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Progresses of mycobacteriophage-based *Mycobacterium tuberculosis* detection

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Abstract: Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide, particularly in developing countries. A rapid and efficient method for TB diagnosis is indispensable to check the trend of tuberculosis expansion. The emergence of drug-resistant bacteria has increased the challenge of rapid drug resistance tests. Due to its high specificity and sensitivity, bacteriophage-based diagnosis is intensively pursued. In this review, we mainly described mycobacteriophage-based diagnosis in TB detection, especially two prevalent approaches: fluorescent reporter phage and phage amplified biologically assay (PhaB). The rationale of reporter phage is that phage carrying fluorescent genes can infect host bacteria specifically. Phage amplified biological assay based on the principle that phages can infect the live *Mycobacterium tuberculosis* in the specimen under suitable conditions and produce plaques. Other phage-based diagnostic methods, such as a combination of the amplified biologically assay and nucleic acid amplification or lateral flow assays, are also actively explored. This review will help us improve the understanding of mycobacteriophages in TB detection and better promote the development of the rapid diagnosis of *M. tuberculosis*.

Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, is an ancient yet recent serious infectious disease of global significance (Lienhardt *et al.*, 2012). According to the TB report released by the World Health Organization in 2019, about 2 billion people are infected with the pathogen of *M. tuberculosis* in the world every year. There are approximately 9 million new cases and nearly 2 million deaths in 2018 (Piuri and Hatfull, 2019; WHO, 2019). Multiple factors contribute to the recalcitrance of tuberculosis, such as HIV coinfection, the emergence of drug-resistant strains with compensatory mutations, and the global demography shift (Raviglione *et al.*, 2012). Laborious, cumbersome, lengthy, and not so precise diagnostic of *M. tuberculosis* and drug resistance impeded the successful control of TB. Each method just showed mixed success, such as bacteriology, immunology, molecular biology, and cytology. The century-old sputum smear-based examination remains the mainstay diagnostic in most resource-limited regions. Pathogens culture-based methodology can improve the specificity, but it is lengthy and has high false-negatives (Davies and Pai, 2008; Pizarro-Bauerle and Ando, 2020). Detection methods based on immunological analysis include antibody from lymphocyte secretion (ALS) assay, enzyme-linked immunosorbent assay (ELISA), the tuberculin skin test (TST), IFN-gamma release assays (IGRAs), and other methods. The detection method combining ALS and ELISA uses ELISA to quickly detect *M. tuberculosis* infection by detecting specific antibodies secreted by lymphocytes. The tuberculin skin test is based on the principle that the specific protein antigen, purified protein derivative (PPD), of the *Mycobacterium tuberculosis* complex causes skin inflammation. Immunological detection methods have the advantages of being relatively fast and high specificity, but there are some limitations such as low sensitivity (Gazi *et al.*, 2015). Tuberculin skin test was flawed by its incapable to distinguish other mycobacteria infection, in particular the vaccination of BCG (Goldstein *et al.*, 2002; Oettinger *et al.*, 2003; Andersen *et al.*, 2000). Diagnostic tests capable of differentiating infected from vaccinated

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animals (DIVA) have been developed, but these tools are too expensive and not sensitive enough to be used globally (Chandran *et al*., 2019). Molecular biology methods based on DNA amplification techniques such as PCR, loop-mediated isothermal amplification, and ligase chain reaction (LCR) appear to be more efficient and less time-consuming. Other novel methods based on the number of lymphocyte subsets and microRNA are emerging too.

Generally speaking, the drawback of the above methods is low sensitivity, which necessitates the enrichment procedure for the detection of a single bacterium or few bacteria. This will greatly prolong the detection time. Molecular biology-based methods can be fast but need more resources and higher cost, which restricted its wide application in most regions. Another disadvantage of a molecular-based procedure is its incapability to differentiate the viability of bacteria (Lu *et al*., 2013), though mRNA detection can compensate such a shortage. More simple, rapid, cost-effective methods are needed. The comparison of different detection methods of *M. tuberculosis* is listed in Tab. 1.

The detection of drug resistance to direct the treatment regimen represents another difficult predicament for tuberculosis control. Multi-drug resistance TB (MDR-TB), defined as the resistance to the rifampin and isoniazid (Silva, 2011; Wei *et al*., 2019), extensive drug-resistance TB (XDR-TB), defined as resistant to any fluoroquinolone and at least one injectable second-line drugs (kanamycin, capreomycin, or amikacin) in addition to resistant against rifampin and isoniazid (Control CfD & Prevention, 2006), even totally drug-resistant TB (XXDR-TB), defined as resistant to nearly all first- and second-line drugs are a global concern (Velayati *et al*., 2009; Loewenberg, 2012; Udwadia *et al*., 2012). The classical gold standard tests are based on the cultivability of *M. tuberculosis* in the presence of drugs with different concentrations. The bacterial growth state reflects the degree of drug-resistance. Though accurate and reliable, it is time-consuming and laborious. Newer culture-based resistance detection methods rely on metabolic activity or growth characteristics, which quickens the process of test. Detection of causal resistance mutation is a good alternative, but not affordable to each and every hospital, and many resistant strains without a clear-cut causal mutation (Palomino *et al*., 2014). GeneXpertMTB/RIF test can detect rifampicin-resistant *M. tuberculosis* in sputum samples within a few hours by the nucleic acid amplification technique (NAAT), but the technique is costly and cannot distinguish between live and dead cells, which

