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The Use of Phage for Detection, Antibiotic Sensitivity Testing and Enumeration

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1. Introduction

Bacteriophage are bacterial viruses which may attack and destroy bacterial cells. While many have a narrow host range and are used in sub-typing techniques, some infect many members of a genus or species and these have been used to develop rapid detection methods for a variety of bacterial pathogens (Rees and Loessner, 2008). The use of bacteriophage (or phage) in assays for detecting bacteria was first reported over half a century ago when an assay to detect *Salmonella* using phage Felix 01 was described by Cherry et al. (1954). Since then other bacteriophage-based detection methods have been developed that take advantage of the specificity of the host-phage interaction and its ability, once inside the host, to replicate rapidly. This is particularly useful when studying slow-growing organisms such as *Mycobacterium tuberculosis* and other slow growing mycobacteria such as *Mycobacterium avium* subsp. *paratuberculosis* (see Stanley et al., 2007; Grant & Rees, 2009, Botsaris et al., 2010). This chapter will provide an introduction to phage biology and will then describe the different phage-based detection methods that have been described for *M. tuberculosis* – including one that has been developed into a commercial product. In addition adaptations of the phage test are described that allow the antibiotic sensitivity of isolates to be rapidly determined, and also how a further modification can be used to allow rapid estimation of viable cell number. However, all these indirect methods have their limitations and these too will be discussed in each case.

2. Mycobacteriophage

Mycobacteriophage, which are phage that infect any members of the *Mycobacterium* genus, were first isolated and characterized by Gardner and Weiser in 1947 and further investigation was prompted in the 1950s by their utility in typing of clinical isolates. Phage typing is a method used to sub-type members of a bacterial species based the sensitivity of a particular host strain to a panel of bacteriophage that have been shown to have a limited host range within the group (see Rees and Loessner, 2008, for a description of this method). So far over 200 different mycobacteriophage have been described, infecting a broad variety of mycobacterial hosts and these have been isolated from a variety of environmental sources, such as soil or surface water (Froman et al., 1954; Caroli & Avio, 1975), and stool

and resection specimens of patients with tuberculosis or sarcoidosis (Mankiewicz, 1961; Mankiewicz & Liivak, 1967). The collection of Mycobacteriophage is continually being expanded due to an education programme developed by researchers at the University of Pittsburgh, supported by the Howard Hughes Medical Institute Science Education Alliance. This has recently resulted in the publication of a multi-author paper describing the isolation, sequencing and comparative genomic analysis of 18 new mycobacteriophages isolated from geographically distinct locations by freshmen attending Universities across within the United States (Pope et al., 2011).

Like all viruses, phage consist of a nucleic acid core and a protein coat, and considerable variation in structure has been reported. However all mycobacteriophage that have been described to date are double-stranded DNA viruses consisting of icosahedral heads with a tail which may be either short or long and either flexible (Siphoviridae) or contractile (Myoviridae) (Hatfull et al., 2008; see Figure 1). These tail structures play an essential role in host cell recognition and penetration of the bacterial cell wall structure. The comparative genomic analysis of mycobacteriophage reveals that they have relatively large genomes (average length approx. 70 kbp), contain large numbers of previously unidentified genes, and are highly diverse at both the nucleotide and amino acid sequence levels (Pedulla et al., 2003; Hatfull et al., 2010; Pope et al., 2011). Once inside the host cell the phage take over the host cell biosynthetic machinery and use this to replicate themselves, usually producing hundreds of progeny phage per infected cell (Figure 2). At the end of the replication cycle many phage produce enzymes (lysins) which degrade the cell wall and result in the lysis of the host cell and release of the progeny phage. However some phage do not always enter this lytic cycle. Instead, after entering the cell, they can enter a dormant state resulting in a latent infection. This state is known as lysogeny, but these lysogenic (or temperate) phage can be induced back into the lytic cycle, often in response to environmental conditions that either damage cellular structures or induce a stress response in the host cell (Figure 2). Both lytic and lysogenic Mycobacteriophage have been identified.
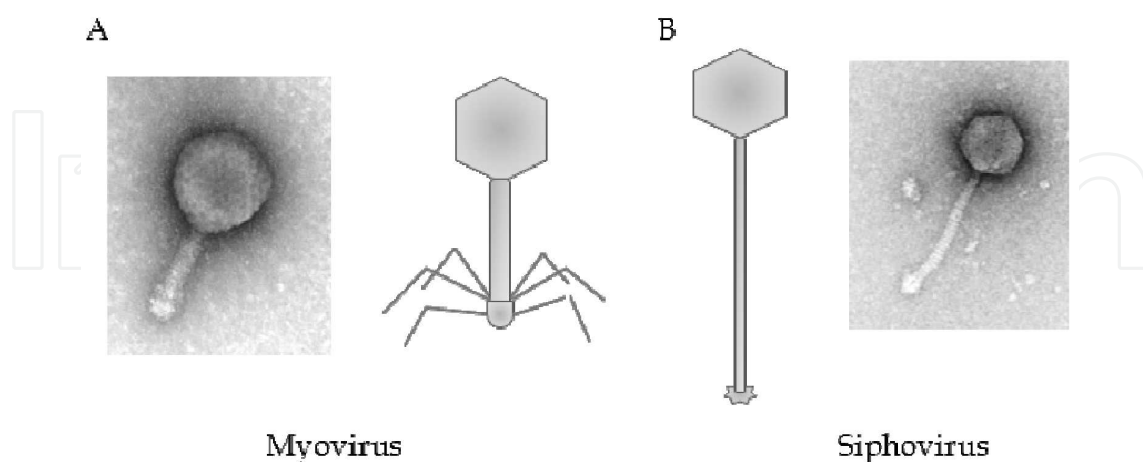

