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Phage in the diagnosis and treatment of tuberculosis

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1. ABSTRACT

The serious global TB epidemic coupled with limited diagnostic and therapeutic technologies necessitate the study of the role phage in TB treatment. Mycobacterium phage have been used for TB diagnosis, but the accuracy of such methods needs to be improved. Phage have various advantages in treating many kinds of bacterial infection, and coupled with the abuse and misuse of antibiotics, and the increasing prevalence of drug-resistant bacteria, they have been studied as a novel therapy to support antibiotics. The study of phage in TB therapy has developed from the selection of appropriate phage to the simultaneous use of multiple phage and even the use of purified lyase proteins. Though phage have great potential in TB therapy, the technology is still in the *in vitro* and animal experiment stages, and needs further study.

2. INTRODUCTION

The global incidence of tuberculosis (TB) is increasing, and existing methods of diagnosis and treatment have been limited and have not effectively stopped the TB epidemic. Existing diagnostic technologies are not perfect. For example, sputum smear microscopy (SSM) diagnosis is the most widely used diagnostic test for TB as it is simple and fast, but the sensitivity is low and it cannot distinguish between viable and dead bacteria. Compared with SSM, the diagnostic accuracy of bacterial culture is better, although it is time-consuming. Nucleic Acid Amplification Tests (NAATs) have high sensitivity but low specificity (1). The tuberculosis-specific ELISPOT assay (T-SPOT.TB) can give an overall measurement of the antigen load on the immune system, and this can reveal the presence of subclinical disease. Because this assay does not rely on the production of a reliable antibody response or

the recovery of the pathogen, the technique can be used to detect conditions such as latent tuberculosis (2). In addition, the outlook for TB treatment is not positive due to the rising prevalence of the increasingly serious multi-drug resistant tuberculosis (MDR-TB) and extremely drug-resistant tuberculosis (XDR-TB) strains. Therefore, novel technologies and medicines for TB prevention and therapy are urgently required.

Due to these concerns, phage have attracted the attention of TB researchers because their biological characteristics have advantages in treating many drug-resistant bacterial infections. The purpose of this presentation is to review the research of phage and their potential use in TB therapy in order to inspire novel TB therapies.

3. BIOLOGICAL CHARACTERISTICS OF MYCOBACTERIUM PHAGE

Mycobacterium phage (M. phage) are viruses that specifically infect mycobacteria. Bacterial hosts include the fast-growing *Mycobacterium smegmatis*, the slow-growing *Mycobacterium tuberculosis*, and *Mycobacterium avium*. The host range of each phage varies. For example, phage DS-6A specifically infects *M. tuberculosis* (3), while phages TM4 and D29 infect both *M. tuberculosis* and *M. smegmatis* (4,5).

Since Gardner and Weiser isolated and identified phage for the first time in 1947 (6), more than 250 kinds of M. phages have been identified, of which 80 have had their genome completely sequenced and comparative analyses of the sequences have been conducted (7–10). The nucleic acid molecules of these 80 phages are all dsDNA, and the phages can be classified morphologically by the presence of a contractile or a non-contractile tail (7). Currently, these 80 phages contain 5 independent genotypes (Giles, Corndog, Wildcat, Omega and TM4) and 9 communities (clusters A to I) (10). Phages D29, TM4, L5 and DS-6A in particular have attracted the attention of TB researchers.

4. THE USE OF MYCOBACTERIUM PHAGE IN TB DIAGNOSIS AND DRUG RESISTANCE TESTING

The timely and accurately diagnosis of TB is important in order to administer appropriate interventions to patients, in order to reduce morbidity and mortality, and to effectively inhibit the production and dissemination of drug-resistant TB (11). An inexpensive, simple, reliable and fast detection method is urgently needed in countries with a high TB burden, and where the medical facilities are poor (12). The use of phage for TB diagnosis is a viable option in these countries. It is based on the phage amplified biologically (PhaB) assay described by Wilson *et al.*, which is a technology that indirectly detects live *M. tuberculosis* in clinical samples (13). Briefly, the PhaB assay depends upon the ability of lytic phage D29 to infect both *M. tuberculosis* and *M. smegmatis*. The phage enter the cells and undergo a lytic cycle, which can subsequently be demonstrated in a quantitative manner by the production of plaques when the infected *M. tuberculosis* is mixed with a heavy suspension of *M. smegmatis* in solid medium. Antibiotic pretreatment of susceptible *M. tuberculosis* organisms should render these organisms incapable of supporting a lytic cycle, and hence plaques should not be produced in the indicator cells. To ensure that only intracellular phage are carried over into the *M. smegmatis* cultures, extracellular viruses are destroyed with the phagicidal agent ferrous ammonium sulfate. Controls with no *M. tuberculosis* cells are included with each batch of isolates. Final plaque counts on untreated plates vary (Figure 1).