TABLE 1

Comparison of different detection methods of *M. tuberculosis*

Method	Technique	Detection time and cost	Characteristic	Reference
Cytological detection	Culture and colony counting	5–7 days, cheap	High specificity, It can distinguish living and dead cells, no need of professional laboratory instruments lengthy, high false negative rate, need of pre-enrichment, need of professional laboratory personnel	(Farooq *et al*., 2018)
Immunological detection	Antibody from lymphocyte secretion (ALS) assay, The tuberculin skin test (TST), IFN-gamma release assays (IGRAs), lateral flow immunoassay (LFIA), mass spectroscopy (MS), etc.	0.5–4 h, expensive	High specificity, rapid, It cannot distinguish living and dead cells, low sensitivity, need of pre-enrichment, need of professional laboratory personnel, need of professional laboratory instruments	(Farooq *et al*., 2018; Gazi *et al*., 2015)
Molecular biology detection	PCR, Loop-mediated isothermal amplification, Ligase chain reaction (LCR), etc.	1–4 h, expensive	Rapid, accurate, high specificity, It cannot distinguish living and dead cells, need of pre-enrichment, need of professional laboratory personnel, need of professional laboratory instruments	(Farooq *et al*., 2018; Gazi *et al*., 2015)
GeneXpertMTB/RIF	–	a few hours, expensive	High sensitivity, easy to operate, rapid diagnosis of drug-resistant *M. tuberculosis* It cannot distinguish living and dead cells, need of professional laboratory personnel, need of professional laboratory instruments	(Liu *et al*., 2016)
Bacteriophage based detection	Phage amplified biologically assay (PhaB) Fluorescent reporter phages, etc.	2 h–5 days, cheap	High specificity, high sensitivity, easy to operate, It can distinguish living and dead cells, no need of professional laboratory instruments, no need of pre-enrichment, need of professional laboratory personnel, limitation of natural phage defects	(Farooq *et al*., 2018; Piuri and Hatfull, 2019)

limit the application of this technology (Rondon *et al*., 2018; Talwar and Talreja, 2016). Moreover, phenotypic heterogeneity is a barrier to the detection and treatment of *M. tuberculosis*, requiring longer and more effective treatment to completely remove bacteria (Jain *et al*., 2016).

New methods of mycobacteria diagnosis are urgently needed. Mycobacteriophage represents a good alternative. As a tool for detecting bacteria, phage has many advantages such as low-cost, shorter detection time, being simple to operate, infecting only live bacteria, and reducing the occurrence of false positives. Mycobacteriophage has been used in the detection and analysis of mycobacteria many years ago. Two main categories of methods are developed (Viñuelas-Bayón *et al*., 2017). The first one is phage amplified biologically assay (PhaB), which uses sensitive host bacteria (such as *M. smegmatis*) to proliferate progeny phages to infect *M. tuberculosis*. Some detection tools based on mycobacteriophage have been commercialized such as FASTPlaqueTB^{TM} (D29 was used for *M. tuberculosis* detection in sputum), FastPlaque-Response (used for rifampicin-resistant strains detection). Sensitive host bacteria as an indicator are indispensable for these methods. Reporter phage represents another direction. The Bronx Box based on film or fluorescent reporter phage can shorten the detection time of bacterial drug resistance (for Isoniazid, rifampicin, capreomycin, and ethambutol) to 3 days. More engineered phages are emerging for this end.

Fluorescent Reporter Phage

Phage can infect host bacteria specifically. The gene encoding fluorescent protein can be integrated into phage genomes, thereby consistent fluorescence might be spotted during phage infection. The presence of fluorescence and strength can indicate the existence of pathogens or drug-resistant pathogens.

Three stages can be roughly divided for the development of fluorescent reporter bacteriophages: 1. The early phase: This stage aimed to develop a bacteriophage capable of detecting *M. tuberculosis* or drug-resistant *M. tuberculosis*. Specificity and sensitivity are further goals. The firefly luciferase gene is the major fluorescent gene used in this stage. 2. Clinical application: Practical clinical application of developed mycobacteriophage is the goal of this stage, especially for direct detection of *M. tuberculosis* or its drug resistance in sputum. Fluorescent protein genes or similar genes (FGFP, ZsYellow, mVenus, etc.) are actively used in this stage. 3. Future detection phase: The goal at this stage is to develop phages that can detect the ratio of active and dormant *M. tuberculosis* in sputum directly.

The early phase of fluorescent reporter phages

W.R. Jacobs firstly demonstrated that luciferin can permeate the mycobacterial cell wall and be catalyzed to generate protons by firefly (Jacobs *et al*., 1993). Both components have been engineered to yield the first luciferase reporter phage (LPR)—phAE40 with mycobacteriophage TM4 as the scaffold. The minimum 10^4–10^5 CFU/mL bacteria to produce significant signal limited the detection threshold of phAE40 (Sarkis *et al*., 1995).

