations in the pncA gene, while Ethambutol resistance results from changes in the embCAB gene (Karmakar et al., 2020; Sun et al., 2017). Bedaquiline resistance is due to efflux pump overexpression and mutations in the atpE gene (Nguyen et al., 2018). Delamanid resistance involves disruptions in multiple genes like fbiA, fbiB, and fbiC (Nguyen et al., 2020). Fluoroquinolones and Linezolid resistance are associated with mutations in gyrA and the 50S ribosomal subunit, respectively (Chien et al., 2016; Nambiar et al., 2021).

TABLE 1: An overall summary of available anti-tuberculosis drugs and *M. tuberculosis* resistance mechanism

SL. NO.	DRUG NAME	DRUG TARGET	MUTATION TYPE	GENE INVOLVED	RESISTANCE EFFECT	REFERENCE
1	Rifampicin	RNA polymerase, β-subunit	Target based	rpoB	The drug cannot bind to RNA polymerase	(Kumar & Jena, 2014)
2	Isoniazid	Enoyl- (acyl-carrier-protein) reductase	Target based	inhA	The drug cannot bind to NADH-dependent enoyl-acyl-carrier protein reductase.	(Tseng et al., 2015)
			Activator based	katG	S315T mutation blocks drug activation	
3	Pyrazinamide	30S ribosomal unit	Activator based	pncA	Protein structural variant limits drug activation	(Karmakar et al., 2020)
4	Ethambutol	Arabinosyl transferase	Target based	embCAB	Amino acid replacement prevents cell wall polymerization	(Sun et al., 2017)
5	Bedaquiline	ATP synthase	Efflux pump	Rv0678	MmpL5-MmpS5 efflux pump overexpression	(Nguyen et al., 2018)
			Target based	atpE	The drug is unable to bind to the c-subunit of ATP synthase	
6	Delamanid	Mycolic acid synthesis	Activator based	FbiA, fbiB, fbiC, fgd, Rv3547	Disrupt gene function and prevent drug bioactivation	(Nguyen et al., 2020)
7	Fluoroquinolones	DNA gyrase and DNA topoisomerase	Target based	gyrA, gyrB	The drug cannot bind to the altered DNA gyrase subunit	(Chien et al., 2016)
8	Linezolid	50S ribosomal subunit	Target based	reply	Drug lacks binding efficiency to 50S ribosomal subunit	(Nambiar et al., 2021)

3. Phage therapy: the timeline

Phage therapy since its very beginning has been the source of conflicts for many, starting from its origin to its implementation. Traditionally Herelle and Twort are believed to be the pioneers of phage therapy. Earlier in 1896, a British chemist, Ernst Hanbury Hankin first observed the antibacterial properties in the river waters of India. He studied the presence of some "heat-labile, filterable, antibacterial properties" in the waters of Ganges and Jumna that caused a reduction in the number of cases of cholera (Kochhar, 2020). In 1915, Fredrick Twort, a British microbiologist, noted the presence of a "filterable agent" that caused the breakdown of a pure culture of bacteria (Twort, 1915). However, Hankin and Twort both found that there was a phenomenon causing bacterial lysis but none of them could reach a clear conclusion. In 1917, Felix D'herelle, a Canadian microbiologist independently discovered the idea of phage therapy at the Pasteur Institute of Paris. During his research, he discovered that the filtrate of the patients' fecal samples included an "anti-Shiga microbe." This was a first step towards the identification of an obligatory bacteriophage, or "bacteria eaters," as Herelle put it. In contrast to Twort, Herelle actively pursued his research in this direction and strongly supported the idea that bacteriophages were 'live viruses', and not enzymes secreted by microorganisms as many researchers thought. Eventually, the priority dispute was settled and the scientific community accepted the discovery of bacteriophages. They named it the 'Herelle – Twort phenomenon' or 'the bacteriophage phenomenon' (Rodrigues et al., 2021).