The PhaB assay is potentially rapid and simple, taking as little as 48 h, compared with the 4 to 8 weeks required for conventional culture methods. The assay involves the use of simple equipment and, as the assay proceeds, the number of viable infectious *M. tuberculosis* cells declines, so that the assay actually becomes safer as it continues (12). Based on the PhaB assay, the FASTPlaque TB™ kit, luciferase reporter phage, and fluorescein-labeled phages have already been used as diagnostic technologies.

5. FASTPLAQUE TB™ KIT

FASTPlaque TB™, also known as PhageTek MB™, is a commercial diagnostic kit researched and developed by Biotec Laboratories Limited, and takes as little as 24 h. Samples of sputum, serum and tissues can be used. Rifampicin resistance can be tested by FASTPlaque-RIF™ or FASTPlaque-MDRi™, which are only used for culturing and isolating strains. The newly developed FAST Plaque-Response™ can directly test sputum samples (14). A meta-analysis reported that the variability of the FAST Plaque-Response™ kit in drug resistance analysis is so large that the specificity was between 73% and 100%, and the sensitivity was between 81% and 100% (14). The large variability of these results may be due to many factors. For example, the accuracy of detection in clinical samples is lower than that in pure culture, which may be due to pollution from contaminating fungi; the results of academic studies may be pessimistic compared with results from the company (14); finally, the growth conditions of *M. tuberculosis* may influence the test results. Thus, the widespread clinical application of the FASTPlaque TB™ kit should be on the basis of standardized operating procedures and advanced methods for pre-processing clinical samples, in order to achieve consistent and highly accurate diagnoses.

6. LUCIFERASE REPORTER PHAGE (LRP)

The first luciferase reporter phage (LRP) was constructed by integrating the firefly luciferase (FFlux) gene into phage DNA by Jacobs *et al.* (15). Recombinant phage infect host bacteria and then express luciferase, which decomposes luciferin, resulting in fluorescence which can be observed by fluorescence microscopy, indicating the presence of *M. tuberculosis*. LRP reconstruction methods have since been improved, with increasingly stable and active luciferase. The first