FFlux gene was inserted into the left arm of the genome where the gene encoding the virion structural protein located to improve the expression level of firefly luciferase. This is the second generation LRP derived from L5 designated as phGS18 (Sarkis *et al*., 1995). L5 is a temperate phage in which firefly luciferase can be consistently expressed during infection of host bacteria. The inability of phGS18 to infect *M. tuberculosis* prevented it from the clinical application. Highly homology to L5, D29 can efficiently infect *M. tuberculosis* and *M. tuberculosis* complex. LRP derived from D29 (such as phBD8) was constructed successfully. It was found that phBD8 and phAE40 have similar sensitivity towards host strain except for the L5 lysogeny (Pearson *et al*., 1996).

LRPs based on phage TM4 (temperature-sensitive phage) were constructed to meet the clinical ends such as phAE85, phAE88. The lower detection limit of phAE88 reaches 120 CFU/mL (BCG), and only one day is required to read out (Carriere *et al*., 1997). The sensitivity and specificity of LRP can be improved by the modification with the addition of p-nitro-a-acetylamino-b-hydroxy propiophenone (NAP) (Riska *et al*., 1997). Film exposure-based LRP, coined as Bronx Box, was constructed with phAE85 (Riska *et al*., 1999). The promoter Phsp60 in phAE85 replaced with a more powerful one, Pleft from phage L5, produced novel LRP-phAE142, which showed higher sensitivity. It takes approximately 12 days from the sputum sampling to yield drug resistance results if phAE142 was used for diagnosis (Bardarov *et al*., 2003). A comparison between LRP detection based on phAE142 (Bronx box or luminometry) with PM method (the agar proportion method), MGIT960, and BACTEC460 showed that both methodologies have similar performance, and LRPs was faster and less costly (Banaiee *et al*., 2001; Hazbon *et al*., 2003; Bardarov *et al*., 2003).

Clinical application of improved fluorescent reporter phages

To overcome the drawbacks of the reporter phages with firefly luciferase gene in the clinical application (Hazbon *et al*., 2003), such as lower fluorescence not easily for drug-resistant strains detection directly in the sputum and high technical threshold, new fluoromycobacteriophages (phAE87::hsp60-EGFP and phAE87::hsp60-ZsYellow) with enhanced fluorescent reporter genes gfp or ZsYellow were constructed (Piuri *et al*., 2009), which had application potential for rapid diagnosis and drug susceptibility testing (DST) of TB (Rondon *et al*., 2018).

This fluoromycobacteriophage can detect or screen MTB and drug-resistant MTB simply by fluorescent microscopy or flow cytometry. The detection sample can be fixed by paraformaldehyde, which makes the fluorescence be maintained at least two weeks, providing enhanced biosafety, but still failed to detect sputum directly (Rondon *et al*., 2011). This obstacle was not overcome until the construction of high-intensity fluoromycobacteriophage Φ^2 GFP10 based on phAE159 (Jain *et al*., 2012). The deletion of 6.0kb nonessential genes greatly improved the cloning size of phAE159. Strong promoter and the multiple reporter genes can be cloned to the phage skeleton. The deletion of the TM4 gp49 gene in phAE159, involved in inhibiting

bacteriophage superinfection, will enhance the per-cell signal due to a higher multiplicity of infection (MOI). This improvement will increase the intensity of fluorescence of cells infected with Φ^2GFP10 approximate 100-fold. In the year 2015, they test the effect of novel reporter phage to detect *M. tuberculosis* and rifampicin resistance. Compared with Gen Xpert MTB/RIF, Φ^2GFP10 has higher sensitivity for *M. tuberculosis* and RIF resistance, including in acid-fast bacillus smear-negative sputum (O'Donnell *et al.*, 2015). The engineered fluoromycobacteriophage can directly detect *M. tuberculosis* in clinical sputum samples. The cost is very low, just around 2 dollars per sample (Jain *et al.*, 2012; Yu *et al.*, 2016). After that, new fluoromycobacteriophage was constructed. Jain and his colleagues constructed a new dual fluorescent reporter bacteriophage Φ^2DRMs by fusing strong promoters of up-regulated genes found in persisted cells and the red fluorescent protein tdTomato (Jain *et al.*, 2016). It can encode an active green fluorescent marker and emit a strong red fluorescence. Fluoromycobacteriophage Φ^2DRMs can not only flexibly select the delivery time of the reporter gene and the gene promoter that controls the expression of fluorescent protein, but also can identify the cells that may persist and the heterogeneity of the cell population that survives in antibiotic treatment, which provide help for downstream analysis and treatment (Jain *et al.*, 2016). Another new fluoromycobacteriophage mCherrybombΦ carrying the mCherry gene of codon-optimized usage has higher detection sensitivity and shorter test time (Estefanía *et al.*, 2016; Piuri and Hatfull, 2019), which can detect *M. tuberculosis* and determine the drug resistance of RIF in 3–5 days from sputum collection. The cost of testing is only $0.25 each time. Fluoromycobacteriophage mCherrybombΦ can also be used to determine the resistance of *M. tuberculosis* to fluoroquinolones such as ofloxacin and levofloxacin (Rondón Salazar, 2017). In addition, p-nitrobenzoic acid (PNB) can be used to distinguish between *M. tuberculosis* complex and non-tuberculous mycobacteria strains to improve detection accuracy during fluorescence detection and analysis (Rondon *et al.*, 2018). As a selective inhibitor, PNB can inhibit the growth of *M. tuberculosis* while non-tuberculous mycobacteria strains are resistant to it (Sharma *et al.*, 2010). Fluoromycobacteriophage Φ^2GFP12 and Φ^2GFP13 based on DS6A, a specific phage infecting *M. tuberculosis* complex, were subsequently successfully constructed, providing important new tools for the research and detection of mycobacteria (Mayer *et al.*, 2016).