Fig. 1. Common Morphotypes of Mycobacteriophage
Myoviridae (panel A) typically have short, rigid contractile tails which often have associated tail fibres. Siphoviridae (panle B) have longer, flexible, non-contractile tails which termimates in a base-plate structure and tail fibres are absent. Both types of phage have double stranded DNA genomes. The majority of Mycobacteriophage are Siphoviridae.
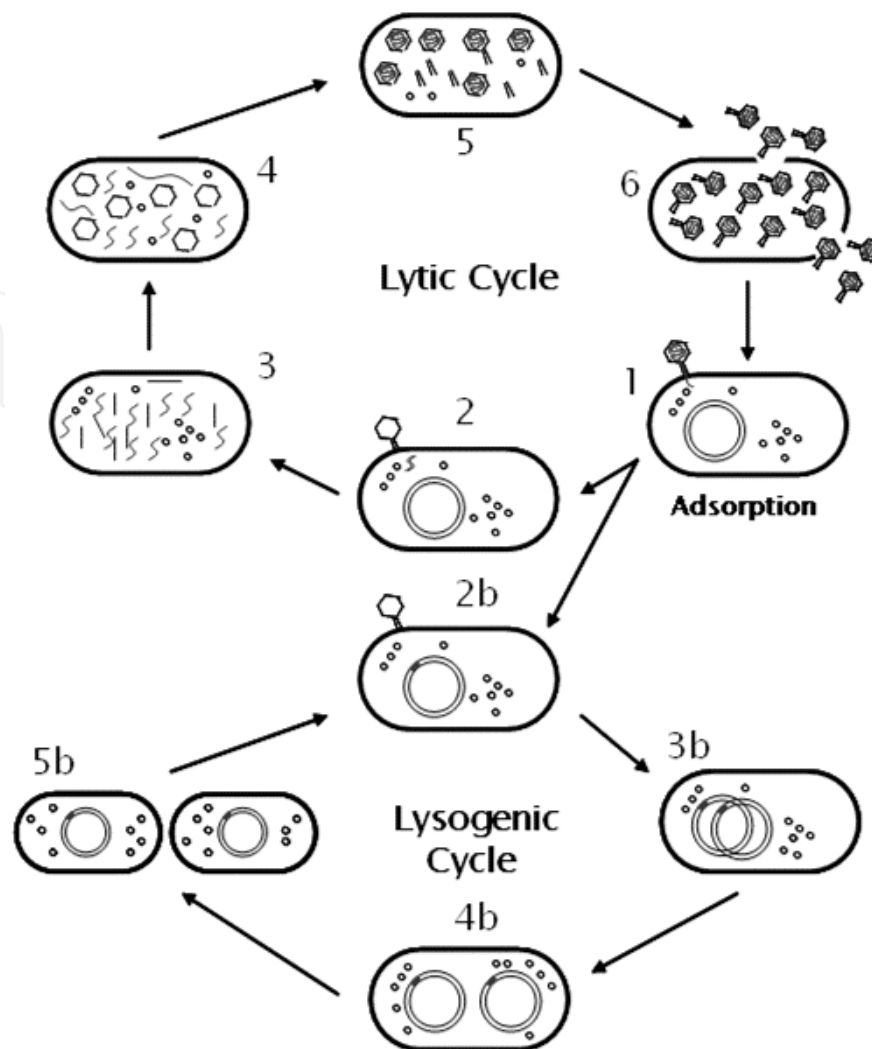
Fig. 2. The Bacteriophage Replication Cycle

Schematic representation of the Lytic and the Lysogenic Cycles. Lytic Cycle: Infection is mediated via receptors found in the phage tail structures. During infection the bacteriophage inserts the genomic DNA located inside the virus capsid into the cell (1 & 2) and then it takes over the cell replication machinery and directs the synthesis of bacteriophage nucleic acids and proteins (3 & 4 and 5). Finally the phage produces enzymes (lysins) that breaks open the cell and the mature bacteriophage particles are released (6). Lysogenic Cycle: adsorption occurs in the same way (1) but the bacteriophage DNA is not replicated and instead integrates into the host cell's genome (2b). It is then replicated along with the host cell DNA at cell division and the lytic genes are not expressed (3b and 4b). When the cell divides a copy of the bacteriophage DNA is transferred along with the host chromosome (5b). Following induction into the lytic phase, the integrated phage DNA is excised from the host cell genome and the lytic genes are then expressed leading to phage replication.