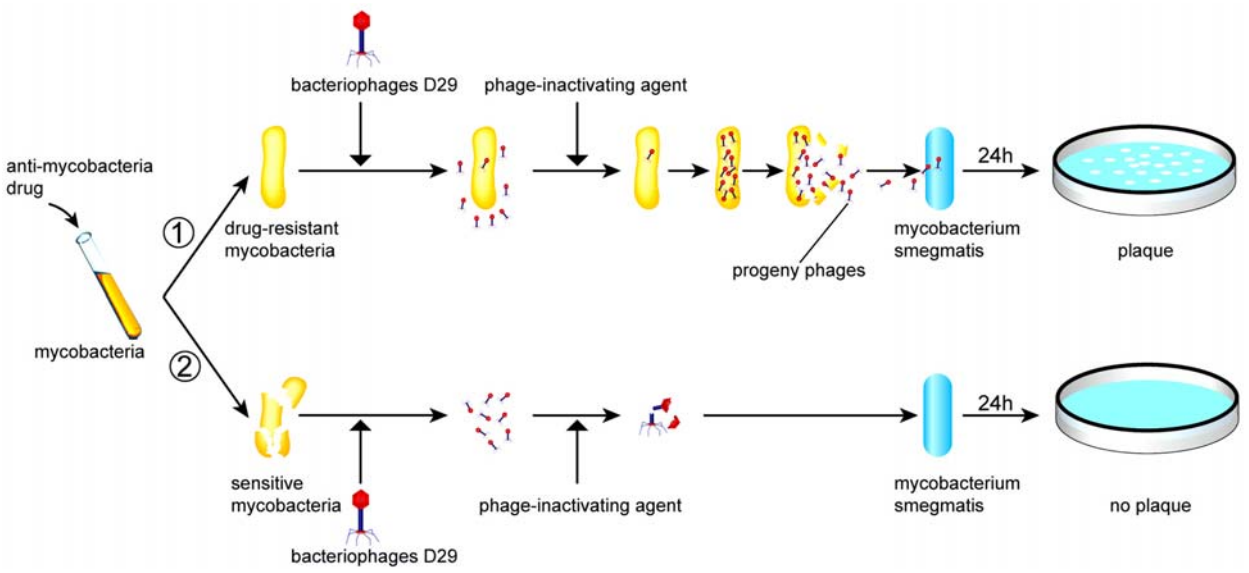

Figure 1. The phage amplified biologically (PhaB) the assay for TB diagnosis. The PhaB assay depends upon the ability of lytic phage D29 to infect both *M. tuberculosis* and *M. smegmatis*. The phage enters the cells and undergoes a lytic cycle, which can subsequently be demonstrated in a quantitative manner by the production of plaques when the infected *M. tuberculosis* is mixed with a heavy suspension of *M. smegmatis* in solid medium. Antibiotic pretreatment of susceptible *M. tuberculosis* organisms should render these organisms incapable of supporting a lytic cycle, and hence plaques should be not produced in the indicator cells. To ensure that only intracellular phage are carried over into the *M. smegmatis* cultures, extracellular viruses are destroyed with the phagicidal agent ferrous ammonium sulfate. Controls with no *M. tuberculosis* cells are included with each batch of isolates. Final plaque counts on untreated plates vary.

generation LRP phAE40 was derived from phage TM4, and can detect as few as 10,000 bacteria/ml (15). Subsequently, phGS18 was derived from lysogenic phage L5, which has a higher sensitivity than phAE40 but cannot infect *M. tuberculosis* (16). Therefore it was replaced by D29 derivatives (17). The recombinant products of TM4 and D29 showed good luminescence, but their sensitivity is not satisfactory because phage infection causes the host bacteria to die, and these dead bacteria cannot fluoresce. Using temperature-sensitive mutant lysogenic phage Che12 and TM4, Bardarov separately developed phAETRC16 and phAE159 (18), for which the detection rate of *M. tuberculosis* was 5 × 105 /ml and 4 × 104 /ml, respectively (19). A meta-analysis was conducted combining the results of eight studies published between 1999 and 2008 addressing the clinical application of LRP. This showed that LRP have a good accuracy. Among these eights studies, seven reported that LRP achieved 100% sensitivity. Four reported 100% specificity, while the remaining four reported a specificity of more than 89% (14). Comparing the meta-analysis results of LRP and the FASTPlaque TB™ kit, it was concluded that LRP are more accurate, and have a greater specificity and higher stability than the FASTPlaque TB™ kit for the diagnosis of TB.

In addition, Dusthackeer *et al.* constructed phAETRC21 from phAETRC16, which expresses FFlux driven by the isocitrate lyase (icl) promoter. They later constructed phAETRC201 and phAETRC202 from phAE159, which were driven by hsp60 and alpha-crystallin (acr), respectively. Among these phages, phAETRC201 has the highest sensitivity and was able to detect 81 cfu/ml of the $H_{37}RV$ standard strain – far lower than the detection limit of 10^5 cfu/ml for the clinical strain. Interestingly, phAETRC201 and phAETRC202 can detect dormant *M. tuberculosis*. In a previous study, both phAETRC201 and phAETRC202 showed a high luminescence in Wayne's dormant *M. tuberculosis* model under anaerobic conditions (19), which is promising for the diagnosis of latent *M. tuberculosis* infection. This technology is in the experimental stage, and the test conditions need to be improved.

7. FLUOROMYCOBACTERIOPHAGE

Fluoromycobacteriophage resulted from the development and optimization of LRP whereby the luciferase gene in the phage genome is replaced by gfp or ZsYellow, which are subsequently expressed in host bacteria. These bacteria then fluoresce without the addition of exogenous luciferase substrate. Furthermore, after fixing with paraformaldehyde, the specimens maintain fluorescence for more than 2 weeks without obvious exhaustion. This advantage is beneficial for the transport and preservation of specimens (4). Piuri *et al.* constructed rifampicin- and streptomycin-resistant strains, and then incubated them with the corresponding antibiotics for 16 h. They found that the fluoromycobacteriophage still fluoresce and the limit of detection was 100 cfu/ml (4). Later, another study found that the sensitivity of this method was 94% for Isonicotinylhydrazine (INH), Rifapin (RFP) and Streptomycin (SM), with specificities of 90% for INH, 93% for RIF, and 95% for SM. These results were

available in 2 days for RIF and STR and in 3 days for INH, with an estimated cost of $2 to test all three antibiotics (20).