Until the discovery of penicillin and other drugs, in Western countries therapeutic phage preparations were used against streptococci, staphylococci, *E. coli,* and other bacterial infections. Gradually, the commercial use of antibiotics increased with an end to the phage era (Cook & Wright, 2022). Nonetheless, the Soviet Union, Georgia, and Poland actively implied phage therapy in various applications and are still used in routine. The phage institute in Tbilisi, established in 1923 by D'herelle and Georgi Eliava, prepares phage formulations to treat bacterial infections conversely to antibiotic usage (Myelnikov, 2018). However, in earlier times, due to a lack of significant evidence and deficient documentation, phage therapy lost its significance. The widespread occurrence of phage therapy substantially failed because of the inadequacy of clinical practice and regulatory guidance. Over the past decade, phage therapy still holds therapeutic potential and is considered to be a promising medicinal product under a legal framework, suggested by the European Medicines Agency (EMA) (Fauconnier, 2019). Various stakeholders have been compelled over phage safety and efficiency and thus have imposed disagreement. In spite, several trials are in process and are often used to treat patients. According to a study by Dedrick in 2019, the modified phage treatment of a patient infected with resistant *Mycobacterium abscessus* with significant clinical improvement (R. M. Dedrick et al., 2019). Successful cases also emerged related to eliminating mycobacterium species using different types of phages. Henceforth, the fundamental advantage of mycobacteriophages has paved the way for reviewing them as potential treatment applications in a drug-resistant era for tuberculosis (S. Aslam et al., 2020; Carrigy et al., 2019; Kalapala et al., 2020).

4. Potential mycobacteriophage against tuberculosis infection

Phages are obligate parasites, dependent completely upon their host for their survival. Phages are composed of proteins or proteolipids as capsid and core containing either deoxyribonucleotide acid (DNA) or ribonucleotide acid (RNA). Owing to their high specificity, they bind to diverse receptors on a susceptible host cell and remain at their site of infection. The relationship between the predator-prey exterminates the targeted strain only. The *M. tuberculosis* infection is one of the most common yet difficult to control because evolution has made *M. tuberculosis* multidrug-resistant. Ongoing research suggests that certain mycobacteriophages can be used to curb the

infection, due to their selective nature against their *Mycobacterium tuberculosis* host. There are several mycobacteriophages investigated, although three of them namely, D29, TM7, and DS6A are proven to be effective in preventing *M. tuberculosis* proliferation (Shield et al., 2021).

4.1 D29 mycobacteriophage

D29 is a lytic bacteriophage that contains two lytic proteins i.e. Lysin A (LsyA) and Lysin B (LysB) encoded by gene gp10 and gp12 respectively. LysA hydrolyzes and disrupts the peptidoglycan layer whereas LysB disperses the mycolic acid on the bacterial cell accompanied by holin protein, which is essential for host cell lysis (Bavda & Jain, 2020). These proteins have shown effective anti-mycobacterial activity against multidrug-resistant (MDR) tuberculosis strains. LysB protein effectiveness was evaluated in both in vitro and in vivo settings. In vitro, set-up showed LysB to be a significant anti-mycobacterial molecule even in low concentration (3µg/ml). On the other hand, an in-vivo study was conducted to ensure the LysB protein activity within the macrophages for the intracellular *Mycobacterium tuberculosis*pathogen, which demonstrated promising results with no cytotoxicity effects (Singh et al., 2023). Silva et al (Silva et al., 2023) prove the assistance of nanotechnology as a delivery system for targeting D29 mycobacteriophage both for active and latent TB infection with *Mycobacterium smegmatis* strain. The liposome-mediated D29 phage action remarkably eliminated high bacterial load with a single phage dose, which could be beneficial even for patients with compromised immune systems.

4.2 TM4 mycobacteriophage

TM4 is a double-stranded DNA bacteriophage, that performs lytic activity on Mycobacterium strains. The TM4 genome encodes a variety of proteins that are analogous to haloperoxidases and glutaredoxins function. Researchers have examined the liposome-mediated delivery of TM4 phage, catering to direct access within the intracellular infection site in the monocytes and macrophages (Azimi et al., 2019). The study conducted by Danelishvili et al provides instances from both in-vitro and in-vivo efficacy of TM7 phage for *Mycobacterium avium* strain. The treatment with TM4 mycobacteriophage accounted for a 100-fold reduction in the number of bacteria residing inside the spleen and in-vitro analysis exhibited 77% lytic activity on the isolates (Danelishvili et al., 2006).

4.3 DS6A mycobacteriophage

DS6A is a highly distinct mycobacteriophage, which has the potential to form plaques on members belonging to the *Mycobacterium tuberculosis* complex (MTBC). However, DS6A cannot form plaques on the nontuberculous mycobacteria (NTM), reflecting an efficient lysogenic system and can assure superinfection immunity. (Mayer et al., 2016). A phage cocktail comprising DS6A, GR21/T, and My327 was administered subcutaneously in *M. tuberculosis-infected* guinea pig, where DS6A was affirmed to be an effective phage resulting in a decreased count of bacilli in the spleen and hilus (Hosseiniporgham & Sechi, 2022). A second in-vivo evaluation offers proof of the powerful effect of DS6A in slowing the growth of *M. tuberculosis.* The intravenous delivery of DS6A phage lysed the *M. tuberculosis* strain H37Rv in primary macrophage augmented with a pulmonary condition in humanized mice (Yang et al., 2024).