3. Use of phage in detection assays

Mycobacteriophage have been used to detect slow fastidious mycobacteria, such as members of the TB complex. The main two techniques developed for detection of

mycobacteria using phage are recombinant Reporter Phage and the Phage Amplification Assay (PAA), also known as Phage Amplified Biologically (PhaB) assay. These two methods differ in how the host cell is detected at the end of the assay and both are described in more detail in the following sections.

3.1 Reporter Phage assays for mycobacteria

The Reporter Phage detection method uses genetic engineering to introduce a reporter gene into a phage genome. Since genes are not expressed inside the virion particle, no signal is produced from the reporter gene until it enters a host cell during infection. At this point the reporter gene is expressed along with the phage replication genes and this can be detected to indicate that an infection event has occurred (see Figure 3). The limitation here is the packaging constraint of the phage being used; this is the natural limit on the size of phage genome that can be packaged into a phage head. If the size of the gene being introduced into the phage exceeds the packaging constraint, the recombinant phage particles are defective. However the firefly luciferase reporter gene (*Fflux* or *luc*) has been successfully introduced into a number of mycobacteria phage (Jacobs et al., 1993; Sarkis et a., 1995; Pearson et al., 1996; Riska et al., 1997). This enzyme requires a source of ATP to produce light (bioluminescence), and this is provided by the infected cell. When the luciferin substrate for the enzyme is added (exogenously) the light can be sensitively detected by a luminometer.

The first Mycobacterial Reporter Phage described were based on the lytic phage TM4 since it was argued that a lytic phage would not be able to enter the dormant lysogenic state and therefore would produce high levels of reporter signal. However but it was found that these lysed the *Mycobacterium* cells too rapidly so that only low levels of luciferase were produced. This reduced the limit of detection when using this phage to approximately 10^4 mycobacterial cells (Jacobs et al., 1993). The same group also constructed a reporter phage using the temperate phage L5 and found that when the phage integrated into the chromosome there was prolonged expression of the reporter gene and this then reduced the limit of detection to approximately 10^2 cells after a 40 h incubation period, or 10^3 cells after 20 h (Sarkis et al., 1995). Although this demonstrated for the first time that reporter phage could be used to rapidly and sensitively detect Mycobacteria, this phage had a limited host range and therefore could not be developed as a practical test for *M. tuberculosis.* Instead the TM4-based reporter phage was improved by changing the site of insertion of the *Fflux* gene in the phage genome and isolating mutants of the phage to improve host range. This lead to the isolation of a TM4-based reporter phage that could detect as few as 120 *Mycobacterium bovis* BCG after 12 h of incubation (Carriere et al., 1997) which is significantly faster than any culture-based method can achieve and when tested on clinical samples it was shown that smear-positive sputum samples could be detected within 24-48 h (Riska et al., 1997). One limitation of the Reporter Phage assay for direct identification of *M. tuberculosis* in clinical samples is that these phage are able to infect a number of species of *Mycobacterium*, for instance TM4 also infects *M. bovis* and members of the *M. avium* complex (Timme and Brennan, 1984). This results in reduced specificity of the assay and hence produces false-positive test results. To overcome this, the reporter phage have been used in combination with p-nitro-a-acetylamino-b-hydroxy propiophenone (NAP) which specifically inhibits the growth of *M. tuberculosis* complex bacteria (Eidus et al. 1960). Parallel samples, with and without the addition of NAP, are used and the results of the Reporter Phage assay