In summary, M. phage have the potential to diagnose TB and detect drug-resistant *M. tuberculosis* if the accuracy of the tests can be improved. Measures, such as improving specimen preparation techniques, the ability to identify *M. tuberculosis* and non-*M. tuberculosis*, and quality assurance during the operation, may be helpful for optimizing and standardizing this technology.

8. MYCOBACTERIUM PHAGE IN TB THERAPY

Phage therapy is based on the lifestyle characteristic of phage whereby they specifically infect and lyse bacteria thereby killing these pathogenic hosts. Before antibiotics were discovered, phage therapy was already a research topic, especially in Eastern Europe and the Former Soviet Union, where researchers carried out extensive research mainly involving *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella*, *Pseudomonas aeruginosa* and other bacterial pathogens. As antibiotics became the dominant form of antimicrobial therapy, phage therapy research became stagnant. Due to the abuse and misuse of antibiotics over recent years, drug-resistant bacteria are increasingly common, and the treatment of bacterial infections such as *M. tuberculosis* is increasingly difficult. MDR-TB and XDR-TB are spreading globally, and becoming a serious threat to human life. MDR-TB is resistant to both RMP and INH, while XDR-TB is additionally resistant to second line anti-TB medicines including quinolones, and at least one of capreomycin, kanamycin, and amikacin (21). Therefore, phage have again attracted attention from researchers as a novel therapy to support antibiotics. Previous studies showed unique advantages of phage in treating infections caused by *E. coli*, *S. aureus*, and *P. aeruginosa*, and clinical trials have been carried out (22, 23). These advantages may inspire new breakthroughs in TB therapy.

9. EARLY STUDIES OF TB THERAPY

Previous studies of M. phage-treated *Mycobacterium bovis* or *M. tuberculosis* infections of animal models showed that these animals died of toxic shock caused by endotoxin release from bacterial lysis (23, 24). Sula *et al.* reported that *M. tuberculosis*-infected guinea pigs were treated with phage DS-6A, and lesions in the liver, spleen and lung later recovered (25). Zemskova found that phage had a therapeutic effect on hematogenous disseminated TB, though the effect was weaker than INH alone (26), although phage could inhibit granuloma formation.

Therefore, M. phage have an advantage in TB therapy, though the therapeutic effect is not yet as good as expected. Factors limiting the phage therapeutic effect will now be described.

10. APPROPRIATE PHAGE FOR TB THERAPY

Phage used for TB therapy should have the following characteristics. First, phage should be able to completely lyse the target bacteria. Second, the phage should be most virulent against *M. tuberculosis*, such as TM4 and D29, even though the phage may infect many hosts. Third, the phage should be non-pathogenic. Though many modern microbiology and phage therapy studies have shown phage to be non-pathogenic to humans, it is important to ensure that the phage are not capable of gene transduction, and that they do not carry antibiotic resistance genes (27). Thus, it is necessary to make a systematic toxicological analysis and conduct the necessary studies at the molecular level on the candidate phage before large-scale clinical trials. Fourth, the phage should be of low immunogenicity, as exogenous microorganisms may trigger an immune response. A previous study showed that phages were cleared by the reticuloendothelial system when they entered the human body through the circulatory system, resulting in phage titers at the site of infection that were not sufficiently high to have a maximal therapeutic effect. Therefore, novel phages with low immunogenicity should be obtained by screening phage mutants (29), by genetic reconstruction (30), or by coating them with biocompatible materials, in order to escape removal by the immune system.