4.4 Engineered mycobacteriophage

ZoeJ is a type of bacteriophage that can infect a wide range of mycobacterium strains. ZoeJΔ45 is a modified version of ZoeJ that does not have the gene 45, which encodes a repressor molecule. This modification allows ZoeJΔ45 to create clear plaques on the bacterial lawn of *M. smegmatis*. As a result, the Δ45 derivative is well-suited for carrying out lytic functions and has a broad host specificity(R. Dedrick et al., 2019). On the other hand, mycobacteriophage BP is unable to infect *M. tuberculosis* as compared to *M. smegmatis* and also forms plaques with lesser efficiency. BPsΔ33HTH_HRM10 is yet another derivative of mycobacteriophage BP, created to be engineered lytic mycobacteriophage with host range mutant. The phage cocktail involving Muddy, ZoeJ, and

BPs did not exhibit the expected cure rate and, therefore cannot be used as a promising alternative for tuberculosis treatment. In contrast, the combination of Muddy, ZoeJΔ45, and BPsΔ33HTH_HRM10 successfully killed *M. abscessus* GD01. Similarly, D29_HRM^{GD40} and BPsΔ33HTH_HRM10 engineered mycobacteriophage was subjected to a 26-year-old suffering from cystic fibrosis and advanced bronchiolitis due to *M. abscessus* infection, showed phage sensitivity (Lv et al., 2023; Wetzel et al., 2023). Dedrick et al. evaluated the phage cocktail efficiency with Muddy, ZoeJΔ45, and BPsΔ. It was administered intravenously and proved a ten-fold reduction in *M. abscessus* load within the initial month until the involvement of the strong immune response of IgG, IgM, and IgA leading to a subsequent decline in treatment efficacy in two months (R. M. Dedrick et al., 2021).

5. Mechanism of Mycobacteriophage Infection

A phage that infects M. tuberculosis follows a two-step mechanism for mycobacteriophage infection. First, the phage attaches itself to specific receptors of the bacillus Various phage proteins have been identified in the recognition of mycobacteriophage-mycobacteria. Specifically, phage L5's minor tail proteins such as gp6 and lysin protein gp10, along with homolog proteins in D29, appear to recognize and attach to mycobacterial surface receptors. Additionally, phage Rosebush's gp42 protein, which is a tail component, plays a crucial role in infection by breaking down sugar-containing molecules on the surface of the mycobacterial cell envelope (Allué-Guardia et al., 2021). After which it injects its DNA into the bacillus. Once inside, the phage DNA adopts a circular shape and the environment in which it finds itself will determine whether it undergoes a lytic or lysogenic cycle.

If the lytic cycle is initiated, new phage DNA and viral proteins are produced and assembled into new viral particles. These particles are then released when the M. tb bacillus ruptures.

Alternatively, If the lysogenic cycle is initiated, the phage genome integrates itself into the bacterial chromosome, becoming a prophage. This prophage will replicate along the tuberculosis genome and will be transmitted to the progeny, acquiring new properties encoded in the prophage (lysogenic conversion). Under certain conditions, the prophage DNA will detach from the bacterial chromosome, and the lytic cycle will be initiated.

Mycobacteriophages are viruses that can target the peptidoglycan layer of bacteria. In the case of *M.tb*, the cell envelope is complex and has a thick mycelial-arabinogalactan core that is responsible for the virulence of the pathogen (Gigante et al., 2017). Mycobacteriophages use phages like D29, which have LysA and Lys B holin enzymes that can cleave through this layer and kill the bacteria. They do this by encoding enzymes, lysins, and endolysins that can cleave the ester bonds between mycolic acids and arabinogalactan, ultimately leading to the death of the bacteria (Bavda & Jain, 2020). Another mechanism involves the existence of secondary metabolites such as superoxide radicals. These radicals induce apoptotic cell death, which is another way to kill the bacteria.