compared. If light is produced from both samples then the organism detected is not M. tuberculosis. However if light is suppressed in the sample containing NAP, this indicates that *M. tuberculosis* cells have been detected (Riska et al. 1997). Using this combination of tests, Banaiee et al. (2001) reported that 94% of strains tested could be correctly identified.
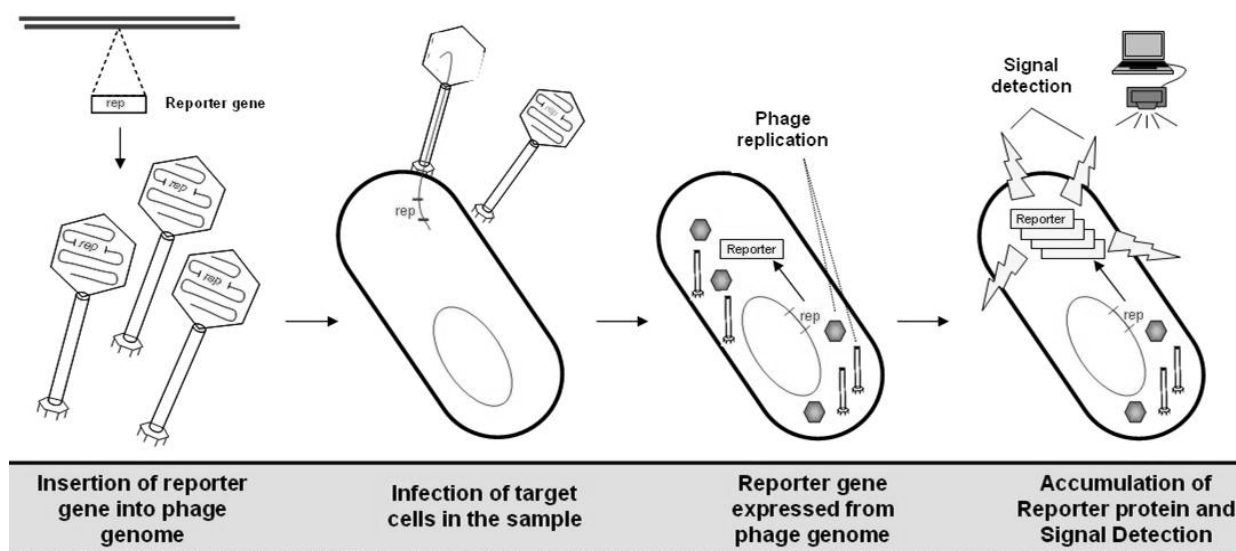

Fig. 3. General Schematic for Reporter Phage Assays
Schematic representation for Reporter Phage Assays. Following the insertion of the reporter gene into the phage genome the phage infects the targeted host cell. The reporter gene is then expressed from the phage genome and as the reporter protein accumulates the signal is detected.

Although these initial reports were promising there have been very few recent reports of the use of reporter phage as a diagnostic tool for TB. Recently, Dusthackeer et al. (2008) reported the construction of a new *Fflux* Reporter Phage using the temperate phage, Che12 – again arguing that integration of these phage would produce more sustained light levels – and also using promoters to direct the expression of the *Fflux* that were predicted to be more highly expressed in dormant bacilli (isocitrate lyase (*icl*) and alpha crystallin protein (*acr*)). They also re-engineered the promoter sequences gene in the defective TM4-based phage phAE129 (Carriere et al., 1997) to determine if light output could be improved. Interestingly, by comparing the results obtained for the two phage they showed that while these promoters did increase light output when the phage infected dormant cells, the Che12 Reporter Phage only poorly infected dormant bacilli. This is probably because it lacks a peptidoglycan hydrolase TM3 motif found in the TM4 tape- measure protein and this is required to allow phage to efficiently infect *M. smegmatis* in stationary phase when it has a thicker or more highly cross-linked peptidoglycan layer.

Two new Reporter Phage constructs have also been described which have been engineered to contain genes encoding the fluorescent proteins GFP or ZsYellow (Piuri et al., 2009). These were introduced into the conditionally-replicating TM4 derivative phAE87 under the control of the constitutive *M. bovis* BCG Hsp60 promoter to create the fluorophage phAE87::*hsp60-EGFP* and phAE87::*hsp60-ZsYellow*. However these were evaluated for use as a rapid, semi-automoated method for determining antibiotic sensitivity of *M. tuberculosis*

isolates rather than as a detection method (see section 4) and this seems to be now accepted as the most useful application of the Reporter Phage in TB diagnostics.

However there may still be examples where the Reporter Phage may provide a useful tool for the detection of other types of pathogenic Mycobacteria. For instance Sasahara et al. (2004) described the use of Reporter Phage for the detection of the cattle pathogen *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Using phage phAE85 >1000 cells /mL were detectable within 24-48 h. When applied to milk, the authors reported that MAP was detectable at 100 cells/ml in skim milk and 1,000 cells/ml in whole milk. While this is a useful demonstration of the rapid detection of pathogenic Mycobacteria in a food matrix, it is unlikely that this will be adopted as a rapid testing method by the food industry since these Reporter Phage are considered to be GMOs and any laboratory using them is required to work according to the local GM regulations, and the cost of implementing these is not generally compatible with food microbiology testing.

