Mycobacteriophages as Diagnostics

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Contributor: Giovanni Satta

Tuberculosis (TB) is one of the most impactful diseases of the modern era. Current diagnostics are struggling to meet the multifaceted challenges TB presents. Mycobacteriophages (specific phages active against mycobacterial species) are now being utilised to create promising new diagnostic technologies. Here we explore and review contemporary phage diagnostics targeting mycobacteria, while commenting on key areas warranting further investigation and development.

Keywords: mycobacteriophage ; phage ; mycobacterium ; tuberculosis ; TB ; diagnostics ; detection ; molecular biology ; viability assay ; testing

1. Introduction

Mycobacterial infections are responsible for some of the most deadly and difficult to control infections in humans and animals. Tuberculosis (TB), caused primarily by *M. tuberculosis*, is thought to infect over 10 million people each year and causes the death of at least 1.6 million people annually worldwide [1]. The highest burden of these cases is attributed to low–middle-income countries (LMICs). Opportunistic mycobacterial infections in people are also caused by a range of non-tuberculous mycobacteria (NTM), including members of the *M. avium complex* (MAC) and *M. abscessus complex* (MAB). Other more recently identified and rarer NTM diseases include *M. chimaera* infections in cardiothoracic patients following exposure to contaminated heater-cooler units, unusual NTMs infections in immunocompromised hosts and BCGosis, a rare disseminated granulomatous disease, following intravesical *Bacillus Calmette-Guérin* (BCG) immunotherapy and in patients with predisposing genetic conditions such as the Mendelian susceptibility to mycobacterial diseases (MSMD) [2][3]. In veterinary medicine, *M. bovis* is the primary cause of TB in cattle and other animals and causes 140,000 new cases and 11,400 deaths per year globally [1].

Bacteriophages are viruses that infect bacteria and are the most abundant lifeforms on earth [4]. There are two types of phages with distinct lifecycles, lytic phages and temperate phages. Lytic phages infect, replicate and break open their host, whereas temperate phages can enter the lytic lifecycle or establish lysogeny by stably maintaining their DNA in the host either by integration into the host chromosome or as an extracellular replicon, and repressing lytic gene expression [5]. Bacteriophages can have very narrow host ranges, infecting specific subspecies of bacteria, but can also have relatively broad host ranges, capable of infecting several bacteria genera. Bacteriophages' ability to kill their host make them attractive tools to treat infections and, although there is a body of literature on their clinical use in Eastern Europe, the Western world has only started to discover their potential benefits [6].

2. Mycobacteriophages as Diagnostics

TB control is limited by current diagnostics. Clinicians are still reliant on X-rays, microscopy and cultures as universal tools to diagnose TB [7]. Molecular platforms, such as the GeneXpert system, have made a difference to diagnosing TB by shortening the time to detection and improving sensitivity [8]; however, they are not yet considered a universal tool for diagnosis [9], due to the associated cost per test/scale up, need for well-trained/ consistent staff and need of a stable power source [10]. Culturing mycobacteria is generally seen as the gold standard diagnostic; however, many mycobacterial pathogens are slow-growing, for example, *M. tuberculosis* and *M. bovis* can take up to 12 weeks to culture on solid media, and *M. avium* subspecies *paratuberculosis* (MAP) can take up to 16 weeks. Culture is also insensitive, because relatively high numbers of bacilli are required for visible growth. The slow growth and low sensitivity makes the use of solid culture as a diagnostic for TB infections both impractical and inefficient [11]. The introduction of automated liquid culture systems, endorsed by the WHO, has improved the practicality and accessibility of culture as a primary diagnostic, but it still remains slow and relatively expensive [12].

Many of the current diagnostics for TB infections are immunologically based, where the host response is used to diagnose infection. A major problem with this approach is that mycobacteria are generally characterized by their ability to avoid their host's immune system, which can result in the inconsistent detection of infected individuals [13], particularly where the

pathogen effectively evades immunity. Succinctly, the methods may fail to detect infection due to the pathogen's innate evasion of host immune responses.

Molecular methods such as PCR exist to detect mycobacterial pathogens to overcome the reliance on immune response. However, widely used PCR platforms (such as GeneXpert) are expensive and tend not to have the required sensitivity to detect *M. tuberculosis* in a range of matrices due to the inefficient lysis of mycobacteria as well as potential inhibitors that are often found in samples being tested. The development and deployment of rapid, sensitive diagnostics is a cornerstone of strategies to understand, control, and eradicate TB [1]. New diagnostics for mycobacterial infections need to be appropriate for use in LMICs or in agricultural settings—meaning low-cost, simple and robust. Therefore, by developing diagnostics that advance the speed, sensitivity, simplicity and cost of testing, TB control can be strengthened.