DS-6A, D29 and TM4DS-6A have previously been used in TB therapy studies (25, 31, 32). D29 was first used for TB diagnosis, and later showed potential as a TB therapy. It had a good effect in guinea pigs infected with both drug-sensitive and drug-resistant *M. tuberculosis* strains (31, 33). Using multivariate analysis of relative synonymous codon usage
and base composition variation for the sequenced phages Che9c, D29, ω, TM4, Bxz1 and Bxb1 in order to compare their genetic makeup, gene expression and codon usage, researchers predicted that TM4 would have the most highly expressed genome and therefore the strongest ability to kill bacteria *in vivo*, suggesting that TM4 might be the best choice for TB phage therapy (34). Although TM4 has been considered for use in TB therapy, additional novel safe phages that treat TB effectively will need to be selected or constructed through genetic recombination. This would increase the number of alternative phages available, establishing a repository of therapeutic phage and providing more choice for combination therapy strategies to treat TB.

11. PROTECTIVE CARRIER

Since *M. tuberculosis* is an intracellular pathogen and nude phage are likely to be neutralized or removed by antibodies, it is necessary to improve the efficiency of intracellular transport of phage and aid their evasion of the immune system. Protective carriers can play an important role in solving this issue. A previous study showed that nude TM4 could not kill intracellular *M. tuberculosis in vitro*, but the results were different when TM4 resided in *M. smegmatis*. *M. smegmatis* was engulfed by the macrophage and then TM4 was transported into the macrophage. This study showed that the number of *M. smegmatis* decreased 100-fold after 48 h, and *M. tuberculosis* 10-fold after 48 h and 100-fold after 4 days

Table 1. Comparison of the advantages and disadvantages of single-phage agents and multi-phage agents in TB therapy

	Advantages	Disadvantages
Single-phage agent	1. When the pathogenic bacteria have been identified, high dose single-phage agents can be used, resulting in specific lysis. These phage can simply and quickly kill pathogenic bacteria, and rapidly alleviate the disease. Thus, it is appropriate for patients with acute severe infection or disease with rapid and progressive development, such as patients with sepsis, disseminated intravascular coagulation, septic shock, and so on. 2. A short-term and high dose treatment is not appropriate if drug-resistant strains emerge.	1. The host range is narrow and it is difficult to treat many kinds of pathogenic bacteria. Thus, it is not appropriate for newly presenting patients with unidentified pathogenic bacteria. 2. Phage resistance is likely to emerge early.
Multi-phage agent	1. The host range is broad. It is appropriate for patients in the early stages of infection with unidentified pathogenic bacteria by selecting multiple phage covering the local spectrum of bacteria. The phage cocktail can be modified according to drug susceptibility testing when the pathogenic bacteria have been identified. It is appropriate for patients with moderate illness, as a combination of phage has a synergistic effect in TB therapy. 2. Patients can be treated repeatedly since the incidence of phage resistance is low.	1. The formulation and regulation of multi-phage preparations is complex, time-consuming, and the elimination rate of phage is high. 2. It takes a long time for preliminary studies to select the various kinds of phages in order to establish the repository, and to pre-design different options to treat different strains.

(32). This suggested that phage can be used to kill intracellular *M. tuberculosis*. An *in vivo* study further demonstrated that the number of *M. avium* in a mouse spleen in the treatment group decreased by log0.5, while the number in the control group increased by log0.6 after 7 days' treatment with phage (35). Results from these two studies suggest that *M. smegmatis* can act not only as a carrier to transport phage into the cell, but also as a host to promote proliferation of TM4, increasing phage infection rates, as well as providing TM4 with a relatively suitable environment within macrophages to maintain its activity.

A study also showed that microencapsulated phages using two sodium alginate-based methods remained stable at both 4°C and 22°C for up to 14 days with no appreciable drop in titer (36), suggesting that a protective carrier could prolong phage viability and reduce the requirements for alternative preservatives.

12. SINGLE- OR MULTI-PHAGE AGENTS

Bacteria can become resistant to phage just as they can to antibiotics. Receptor proteins in the bacterial cell wall that specifically bind the phage can mutate or be lost, resulting in the bacteria escaping from absorption and infection by phage, and the emergence of phage resistance. The emergence of phage resistance is associated with repeated treatment with a single phage, which could result in the selection of naturally occurring resistant strains (37). One promising solution is to use a mixture of phages. This is similar concept to combination antibiotic therapy. Researchers have found that phage mixtures can inhibit resistant bacteria from arising during the therapeutic use of phage, and the time before emergence of resistant bacteria could be prolonged through optimization of the phage cocktail. They suggested that the best phage combination is composed of more than two kinds of phages that bind to different receptors in the cell wall (38). The characteristics of single- and multi-phage agents in TB therapy determine their use in that the former is appropriate for patients with acute severe infection, or disease with a rapid and progressive development, while the latter is appropriate for patients with moderate illness (Table 1).