Future detection phase of fluorescent reporter phages

Future mycobacteriophage for diagnosis shall be higher sensitivity, higher specificity, and lower cost. If the result can be obtained within 1 h, this technology will be highly desirable (Jain *et al.*, 2011). In addition, the adsorption rate of TM4-derived fluoromycobacteriophage is relatively low and it is not a kind of specific phage infecting the *M. tuberculosis* complex. It can also infect some non-pathogenic bacteria such as *M. smegmatis*, so fluoromycobacteriophages that can specifically infect *M. tuberculosis* deserve further research. It is also a good choice to improve the efficiency of adsorption by isolating mutant phages and constructing more effective fluoromycobacteriophages (Fu *et al.*, 2015). For better applications in developing countries, lower-cost downstream detection instruments are also being developed such as light-emitting diode (LED) fluorescence adapters (Piuri and Hatfull, 2019; O'Donnell *et al.*, 2015). The methods based on fluoromycobacteriophage that are capable of distinguishing live or dead *M. tuberculosis* in the sputum will be developed in the future by combining with other technologies, such as confocal imaging and analyses methods and acid-fast staining (Jain *et al.*, 2012; Jain *et al.*, 2011; MacGilvary and Tan, 2018). In the future, an automated fluorimeter evaluation method is recommended to rapidly determine antibiotic sensitivity in clinical isolates (Rondón Salazar, 2017). In general, the prospect of clinical diagnosis using fluoromycobacteriophage is broad and attractive (Fig. 1). The fluorescent reporter phages mentioned in the article are listed in Tab. 2.

Phage Amplified Biologically Assay (PhaB)

Mycobacteriophage D29 was firstly characterized, with a lytic time of about 13 h in slow-growing *M. tuberculosis* and just 90 minutes in fast-growing *M. smegmatis* (David *et al.*, 1980). Phage amplified biologically assay (PhaB) was developed in 1997 based on this differential lytic time Viable mycobacterium can protect mycobacteriophage within (Wilson *et al.*, 1997). After drug treatment of the mixture of mycobacteriophage and *M. tuberculosis*, only mycobacteriophages infected can survive and enter the lytic cycle. The lawn formed by sensitive host *M. smegmatis* can be used to enumerate the number of phages by the plagues. Compared with the traditional incubation method, this method can reduce the drug resistance detection time of isoniazid and rifampin from 6–8 weeks to only 3–4 days. The detection sensitivity can reach 10^2 CFU/mL. But the specificity depends on the host range of phages. The wide host range of D29, including *M. fortuitum*, *M. phlei*, *M. butyricum*, and *M. aurum*, is a downside. A further selection of phage or engineering is needed (David *et al.*, 1980). Removal of uninfected mycobacteriophage is crucial for the successful application of PhaB. Ferrous ammonium sulfate can effectively remove uninfected phage without compromising the growth and lytic cycle of mycobacteriophages. This improvement makes PhaB more reliable. This method has been successfully applied to sputum specimens (McNerney *et al.*, 1998). For rifampin resistance detection, PhaB is better than the transcription-PCR method (by detecting the level of dnaK mRNA) calibrated with the traditional method (Eltringham *et al.*, 1999). PhaB can be used to detect the resistance to isoniazid, ethambutol, streptomycin, pyrazinamide, and ciprofloxacin with an agreement ratio to the traditional method of 88%, 87%, 96%, 100%, respectively. If 90% reduction is used as the cutoff in plaque counting, the correlations would be better (Eltringham *et al.*, 1999).

Based on PhaB, FASTPlaqueTB^{TM} for the detection of *M. tuberculosis* and FASTPlaque-MDR^{TM} (Biotec Laboratories Ltd., Ipswich Suffolk, U.K.) for drug resistance were developed (Albert *et al.*, 2001; Muzaffar *et al.*, 2002; Trollip *et al.*, 2001) and used for detection in sputum (Albert *et al.*, 2004; Muzaffar *et al.*, 2002) (Tab. 3). Details