Phage-based diagnostics historically consisted of two general areas: phage amplified biologically (PhAB) assay and phage reporter assays (PRAs). PhAB exploits a certain aspect of the phage's natural ability to infect, amplify and break open cells to detect the mycobacteria; PRAs typically involve genetically modified bacteriophages or their hosts so that a fluorescent, luminescent or alternative signal can be detected. A meta-analysis of the PhAB assay found that its main limitation was a high rate of indeterminate/contaminated results (20%) [14]. However, this technique's appropriateness for LMICs has long been recognized [14][15]. PRAs have consistently had high sensitivity and specificity, although the only effort to commercialize the technology—The Bronx Box (Sequella, Rockville, USA)—has been discontinued [16]. Recent PRA endeavors have focused on facilitating the technology by creating affordable detection equipment [17]. PhAB assays and PRAs have been apprised in detail elsewhere [18][19][20]. Therefore, we sought to evaluate other, less explored, phage-based approaches. For more in-depth detail on PhAB and PRA methodologies, readers are encouraged to see the FASTPlaque TB™ (Biotech Labs Ltd., Ipswich, UK) [16][21] and proof-of-concept luciferase reporter phage assays [20][22]. **Table 1** presents a summary of commercial and published phage technologies that have been used to detect mycobacteria.

Table 1. Phage technologies used to detect mycobacterial infections. * MTB, *M. tuberculosis*; MAP, *M. avium* subspecies paratuberculosis.

Commercial Assays Already Available								
Name	**Mechanism of Action**	***Mycobacterium* spp.**	**Phage(s) Used**	**Limit of Detection**	**Sensitivity**	**Specificity**	**Turnaround Time**	**References**
Actiphage® Rapid (PBD Biotech Ltd., Thurston, UK)	**Mycobacteria are isolated from peripheral blood mononuclear cells, then the phage is used as a lysis agent. PCR, detecting mycobacteria, is used as an endpoint.**	**MTB *, MAP *, *M. bovis***	**D29**	**≤10 cell mL^{-1}**	**95%**	**100%**	**6 h**	**[23]**
FASTPlaque TB™ (Biotech Labs Ltd., Ipswich, UK)	**Phage amplified biologically assay. Other kits (FASTPlaque RIF™/MDR™) offer drug susceptibility testing.**	**MTB**	**D29**	**100–300 cell mL^{-1}**	**95%**	**95%**	**48 h**	**[16][21]**
Proof-of-concept Assays								

Commercial Assays Already Available								
Name	**Mechanism of Action**	***Mycobacterium* spp.**	**Phage(s) Used**	**Limit of Detection**	**Sensitivity**	**Specificity**	**Turnaround Time**	**References**
Enzyme detection biosensor	**Phages are used as a lysis agent. The released enzyme (TOP1A) binds and cleaves a surface bound DNA complex. Addition of Mg^{2+} causes DNA circularization and enzyme turnover. The DNA circle is amplified by rolling circle amplification. Then, visualized using fluorescent probes.**	***M. smegmatis***	**D29; Adephagia Δ41, Δ43**	**0.6 million CFU mL^{-1}**	**-**	**100%**	**-**	**[24]**
Phage real-time PCR	**48 h pre-incubation with first- and second-line antibiotics. Then, incubated with phage. Real-time PCR used to detect phage DNA. Extracellular phages are inactivated. Presence of phage indicates cell viability, and thus, resistance. Later adapted so that real-time PCR is directly performed on MGIT broths for clinical applicability.**	**MTB**	**D29**	**-**	**90%**	**99%**	**1 to 3 days (proof-of-concept)/positive MGIT culture plus 3 days (clinical)**	**[25][26]**
Phagomagnetic separation	**Phage-coated paramagnetic beads capture and concentrate bacilli. Bead-bound mycobacteria are separated using magnetism. Mycobacterial DNA is released (phage-mediated lysis) and detected by real-time PCR.**	**MAP**	**D29**	**$LOD_{50\%}$: 10 cell 50 mL^{-1}**	**97%**	**99%**	**7 h**	**[27]**
Peptide mediated magnetic separation	**Bead-bound peptides capture and concentrate bacilli, which are then separated magnetically. Then, the phage-amplified biologically assay, followed by plaque PCR, are used for detection.**	**MAP**	**D29**	**10 cell mL^{-1}**	**-**	**-**	**48 h**	**[28]**
Electrochemical detection of enzymatic action	**Phages are used as a lysis agent. The activity of a released enzyme (beta-glucosidase) is quantified amperometrically.**	***M. smegmatis***	**D29**	**10 cell mL^{-1}**	**-**	**-**	**8 h**	**[29]**