13. DELIVERY METHOD, FREQUENCY AND DOSE

The delivery methods, including nose drops, inhalation, oral, subcutaneous injection, intravenous injection, or direct injection of an abscess, depend on the location of the TB lesions. The process of phage moving between different organs, for example, from the blood into parenchymal organs, occurs with first-class pharmacokinetic kinetics, and this rate is dependent on the phage density, and forward and reverse movements (39). The life cycle of phage *in vivo* includes replication and decay. Decay refers to phage inactivation and the loss of proliferative offspring. The removal of phage can be regarded as movement to different body parts, resulting in decreased numbers overall.

The delivery frequency and dose is dependent on how long the phage can remain effective and maintain an effective therapeutic concentration at the treated sites without serious side effects. In theory, phage should meet this requirement to achieve the best therapeutic effect. The ability of phage to amplify is positively correlated with the concentration of target bacteria because the replication of phage depends on the host. When the bacterial density is large enough, the ability of phage amplification is strong and lysis occurs. At low bacterial densities, the rate of phage loss is faster than the replication rate, or the phage does not amplify, and a higher phage dose may need to be considered (40). The question then arises as to whether administration of large doses of phage will cause side effects or even fatal damage. Since phage are composed of protein and DNA, their toxicity is low (29). In addition, phage particles may be degraded during metabolism, which is different from antibiotic metabolism because phage metabolism would not produce toxic metabolites. Therefore, high-dose treatment will not result in serious toxicity except mild allergies to the phage capsid protein, although these are likely to be uncommon (39).

14. THE THERAPEUTIC EFFECT OF LYASE

Lyase is an enzyme that is expressed in the final stages of the phage life cycle and biodegrades peptidoglycan in the bacterial cell wall, resulting in bacterial lysis and release of progeny phage. As drug-resistant TB becomes an increasingly serious problem, phage lyase has become a potential additional treatment strategy. There are several advantages. First, purified lyase specifically lyses the corresponding host without interfering with normal microflora, unlike antibiotics, which can be non-specific. However, the disadvantage of this specificity is a narrow antibacterial spectrum. Thus, a lyase cocktail

treatment may be appropriate. A previous study found that a combination of lyases had a synergistic effect, as complementarity between these lyases increased the probability of reaching their target cleavage sites on pathogenic bacteria. Furthermore, the protein structure can be engineered resulting in an increased ability to kill bacteria and an altered targeting specificity (42). Second, lyase is not likely to be antigenic. One concern is that pathogenic bacteria may develop resistance to lyase, and that an immune response may be stimulated. Fortunately, lyase resistance has not been described yet (43,44). Though lyase could stimulate the body to produce antibodies, these antibodies are not able to lyse bacteria (45,46).

A significant issue for the use of phage lyase in TB therapy is how it can cross the outer lipid layer of bacteria to access the peptidoglycan. Lyase combined with other chemical medicines such as INH may offer a solution, and the therapeutic effect needs further study.

15. CONCLUSION

In recent years, phage therapy has become important in light of the increasingly serious situation of drug-resistant TB around the world. The use of phage and their lyases show potential in treating bacterial infections, including TB. The rapid development of proteomics, genomics, bioinformatics and other disciplines strongly supports basic phage research. Therefore, it is clear that whilst the study of the use of phage for TB therapy is still in the *in vitro* and animal experimentation stages, further study is necessary. When late-stage clinical trials are in place for drug-resistant TB therapy, a novel method for drug-resistant TB therapy will be available, potentially shortening the chemotherapy treatment.

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Abbreviations: TB: tuberculosis; SSM: sputum smear microscopy; NAATs: Nucleic Acid Amplification Tests; T-SPOT.TB: tuberculosis-specific ELISPOT assay; MDR-TB: multi-drug resistant tuberculosis; M. phage: Mycobacterium phage; PhaB: phage amplified biologically; LRP: luciferase reporter phage; FFlux: firefly luciferase; icl: isocitrate lyase; acr: alpha-crystallin; INH: Isonicotinylhydrazine

Key Words: tuberculosis, multi-drug resistant tuberculosis, Mycobacterium phage, Review

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