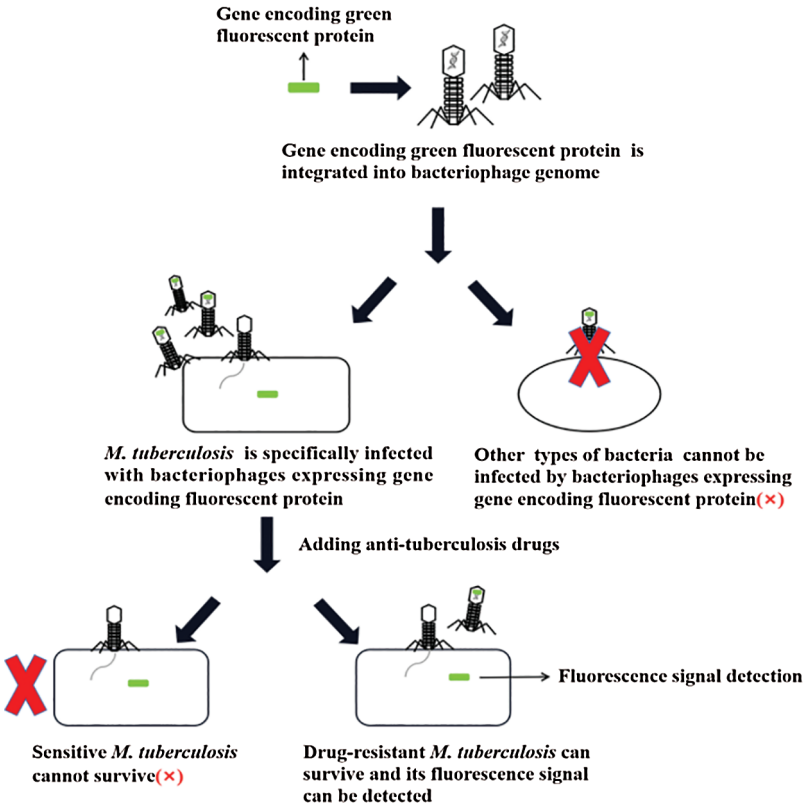

FIGURE 1. Fluoromycobacteriophage for the detection of *M. tuberculosis* and its drug resistance. *M. tuberculosis* is specifically infected by fluoromycobacteriophages. After being treated with anti-tuberculosis drugs, only drug-resistant *M. tuberculosis* can emit fluorescence which is detected by fluorescence microscope.

TABLE 2

Fluorescent reporter phages

Fluorescent reporter phage	Fluorescent protein gene	Bacteriophage carrier	Reference
phAE40	Firefly luciferase	TM4	Jacobs *et al.* (1993)
phGS18	Firefly luciferase	L5	Sarkis *et al.* (1995)
phBD8	Firefly luciferase	D29	Pearson *et al.* (1996)
phAE85	Firefly luciferase	TM4	Carriere *et al.* (1997)
phAE88	Firefly luciferase	TM4	Carriere *et al.* (1997)
phAE142	Firefly luciferase	TM4	Bardarov et al. (2003)
phAE87 (phAE87::hsp60-EGFP, phAE87::hsp60-ZsYellow)	GFP/ZsYellow	TM4	Piuri *et al.* (2009)
phAE159 ($\Phi^2$2GFP10)	GFP	TM4	Jain *et al.* (2012)
phAE159 (Φ^2 DRMs)	GFP,RFP	TM4	Jain *et al.* (2016)
phAE159 ($mCherry_{bomb}\Phi$)	GFP	TM4	Estefanía *et al.* (2016)
phAE159 (Φ^2GFP12, Φ^2GFP13)	GFP	DS6A	Mayer *et al.* (2016)

on the infection process of D29 phage, and optimization of the procedures are thoroughly evaluated (McNerney *et al.*, 2004). Schemes to avoid the formation of plaques on the lawn by uninfected phages are devised to improve the results (Ulitzur and Ulitzur, 2006). The comparison with other methods in the high burden, low-resource city, Kanpur, India, showed mixed results (Prakash *et al.*, 2009). The result will be influenced by the time and velocity of sputum specimen treatment and when the detection began. Earlier treatment will yield better results. Cross-contamination is another concern for detection. This method is helpful for the detection of samples with HIV co-infection. Later the PhaB was improved and made use of a liquid culture media and a multichannel series piezoelectric quartz crystal sensor

TABLE 3

The commercial bacteriophage kits for detecting *M. tuberculosis*

Kit name	Company	Mechanism	Reference
The Bronx Box	Sequella Inc, Rockville, MD, USA	Film exposure based fluorescent reporter phage	Riska *et al.* (1999)
FASTPlaqueTB^{TM}	Biotec Laboratories Ltd., Ipswich Suffolk, UK	Phage amplified biologically assay	Muzaffar *et al.* (2002)
FASTPlaque-RIF	Biotec Laboratories Ltd., Ipswich Suffolk, UK	Phage amplified biologically assay	Albert *et al.* (2001); Albert *et al.* (2002)
FASTPlaque-MDR^{TM}	Biotec Laboratories Ltd., Ipswich Suffolk, UK	Phage amplified biologically assay	Trollip *et al.* (2001)

to detect cellular lysis. This PhaB assay could detect pathogenic bacteria as low as 100 CFU/mL in 30 h (van der Merwe *et al.*, 2014). Compared with traditional *M. tuberculosis* diagnostic tools, FASTPlaqueTB^{TM} has higher sensitivity and high specificity, and can more accurately reflect MTB activity, metabolic characteristics, and the proportion of drug-resistant bacteria (Singh, 2019). PhaB is a very promising mycobacteriophage-based diagnosis method for *M. tuberculosis* in developing countries such as India due to its simplicity, speed, and low cost (Bhattacharya *et al.*, 2015; Singh, 2019). It is helpful to start tuberculosis treatment as soon as possible.

To avoid various factors affecting the sensitivity of PhaB include anti-tuberculosis treatment, sample transportation, environmental conditions, and selection of chemical reagents before the experiment, further research is needed (Fu *et al.*, 2015). PhaB still has a lot of room for improvement. One aspect for the improvement of PhaB is to measure the metabolites of phage and lessen the dependence on sensitive hosts (Chauca *et al.*, 2007). Real-time quantitative PCR can be used to monitor phage amplified DNA (Sergueev *et al.*, 2010; van der Merwe *et al.*, 2014). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) can be used for detecting bacteriophage capsid protein (van der Merwe *et al.*, 2014). In the future, PhaB can be combined with the above detection methods to improve detection sensitivity and speed (Fig. 2).