Commercial Assays Already Available								
Name	**Mechanism of Action**	***Mycobacterium* spp.**	**Phage(s) Used**	**Limit of Detection**	**Sensitivity**	**Specificity**	**Turnaround Time**	**References**
Surrogate marker locus generation module	**16 h pre-incubation with first- and second-line antibiotics. Phage encoded with RNA cyclase ribozyme, under SP6Pol transcriptional control, generate circular surrogate marker locus RNA. This unique nucleic acid sequence is detected by reverse transcriptase PCR. Presence of surrogate marker locus RNA indicates cell metabolic activity, and thus, resistance.**	**MTB**	**phSGM2**	**<100 CFU**	**-**	**-**	**1 to 2 days**	[30]
Peptide-mediated magnetic separation with phage ELISA	**Bead-bound peptides capture and concentrate bacilli, which are then separated magnetically. This concentrate is incubated with phage. Extracellular phages are inactivated. D29-specific ELISA is used as an endpoint.**	**MAP**	**D29**	**~100 PFU mL^{-1}**	**-**	**-**	**<1 day**	[31]
Phage-amplified multichannel series piezoelectric quartz crystalsensor	**Phage-amplified biologically assay performed in liquid broth. The response curve of the reporter *M. smegmatis* is measured using a multichannel series piezoelectric quarts crystal sensor.**	**MTB**	**D29**	**100 CFU mL^{-1}**	**89%**	**95%**	**30 h**	[32]

PhAB assays have also been demonstrated for other mycobacteria, including *M. ulcerans*, *M. avium*, *M. scrofulaceum, M. marinum, M. fortuitum* and *M. chelonae* [34], although these have not been exploited further.

The advantages of phage-based approaches are reflected by improvements achieved in the speed, specificity and sensitivity. All assays give results faster than the “gold standard” eight weeks and consistently high specificity values are reported. However, directly comparing sensitivities is difficult because different comparators have been used. Some studies used culture, whereas others used GeneXpert (Cepheid, Sunnyvale, CA, USA), and fewer still used sputum smear microscopy. Standardizing reference tests would improve confidence when comparing results. We call for more studies directly comparing culture, GeneXpert and sputum smear microscopy to allow more accurate comparisons of diagnostic proof-of-concepts. No studies reported an approximate cost.

The lowest reported limit of detection for TB (≤10 cell mL^{-1}) used Actiphage® (PBD Biotech Ltd., Thurston, UK) [23], whereas the lowest (LOD50%: a 50% probability of detecting contamination at this level) for NTM was 10 cell 50 mL^{-1} by using phagomagnetic separation [27]. These two methods also had the lowest reporting times and highest sensitivities and specificities. Both methods targeted mycobacterial insertion sequence DNA with PCR, showing these to be good targets for accurate diagnostics.

Nucleic acid amplification tests were frequently used endpoints. Low limits of detection were achieved when mycobacterial insertion sequence DNA (IS6110) was targeted with PCR [23][27][28]. Given that multiple copies are present in a single cell, it is clear why they make a good target, especially for detecting small numbers of bacteria. Another method detected phage DNA [25] to good effect in drug susceptibility testing.

Several assays used enzymes to catalyze the generation of their respective biomarkers. Using this method, two endpoints have been explored: the detection of changes in electric current [29][32] and the detection of unique nucleic acid sequences [24][30]. One approach used reporter phages to introduce the enzyme [30], whereas another used an enzyme already present in mycobacteria [24]. These methods had the highest limit of detection, but were still within the range of clinical relevance. The insensitivity of these methods may be due to their use of lytic phages, releasing cell contents and preventing further catalysis.

A reporter phage (Φ2GFP10) was developed to detect TB and rifampicin-resistance in LMICs [22]. During a trial in South Africa, the reporter phage could detect TB with a high degree of agreement in sensitivity and specificity compared to GeneXpert MTB/RIF in both smear-positive and smear-negative sputum samples. The ability to rapidly identify antimicrobial-resistant mycobacteria is also a great benefit, and by using this technology, extensively drug-resistant tuberculosis could be detected [35]. However, one drawback of this method was the need to carry out analysis using FACS, which reduces the ability to use this near to care in all high TB burden countries.