3.2 Phage amplification assays

Unlike the Reporter Phage assays, the PAA or PhaB assays do not use recombinant phage. In these assays a positive indication of the presence of mycobacteria is the formation of plaques at the end of the assay. There have been several variations of these types of assay published but the general principle is a phage protection assay (see Rees and Loessner, 2008). To initiate the assays the sample containing the target cell is first mixed with a high titre of the bacteriophage. The samples are then incubated to allow time for cell infection to occur and for the phage to enter the eclipse phase. At this point any exogenous phage that have not entered an appropriate host cell are destroyed by the addition of a virucide. Various chemicals have been described that can be used as the virucide, but the essential feature of the chemical chosen is fast inactivation of phage particles while having no effect on the viability of the host cells (Stewart et al., 1998). These internalized phage now must be detected, and in its simplest form the phage released at the end of the lytic cycle are detected by the formation of plaques (areas of no growth) in a bacterial lawn. Many of the Mycobacteria phage, such as TM4 and D29, have a broad host range and can also infect the fast growing, non-pathogen *M. smegmatis.* Hence this is often used as a rapidly growing host to produce lawns of bacterial to detect the newly released phage (see Figure 4).

This assay has been produced as commercial kits (the *FAST*plaqueTB™ or Phage Tek MB assays; www.biotec.com) and also "in house" versions have been described (McNerney et al., 2004) and its performance has been extensively reviewed (see Kalantri et al., 2005; Palomino, 2005; Dinnes et al., 2007). The assessment of the performance of these tests have been variable, with some groups reporting that that they perform very well (Albert et al., 2002; Muzaffar et al., 2002; Shenai et al., 2002; Kiraz et al., 2007) while others have reported problems with sample contamination leading to loss of results (Mbulo et al. 2004; Bonnet et al., 2009), and hence decreased sensitivity of the test. This problem has been addressed by the manufacturers by the introduction of an antibiotic supplement containing nystatin, oxacillin and aztreonam (NOA) which suppresses the growth of a wide range of Gram-positive and Gram-negative bacteria and yet does not lead to significant reduction in assay sensitivity while increasing the proportion of interpretable results obtained (Albert et al., 2007; Mole et al., 2007). Although the phage assay is simple to perform, inexpensive and does not require any sophisticated or dedicated equipment, it does require the samples to be
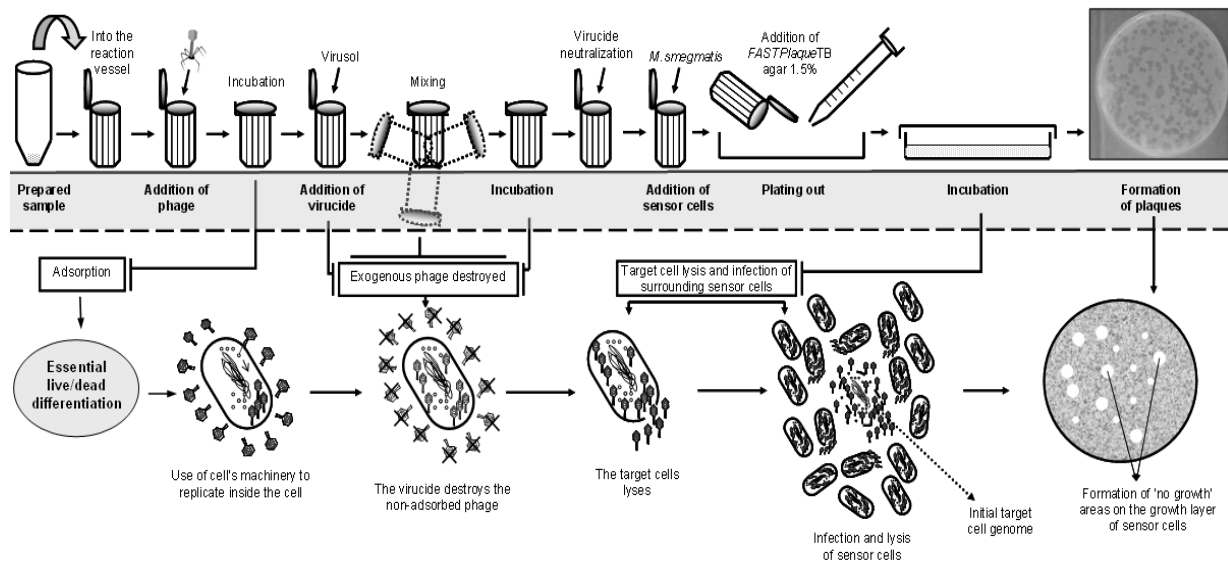
Fig. 4. Diagram of Phage Amplification Assay
Grapic representation of the phage amplification assay. On the top the processing steps of the assay are presented and on the bottom the scientific details of the assay are shown. Following the appropriate sampling preparation the assay is carried out following the procedure described and presented in this figure.

transferred to a dedicated microbiology laboratory. This was perceived to be a disadvantage over conventional sputum smear testing when diagnosis was being performed in clinics in remote areas where reliable, temperature controlled transportation of samples is difficult (Mbulo et al., 2004; Prakash et al., 2009).