Other Phage-Based Diagnostic Methods for *M. tuberculosis* and its Drug Resistance

Integrating phage amplification technology and nucleic acid amplification technology can produce more sensitive and rapid diagnostic methods. Recently, D29 quantitative PCR based on the replicability of phage in *M. tuberculosis* was used to amplify the DNA of D29. This method can distinguish whether the pathogen is resistant to all available anti-tuberculosis drugs within 1–3 days (Pholwat *et al.*, 2012). Actiphage^{TM} is a new detection method based on mycobacteriophage D29. It uses phage D29 to extract DNA from a small number of cells and combines with PCR to detect mycobacteria. This method can identify live pathogenic mycobacteria (LOD ≤ 10 cells/mL) in 6 h (Swift *et al.*, 2019). Now there has been a problem of insufficient sensitivity for the detection methods of *M. tuberculosis* in blood. Actiphage^{TM} can detect low levels of *M. tuberculosis* in early infected blood without sputum, and the results can be provided within 8 h by testing blood samples (Verma *et al.*, 2019). Compared with the original phage amplification method, it improves the detection sensitivity and is suitable for a large number of samples, which is helpful for the early treatment of *M. tuberculosis*. Based on the uniqueness of SP6 polymerase-dependent surrogate marker RNA (it does not exist in nature, known as surrogate marker locus, SML), typical SGM (SML generation module) phage, phS3P6–ProPol was invented. Reverse transcriptase PCR can amplify the SML reporter when phSP6-ProPol infecting *M. tuberculosis*. The report time is just 16 h (Mulvey *et al.*, 2012). Another *M. tuberculosis* detection tool developed by the combination of phage p53 and TaqmanqPCR can complete the detection within 4 h. Compared with the previous detection methods, it has high speed, specificity, and sensitivity, and can detect live bacteria without relying on DNA extraction or purification (Luo *et al.*, 2018). Moreover, bacteriophage can be used to reduce the interference effect of other bacteria. During the overnight culture, the surviving colonizing floras grow faster than *M. tuberculosis*, which interferes with the diagnosis of *M. tuberculosis* (especially on the assay based on bacteriophage). The phage cocktail (a mixture of three kinds of phages) can effectively control the formation of colonizing of bacteria, then decrease the interference of diagnosis (Kumar *et al.*, 2007).

Meanwhile, the phage detection methods of other different bacteria also have certain reference significance for the detection of *M. tuberculosis*. Established technologies such as the phage-based immunomagnetic enrichment method (Favrin *et al.*, 2003), a method combining optimized peptide-mediated magnetic separation (PMMS) and phage amplification (Swift *et al.*, 2013); the bacterial ice nucleation diagnostic (BIND) assay (van der Merwe *et al.*, 2014), a method combining phage lysis and biosensor (Farooq *et al.*, 2018; Franch *et al.*, 2019); the phage-mediated molecular detection method (PMMD), a method combining phage amplification and lateral flow assays (LFA) (Alcaine *et al.*, 2016), the biosorpted phage (Minikh *et al.*, 2010), phage detection methods combining radioactive isotopes and enzyme markers to tag phage have been developed (Pierce, 2011; Willford *et al.*, 2011) (Tab. 4). Detection of released products of host bacteria after the lytic cycle, is another alternative, such as adenosine monophosphate (AMP) (Zhou