6. Phage therapy: Defence Against *Mycobacterium tuberculosis*

Phage therapy is an alternative approach used to combat the growing antibiotic resistance caused by the notorious bacillus. This bacterium is known to hide in macrophages, which defend the body against infection, and can also exist in a latent stage. As a result, completely eradicating it presents a significant challenge. According to a study conducted by Wei et al., bacteriophages can be used to treat *M.tb*, The study used genomics and proteomics to analyze mycobacteriophages and found a protein called PKB (protein kinase-B) that exhibits anti-microbial properties by interacting with the cord factor of *M.tb* (Wei et al., 2013). This alternative is highly effective because of its high specificity to replicate within its host organism and kill the pathogen at the infection site.(Yang et al., 2024). However, one of

the major challenges of this therapy is the phage delivery into the mammalian system where the infectious agent resides. To establish a stable interaction with the mammalian cell system, mycobacteriophage is mediated through a delivery agent *Mycobacterium smegmatis* (Azimi et al., 2019). One of the primary reasons for using mycobacterium *smegmatis* is that it is a nonvirulent fast-growing organism that doesn't cause any infection in people. It works like a medium for other phages like DS6A to attach themselves on the surface followed by its integration into the genome and once complete cycle leads to lysis outside the *M. smegmatis*. The lysed phages once inside the same environment as that of the *M. tb* attach themselves to its surface (Azimi et al., 2019).

Other developments have emerged, such as the use of base polymers and liposome-mediated delivery, which allows for easy penetration through the mucosal membrane and increased specificity to bacteria, even within biofilms (Durr & Leipzig, n.d.).

Further delivery systems include using designer phages like phage cocktails using more than one phage, thereby increasing the host specificity range as done in the T2, T4, and T7 families. In these sorts of delivery systems, certain modifications are made to increase the target specificity of the phages towards the infections. The adjustments made by synthetic biologists do so by modifying the Receptor-binding proteins present at the tip of the tail fibres via homologous recombination (Lin et al., 2012).

7. Integrating CRISPR with Phage Therapy

Bacteriophages can be modified to target extremely virulent and drug-resistant strains of *M. tb* like the Beijing lineage. These strains, which are common throughout Eastern Europe and Asia, pose serious problems for traditional therapies (Ribeiro et al., 2014). Through the use of bacteriophage specificity, CRISPR technology allows phages to be engineered to contain CRISPR-Cas9 components. Researchers can now specifically target particular genetic sequences within M. tb, such as those linked to virulence, antibiotic resistance, and vital metabolic pathways (Duan et al., 2021).

CRISPR-enhanced phages target specific genetic regions within M. tb, disrupting essential biological processes for bacterial survival and pathogenicity. Moreover, crucial targets for CRISPR-enhanced phage disruption are vital metabolic pathways, including fatty acid synthesis (kasA, kasB) and cell wall biosynthesis (embCAB, dprE1). These disruptions ultimately result in bacterial mortality (Hameed et al., 2018). Phages are modified at the genetic level to contain genes that code for enzymes such as biofilm-depolymerase, cell wall hydrolyses, and quorum quenching enzymes. Upon infection, these cells can produce these enzymes upon lysis. The degrading enzymes are usually Cas9 nucleases which create a nucleotide sequence-specific antimicrobial to target the host genome for degradation. The CRISPR method enables the removal of antibiotic-resistant genes (Tao et al., 2022).

Recent studies have demonstrated the feasibility and effectiveness of CRISPR-enhanced phage therapy in combating drug-resistant bacterial infections. Bikard et al. showed that CRISPR-Cas9 could be used to selectively target and kill antibiotic-resistant Staphylococcus aureus. This approach was also found to be effective in Mycobacterium tuberculosis, where CRISPR-Cas9 systems were engineered to cut specific DNA sequences within the bacterial genome, thereby enhancing the bactericidal activity of phages (Bikard & Barrangou, 2017). Yosef et al. also showed in a work how to eliminate antibiotic-resistant bacteria by using lytic and temperate bacteriophages that have been CRISPR-programmed. This dual approach not only targets the bacteria for lysis but also uses CRISPR to cut essential genes within the bacteria, thereby increasing the lethality of the phages and reducing the chances of resistance development (Yosef et al., 2015). Additionally, this technology opens up new avenues for personalized medicine, where phages can be tailored to the specific

genetic makeup of a patient and bacterial infection (Khambhati et al., 2022).

8. Bacteriophage: Applicable Challenges

One of the critical factors that needs to be taken in the course is the lung environment. When M. tb infects the lung, its cell envelope comes into contact with the alveolar lining fluid (ALF), which contains hydrolases that modify the bacterial cell envelope. These alterations can affect M. tb-host cell interactions, with potential impact on the infection and disease outcome. Moreover, during latent infection, M. tb is thought to remain intracellular within the granuloma, where its cell envelope may experience remodelling due to altered metabolic processes within the host cell. The variability of ALF hydrolase expression and functionality in different individuals makes defining phage-M. tuberculosis cell surface interactions are more challenging, depending on the ALF status of a given person at a given time (Allué-Guardia et al., 2021).