The issue of specificity of the phage test arises from the fact that the test utilizes the broad host range of phage D29 to allow both slow growing and fast growing Mycobacteria to be infected. As discussed in relation to the Reporter Phage assays, additional tests are therefore required to determine if the cell detected is TB or a non-tuberculosis *Mycobacterium* spp. For the commercial phage assays a cut-off of 20 plaques is applied, which is the expected number of plaques that will result from the presence of non-pathogenic Mycobacteria that have been fortuitously introduced into sputum, so that samples with fewer than 20 plaques are scored as negative. Patients with active disease generally produced samples with much higher plaque numbers due to growth of the bacteria. However Stanley et al. (2007) have demonstrated that PCR can also be used to increase the specificity of the D29 phage amplification assay.

In this report the assay was being used for the detection of MAP in raw milk samples, but the presence of other pathogenic or non-pathogenic Mycobacteria in these samples could not be ruled out. To overcome this problem, DNA was extracted from the center of the plaques formed at the end of the assay and used for PCR amplification of signature sequences so that the identity of the cell detected by the phage could be determined (see Figure 4). Since *M. tuberculosis* and *M. bovis* were both likely to be present in raw milk samples a multiplex plaque PCR assay was developed to allow simultaneous discrimination between these three organisms. The signature sequences chosen were all multicopy *IS* elements (MAP, IS*900*; TB complex IS*6110* and IS*1081*) with the size of the PCR product

indicating which element had been amplified. Combining the phage assay with PCR provides a significant advantage over direct PCR detection methods since the phage test provides live/dead discrimination while the PCR assay achieves definitive molecular molecular identification of the cell detected.

By reducing sample loss due to contamination by the introduction of antibiotic supplements and increasing the specificity of the phage-based test by combining with PCR, it should be possible that a rapid and robust assay format can be achieved. However the need to perform PCR increases the complexity, cost and time required to complete the assay and the usefulness of this combination assay has yet to be evaluated for clinical cases of TB.

4. Antibiotic sensitivity testing

While the usefulness of phage detection tests for direct identification of *M. tuberculosis* in clinical samples remains questionable, the use of both Reporter Phage and PAA/PhaB assays for determining antibiotic susceptibility of clinical isolates has proven to be more a valuable tool. The principle of these tests was first outlined by Jacobs et al. (1993) using Reporter Phage when they demonstrated that phage growth and gene expression was inhibited if rifampicin was added to antibiotic-sensitive cells whereas if the cells were resistant to the antibiotic, signal generation was unaffected. They showed that the reporter gene signal could be detected within minutes of infection of *M. tuberculosis* with a Fflux Reporter Phage, and by comparing the results of Reporter Phage infection in the presence and absence of the antibiotic, the sensitivity of *M. tuberculosis* isolates could be determined within days. As before, this type of assay is not specific to *M. tuberculosis* and Williams et al. (1999) described the use of *Fflux* Reporter phage to rapidly determination of drug susceptibilities of MAP giving faster results and, in this case, also being used to determine the minimum inhibitory concentration (MIC) of the antibiotics tested more rapidly and accurately.

Similar assays have been developed for the non-recombinant phage assays by showing that phage growth is inhibited, but in this case it is the formation of plaques that is inhibited rather than the signal from a reporter gene (Wilson et al., 1997 and reviewed in Minion and Pai, 2010), and again these have been produced as a commercial test (*FAST*plaque-Response™; www.biotec.com) which can be used on direct patient specimens as well as indirect isolates. Other assay formats using microtitre plates and alternative methods of detecting phage growth have also been described (Gali et al., 2003; McNerney et al., 2007;, which have been designed to shorten the time required for testing. Most recently Bainee et al., (2008) have described a microtitre format Reporter Phage assay that can be semi-automated. Isolates were inoculated in to the wells of the microtitre dish, treated with either rifampicin (RIF), isoniazid (INH) or no antibiotic and then incubated for 40 h before samples were infected with the TM4-based Reporter Phage phAE142. After 3 h of infection the light levels in each well are determined using a microplate luminometer and the sensitivity of the strains determined by comparing the light levels of the treated and control samples. This method was shown to be able to determine both the RIF and INH resistance of clinical isolates of M. tuberculosis with a median test result time of 2 days.

Minion and Pai (2010) carried out a meta-analysis of 31 studies describing the use of the different phage assays used to determine rifampicin resistance and concluded that the

Reporter Phage assays had the highest accuracy (sensitivity = 99.3%, specificity = 98.6%), with in-house phage amplification assays also performing well (sensitivity = 98.5%, specificity = 97.9%). Estimates from studies evaluating the commercial *FAST*plaque kits were slightly lower (sensitivity = 95.5%, specificity = 95.0%); however, this difference was not statistically significant (based on overlapping confidence intervals) from the LRP and in-house assays (Minion and Pai, 2010). However this report did highlight the fact that the phage-based methods currently provide the fastest phenotypic assay for antibiotic susceptibility testing and if levels of technical failure can be reduced these assays could be a useful clinical tool to improve patient outcomes.