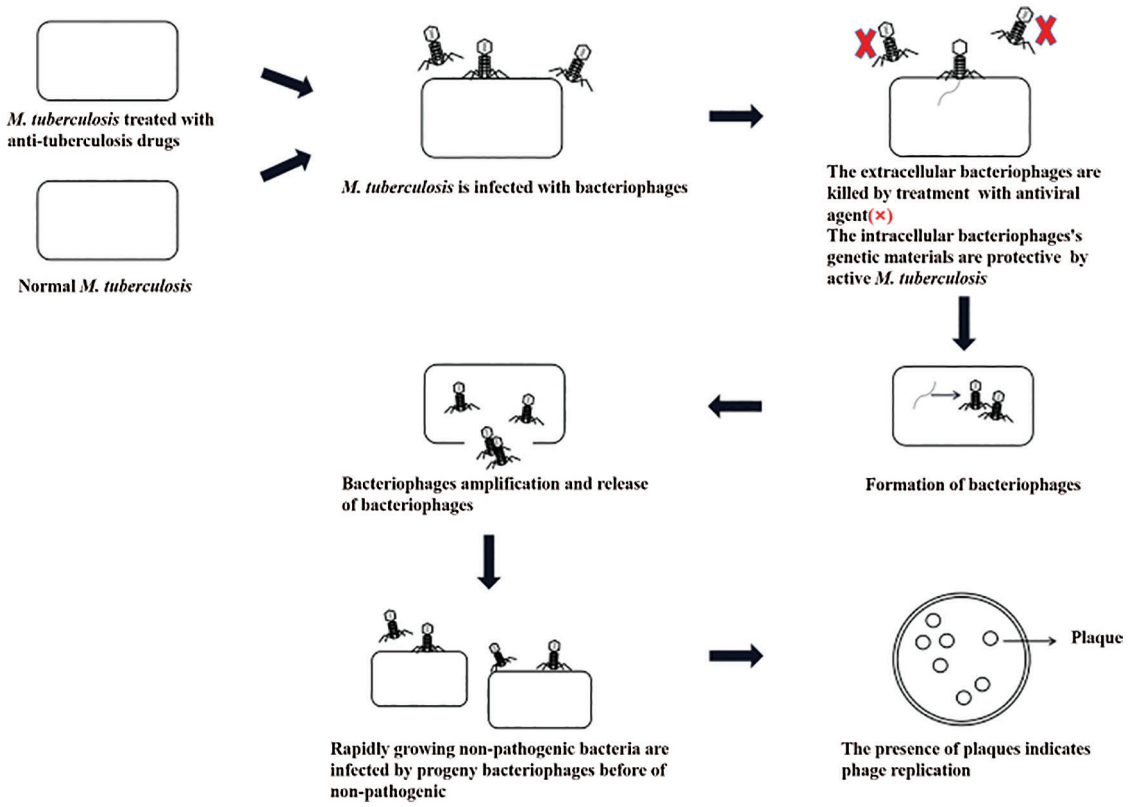

FIGURE 2. Phage amplified biologically assay (PhaB) for the detection of *M. tuberculosis* and its drug resistance. Treated with anti-tuberculosis drugs or not, acitive *M. tuberculosis* is infected by bacteriophages and produces plaques on lawns of rapidly growing non-pathogenic bacteria. The extracellular bacteriophages are killed by treatment with antiviral agent.

TABLE 4

Comparison of other phage diagnosis methods

Method	Mechanism	Characteristic	Reference
Actiphage™	It uses phage D29 to extract DNA from a small number of cells and combines with PCR to detect mycobacteria.	It can distinguish living cells in early infected blood without sputum. It is suitable for a large number of samples and has high sensitivity.	Swift *et al.* (2019)
IMS–bacteriophage assay	It is based on the isolation of target cells using paramagnetic beadscoated with polyclonal antibodies and the subsequent infection of the target cells by lytic bacteriophages.	It is highly specific, cheaper, simple to operate and and can shorten enrichment times for various rapid detection protocols. But some bacteria cannot bind to magnetic beads.	Favrin *et al.* (2003)
PMMS-bacteriophage method	It uses a specific binding agent to capture and concentrate cells, then uses PhaB and PCR to dectect MAP cells in blood.	It can detect MAP present in naturally infected blood and only detect viable cells. It is more rapid but it cannot determine the viability of the cell detected.	Swift *et al.* (2013)
Bacteriophage biosorbents	It immobilize the bacteriophage to isolate target bacteria and detect by surface-plasmon resonance or fluorescence microscope based on the principle of the specific interaction between the host and the bacteriophage-encoded bacterial binding proteins.	It can rapid capture bacteria.	van der Merwe *et al.* (2014)
Labeled bacteriophages	It uses labeled bacteriophages of radioactive isotopes and enzyme markers and IMS to isolate target bacteria and perform colorimetric or luminescence detection.	Highly specific, rapid, simple to operate	van der Merwe *et al.* (2014)
The bacterial ice nucleation diagnostic (BIND) assay	It is based on the principle that bacteria can form nucleation of ice in supercooled water and detects ice formation by making use of a fluorescent freezing-indicator dye.	High specificity and high sensitivity	van der Merwe *et al.* (2014)

(Continued)

Table 4 (continued).

Method	Mechanism	Characteristic	Reference
Phage-based biosensors	A phage -based biosensor is an analytical device that transforms biological response by integrating bio-recognition component (bacteriophage) and a biological material (cell-receptors, organelle, tissue, and bacteria), which use whole-phages and phage components as a detection probe.	It is rapid, simple, reliable, cost-effective, and accurate and has high sensitivity and high specificity.	Farooq *et al.* (2018); Franch *et al.* (2019)
Method combining phage amplification and lateral flow assays (LFA)	It uses bioengineering phage to overexpress protein and enzymatic reporters, and uses phage-amplification based LFA to detect.	It is sensitive, rapid, and easy to use.	Alcaine *et al.* (2016)
Phage-mediated molecular detection	It includes a incubation of the target bacteria with bacteriophages and total RNA extraction of bacteriophage and RT-PCR.	It is rapid, highly specific and sensitive, and can be applied to complex biological tissues.	Malagon *et al.* (2020)
Phage-based surrogate marker loci(SML) assay	It includes a incubation of the target bacteria with bacteriophages, phage/SML amplification and RT-PCR detection.	It is rapid, highly specific and sensitive.	Mulvey *et al.* (2012)