An emerging technology is the use of magnetic microbeads to capture bacilli followed by concentration using magnetic separation. This step does not require centrifugation or filtration and further inroads into automation. Historic efforts used peptide-beads followed by phage lysis [28][31]. The process has recently been streamlined by using phage-beads to capture and lyse in one step and using real-time PCR for the readout [27]. These methods had consistently low limits of detection. Inclusion of this step into other methods may improve accuracy, sensitivity and LMIC applicability.

Few phage-diagnostics have been translated into commercial and clinical use. One way to ease this transition is to demonstrate the assay with clinical samples. Many proof-of-concept studies utilized clinical samples, improving confidence in their applicability [20][25][32][30][36]. A good example of proof-of-concept translation can be seen when the phage real-time PCR assay developed by Pholwat et al. [25] was implemented in a Thailand reference laboratory [26]. This allowed for direct comparisons against standard methods and demonstrated the assay's capability in a high-volume, real-world setting. For the field of phage diagnostics to advance, more proof-of-concepts that are successful need to be developed through the translational pipeline.

Commercial Assays Already Available								
Name	Mechanism of Action	*Mycobacterium* spp.	Phage(s) Used	Limit of Detection	Sensitivity	Specificity	Turnaround Time	References
Colorimetric detection testing phage replication	Mycobacteria are added to a 96 microwell plate with antibiotics and incubated overnight. Phage is added. After incubation, extracellular phage are inactivated. Samples were added to a fresh 96 microwell plate containing reporter *M. smegmatis* and incubated overnight. A redox dye, MTT, was added. Growth of *M. smegmatis* results in a color change. Lack of a color change indicates lysis of *M. smegmatis* by phage, and thus, the viability of mycobacteria in the original 96 microwell plate	MTB	D29	-	91%	99%	>2.5 days	[33]
FAST PHAGE Reporter Phage	A specified mycobacteriophage are incubated with a processed sputum sample and fluorescence indicates the presence of a viable mycobacterial host. Fluorescence is detected by FACS	MTB	Φ^{2}GFP10	<10^4	96%	83%	>2 days	[22]

Developing diagnostic tests for use in LMICs can be difficult, because tests need to be inexpensive to run and have access to a power source, largely limiting their use to reference laboratories. Isothermal amplification steps as well as the development of colorimetric assays have been explored to circumvent these issues and move towards a point-of-care test [24][33]. However, there have been difficulties experienced in achieving low limits of detection.

One inherent limitation of using phages as lysis agents comes from the time they take to lyse mycobacteria. For instance, D29 takes 3.5 h to enter the eclipse phage and burst target cells; a fundamental aspect of phage biology that is seemingly unavoidable. Delaying time to detection can be somewhat mitigated by using faster endpoint detection methods. For example, using colorimetric results as opposed to quantitative when only presence/absence information is needed.

* MTB = *Mycobacterium tuberculosis* – MAP = *Mycobacterium avium subsp. Paratuberculosis*; (-) = No data available.

The inconsistent burst size of phages can create limitations when detecting phage DNA in real time. Exact burst sizes vary; therefore, setting a threshold to differentiate between inoculated phage DNA and amplified phage DNA is tricky, resulting in difficulties in creating specific diagnostics for low levels of bacteria. Resolutions of this problem would advance the field of phage diagnostics.

Difficulties detecting low bacterial concentrations with phages arise from the low likelihood of phages randomly interacting with a single cell within a given space. Efforts to circumvent this include maximizing the multiplicity of infection [23]. Other methods have used magnetic beads [27][28][31], to capture and concentrate the bacteria, facilitating infection. These efforts have largely succeeded, seen in the low limits of detection reported. However, new diagnostics should be mindful of this pit fall.

Phage diagnostics can be improved by standardizing comparator tests and translating more successful proof-of-concepts. Working towards the WHO's diagnostic guidelines [1] in the proof-of-concept stage will ease the transition. The field can improve by focusing efforts on developing point-of-care tests.

Bacteriophage-based diagnostics offer great potential. The advantages of phages are numerous; only viable bacilli are detected whereas specificity is determined by the phage's host range. They can be produced at a low cost, are easy to handle, and their rapid rate of infection can drastically reduce reporting times. Due to their several advantages, phages may fulfil the needs of modern TB diagnostics.

Given that both phage-therapy and phage-diagnostics are becoming more prevalent, their interplay needs to be considered. We should always be mindful of resistance. In isolation, diagnostics circumvent this concern by operating as a closed system; however, resistance derived from therapy will likely impact diagnostics if the same phage is used in both instances. When designing and implementing these therapies and technologies, this needs to be considered.

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