5. Enumeration assays

Perhaps the most overlooked potential of these phage based assays is as a research tool for the rapid evaluation of viable count of laboratory cultures. Simple modifications of the Phage Amplification assay allow cell number to be rapidly determined, with results being available within 24 h. This method was first reported by Stanley et al. (2007) in a paper describing the development of a phage amplification assay for MAP. In this case 10-fold serial dilutions of MAP cultures were prepared and a sample of each dilution tested using the Phage Amplification assay and it was found that the number of plaques detected correlated well with the number of MAP cells in the test sample (Stanley, 2005). Rather than diluting the sample and testing each one, a modification of the *FAST*plaque assay method was devised that allowed the viable count of a sample containing an unknown number of cells. In this case the sample is simply diluted immediately prior to plating so that the plaque number can be accurately counted and then the number of cells present in the original sample determined according to the dilution factor (Botsaris et al., 2009; Figure 5).
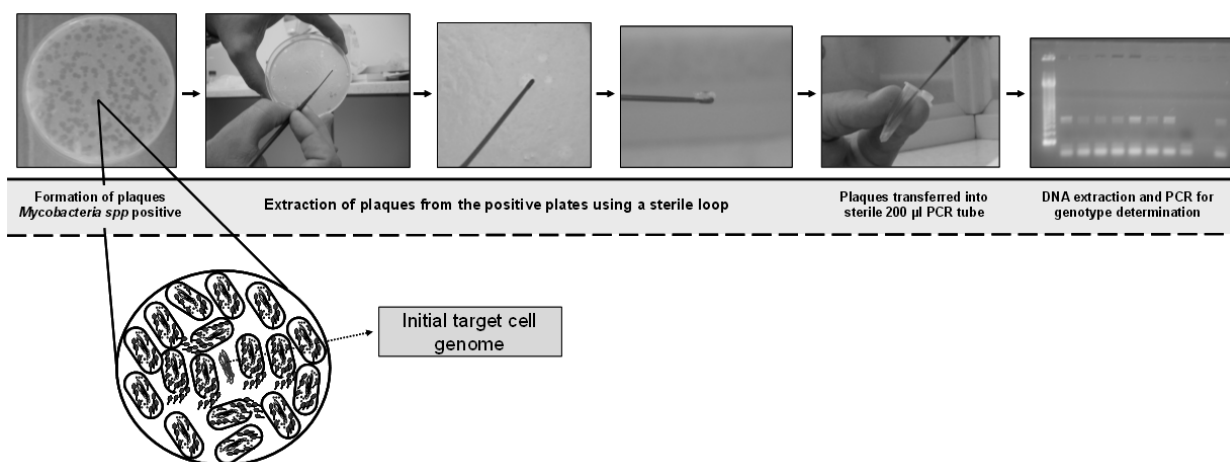

Fig. 5. Extraction of Plaques and Molecular Identification
Schematic representation of the plaque extraction method. The plaques containing the genome of the initial targeted cell are extracted using a sterile loop. The loop is used to cut gently all 4 sides of the plaque area and then the plaque is gently lifted and placed into a PCR tube for DNA extraction.

It is clear that there is not always a 1:1 relationship between the cfu and pfu counts obtained, however results for a given isolate grown under the same conditions seem to be relatively

constant (Stanley, 2005). Foddai et al. (2009) described the optimisation of the MAP Phage Amplification assay to accurately detect and enumerate MAP cells. They investigated the optimal buffer conditions for D29 phage infection of MAP cells, determined the minimal time for D29 incubation with MAP to produce extracellular phage (burst time) and the incubation time required before virucide treatment and assessed the impact of changing these parameters on the correlation between plaque count and corresponding colony count. To achieve a correlation closer to 1:1 between pfu/ml and cfu/ml value when detecting MAP, the authors suggested modifications to the standard protocol including supplementing the medium with 2 mM calcium chloride to enhance phage infection, incubating cells at 37 °C overnight before infection with D29 and extending the time allowed for phage infection to 2 h so that phage successfully infect a higher percentage of cells.
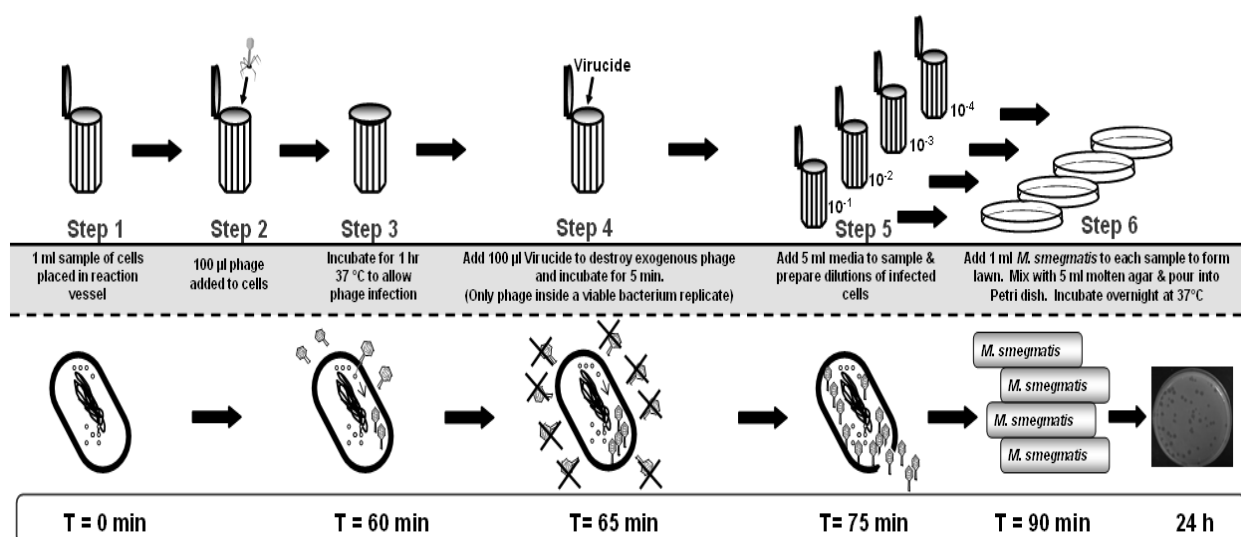