Once the phages have been introduced via different delivery systems inside the mammalian system, the innate as well as adaptive immune system comes into play as it considers the phages as foreign entities and hence tries to eradicate them from the environment therefore the phage titer and the route of administration should be highly optimized to work as an alternative therapeutic approach(R. M. Dedrick et al., 2021). The prospect of phage therapy also has certain concerns which need to be discussed before its optimum application can begin. Firstly, the identification of the appropriate phage for a specific bacterium and its legitimate illustration is intricate. This implies determining a particular phage particle against its targeted bacterial pathogen. To introduce a specific bacteriophage against a specific bacterial strain is a very composite and lengthy process. The enumeration and isolation of each phage particle from a complex sample is a fundamental key. The interaction between the phage and its bacterial host is greatly influenced by its lytic capacity and its dosage. The lytic factors dictate the efficiency rate of phage over its prey. Another regulatory hurdle is deciding the precise concentration of phage particles for each disease and infection.

Hence, it is a convoluted process to obtain a bacteriophage monopoly (Abedon et al., 2011).

Secondly, the theoretical possibility of bacterial resistance against phages may be possible. Moreover, the phage should not contain toxic genes, integrase genes, and other virulent genes. Lysogeny should be ruled out as it might be a channel for horizontal gene transfer. In the lysogenic cycle, the phages tend to integrate their genome within the bacterial chromosome. This amalgamation induces beneficial changes in the bacterial genome, unlocking new virulent elements that hasten the development of resistance (Howard-Varona et al., 2017). Environmental stresses impel the switch from lytic to lysogeny, promoting a competitive advantage to the host. Henceforth, it is not preferential for a phage to uphold the lysogenic cycle.

Thirdly, there might be an inefficient tendency of phages to bind to the receptors of the dead bacterial debris thereby outrunning the adsorption to the actual live bacteria (Aviram & Rabinovitch, 2008). This phenomenon blocks the receptor-binding protein of the phages and limits the availability of phage particles to interact with other receptors. Thus, it outruns the adsorption to the actual live bacteria with a significant reduction in the efficacy of phage.

Fourthly, the versatility of phage administration is known with the provided evidence. However, the ideal dose, precise delivery system, and duration of treatment for a specific phage to function at its optimum still need to be supervised (Wittebole et al., 2014).

Fifth, the viral genome heterogeneity is very high yet not much is known and identified. Due to this reason, we are far from abstracting sequenced genomic data for every phage type. Therefore, it becomes difficult to establish a relevant phage therapeutic approach with incomplete information (Hatfull, 2008).

Lastly, phage being a non-self-particle can stimulate a low level of immune response. Phage triggers specific antibodies in the blood such as IgM, IgG, and IgA. IgM-rich serum decreases the phage activity; IgG-rich serum inactivates the phage whereas IgA acts as a limiting agent influencing the phage activity in the gut region. The serum complement factors present in the serum are thus responsible for the decline in phage feasibility and substantially lower phage administration (Hodyra-Stefaniak et al., 2015; Van Belleghem et al., 2018).

Conclusion

Phages are the most abundant species in the biosphere constituting 10^{31} phage particles i.e., tenfold more than the bacterial population This leads to the theoretical possibility of having at least one distinct phage particle against each bacterial species. The global scenario has highlighted the evident use of mycobacteriophage therapy to combat the emerging problem of resistance mechanism exhibited by the *Mycobacterium tuberculosis* complex. The reimplementation of bacteriophages can become an essential tool serving advantages in curbing present-day ailments. However, certain setbacks cannot be ruled out because of the lack of profound research in phage physiology and clinical practicality. The lack of proper documentation and regulatory guidance has limited the widespread acceptance and consideration of phage therapy. This treatment is typically only considered when antibiotic treatment is inadequate. Challenges related to mammalian cell safety and the lack of standardized protocols have hindered the success of phage therapy in clinical trials. However, genetic engineering could be used to address these challenges and simplify the process. Therefore, it is essential to conduct in-depth studies to explore the potential of phage therapy as a substitute for treating patients with multidrug-resistant bacterial infections liketuberculosis.

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MD, SBM and DC have contributed equally in writing the review article. FC reviewed it and made the necessary corrections.

Conflict of interest

"The author(s) declare that there are no conflicts of interest "

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