et al., 2001), β-D-galactoside (van der Merwe *et al.*, 2014). Phage-mediated molecular detection (PMMD) is a new type of molecular detection method, based on highly transcribed RNA carried by phage. Its principle is to perform the total RNA extraction and PCR reaction after co-cultivation of the sample and natural phage. Phage-mediated molecular detection (PMMD) has high sensitivity, efficiency, and specificity, which can be used to detect bacterial antibiotic resistance. At present, PMMD has been applied to the detection of *Staphylococcus aureus* (SA) and Bacillus charcoal, providing a reference for the detection of mycobacteria (Ma *et al.*, 2020). Phage-based biosensors are also considered promising detection tools. The diversity of bacteriophages makes the biosensor theoretically detect almost all bacterial strains. As a new detection tool, it has the advantages of simplicity, reliability, accuracy and low-cost-effectiveness (Richter *et al.*, 2018; Wei *et al.*, 2019; Zhang *et al.*, 2018). As a detection method, the detection methods based on mycobacteriophage is sufficiently sensitive and specific, rapid, simple, and relatively cost-effective. Research evidence also indicates that the phage-based detection methods are safe, but there are other limitations that limit its application such as the necessity of using phage cocktails, complex pharmacokinetics, and the possibility of the evolution of bacterial resistance. Synthetic biology will address these shortcomings. It can also accelerate the engineering of phages and greatly improve the diagnostic methods and application prospects of phages (Lu *et al.*, 2013). The continuous development of biotechnology such as nanotechnology also provides a new direction for phage detection. Synthetic biology provides a powerful tool for transforming natural bacteriophages. Using bacteriophage genome engineering methods, bacteriophages can be modified to overcome the defects of natural bacteriophages, such as difficulties penetrating biofilms and limited host range, and to be better used for phage detection (Citorik *et al.*, 2014). Using phages engineered to express biofilm matrix-degrading enzymes can overcome the barriers to invasion. The host range can be expanded or reduced to improve the detection specificity by changing the specific receptor on the phage surface. Temperate bacteriophages can also be transformed into lysed phages to increase the utilization of the detected phages by engineering methods such as the deletion of repressors (Lemire *et al.*, 2018). The combination of synthetic biology and nanotechnology provides a new idea for phage detection. For example, the surface protein of filamentous bacteriophages is combined with nanoparticles to make a biological probe (Lee *et al.*, 2013). Biological probes combining T7 bacteriophage with magnetic nanoparticles can successfully isolate bacteria from complex samples and improve detection efficiency (Chen *et al.*, 2015). The phage detection method combining synthetic biology and nanotechnology has good development prospects and will be faster, more specific, and more in line with people's needs in the future.

Concluding Remarks

Tuberculosis, as one of the world's top ten infectious diseases, still causes many infections and deaths in the world every year. Early diagnosis of *M. tuberculosis* is very important. The diagnostic technology methods that have been developed mainly include bacteriology, immunology, molecular biology, and cytology. Some new *M. tuberculosis* diagnosis methods have been noticed, such as the rapid culture system of mycobacterium BACTEC, MGIT (BD, USA), nucleic acid amplification technologies MTD (Gen-Probe, USA), AmPlicor (Roche, Switzerland), Genexpert (Cepheid, USA) and LAMP (Eiken Chemical, Japan), and interferon-gamma release assay (IGRA) that can be used to evaluate latent *M. tuberculosis* infection, etc. However, these methods generally have some disadvantages such as long time, high cost, and low sensitivity. The emergence of extensive drug-resistant bacteria (XDR) and multi-drug-resistant bacteria (MDR) is a huge challenge for rapid diagnosis and treatment. Phage has been an indispensable player in the development of biology. As natural enemies of bacteria, bacteriophages have always attracted wide attention from researchers. Since the

discovery of mycobacteriophages, at least 1,901 mycobacteriophages have been isolated and sequenced as of May 13, 2020 (https://phagesdb.org/). Diagnosis methods for *M. tuberculosis* based on mycobacteriophages are gradually developing, and their characteristics of rapidity, specificity, sensitivity, and relatively low cost are causing widespread concern. It has been proved that phage-based diagnosis can effectively and rapidly detect *M. tuberculosis* in clinical samples. In addition to mycobacteriophage D29, TM4 and DS6A, other mycobacteriophages are also developed and most of them are used for tuberculosis treatment (Azimi *et al*., 2019; Tang *et al*., 2019). As more and more mycobacteriophages are isolated and sequenced, more detection methods based on different mycobacteriophages will be developed in the future.

In this review, we summarize the development of phage-based diagnostic tests for *M. tuberculosis* including fluoromycobacteriophage and PhaB. Fluoromycobacteriophage utilizes the characteristic that fluorescent protein genes were integrated into bacteriophages which specifically infect the host bacteria. The presence of fluorescence can indicate the status of *M. tuberculosis*. The technology is mainly divided into three stages of development including the early stage of fluorescent reporter phages, the clinical application of improved fluorescent reporter phages, and the future detection phase of fluorescent reporter phages. The phage biological amplification method is mainly based on the ability of viable *M. tuberculosis* to protect mycobacteriophages. Only phages infected with *M. tuberculosis* can enter the lysis cycle, and the existence of active *M. tuberculosis* is detected by the number of plaques. Other phage-based detection methods such as combining phage amplification technology with nucleic acid amplification technology and phage biosensors have also been developed in succession. In short, phage technology provides tools for people to detect pathogens more quickly and sensitively, which is quicker than pathogen culture-based detection and drug-resistant tests. It is estimated that there are 10^{31} types of bacteriophages on earth. A rich bioresource of bacteriophages will be a solid basis for this technology. The rise of synthetic biology and nanotechnology will also lay the foundation for this. In the future, more phages and detection methods will be developed.

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