Fig. 6. Modification of the PAA for Enumeration of cells
Enumeration of cells using the PAA. 1 ml of the sample is infected with phage. Following adsorption exogenous phage are destroyed by the addition of a virucide which is then neutralised. Serial dilutions are made and plated on a lawn of M. smegmatis cells. Plaques (representing the target cells) are enumerated after overnight incubation.

A limited number of reports of the use of phage for enumeration have appeared in the literature – all for MAP rather than for *M. tuberculosis* – but these do demonstrate the usefulness of the method, especially when following inactivation kinetics. Altic et al. (2007) used the FPTB assay in UV inactivation studies and the authors observed that the colony counts were consistently 1 to 2 $\log_{10}$ higher than the plaque counts. However the rate of inactivation measured by both culture and Phage Amplification Assay were identical, so that even though a proportion of the population was detected, these cells were inactivated in the same rate as the rest of the culture. Similarly Donaghy et al. (2009) used the Phage Amplification assay to monitor UV inactivation of MAP using a novel pilot-scale UV treatment for milk. Again there was a discrepancy between the cfu and pfu values obtained, and some evidence of large differences in the infectivity of different strains of MAP, but despite this the inactivation curves obtained were identical and the pfu data was available within 24 h whereas the culture results required up to 18 weeks for growth of the colonies. This demonstrates the power of the phage-based assays to provide rapid data to allow

development of experimental design to initiate new areas of research. Once the normal pfu:cfu ratio has been established for a particular strain grown under a particular condition, the phage assay can be used to rapidly determine the number of viable cells present in a sample without the need for extended periods of incubation.

6. Conclusion

The use of bacteriophage to type bacterial cells has been accepted as a standard microbiological method for many decades. First reports of the use of this technique for Mycobacteria appear in the 1960's and it continued to be used as a standard method for investigations of epidemiological investigations until the 1980's (see Snider et al., 1984). However the advent of molecular methods of identification and subtyping has made such phage-based methods obsolete. The difficulty of culturing with these slow growing and fastidious bacteria may have driven the development of alternative methods for detection and identification. To this end the rapid growth of phage within these cells has been exploited to produce a range of different assay methods and assay formats, all with the aim of producing rapid, simple, economic tests that can be adopted in areas where more expensive molecular diagnostic tests cannot be supported. However to date none of these has gained widespread acceptance in the clinical setting. Perhaps this is because all of these methods still require a degree of staff expertise to be able to perform the tests and therefore they do not yet fulfil the criteria required to be of use in less developed countries. However there is potential for these assays to be used effectively as an adjunct to standard culture methods to further our understanding of Mycobacteria, and perhaps as our understanding of the phage-host interaction increases we may be able to solve some of the limitations of the current phage based assays and allow them to eventually realise their potential in combating human disease.

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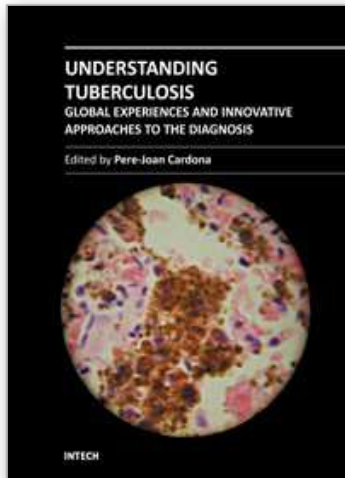
Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis
Edited by Dr. Pere-Joan Cardona

Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

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