Original Article

A Rapid Viability and Drug-Susceptibility Assay Utilizing Mycobacteriophage as an Indicator of Drug Susceptibilities of Anti-TB Drugs against *Mycobacterium smegmatis* mc^2 155

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Abstract

Background: A rapid in-house TM4 mycobacteriophage-based assay, to identify multidrug resistance against various anti-tuberculosis drugs, using the fast-growing *Mycobacterium smegmatis* mc^2 155 in a microtiter plate format was evaluated, based on phage viability assays. **Methods:** A variety of parameters were optimized before the study including the minimum incubation time for the drugs, phage and *M. smegmatis* mc^2 155 to be in contact. An increase in phage numbers over 2 h was indicative that *M. smegmatis* mc^2 155 is resistant to the drugs under investigation, however when phage numbers remained static, *M. smegmatis* mc^2 155 found to be sensitive to the drug. **Results:** The study confirmed that the data are statistically significant and that *M. smegmatis* mc^2 155 is, in fact, sensitive to isonazid, iifampicin, pyranzaimide, and ethambutol as phage numbers doubled over 2 h ($P = 0.015$, 0.018, 0.014, and 0.020). The study also confirmed that *M. smegmatis* mc^2 155 is resistant to the drugs ampicillin, erythromycin, amoxicillin streptomycin as phage numbers remain static over the same 2 h period ($P = 0.028$, 0.052, 0.049, and 0.04). This drug-susceptibility profiling of eight different drugs against *M. smegmatis* mc^2 155 was detected in as little as 1½ days with a cost of ~ one euro and fifteen cent to test four drugs. **Conclusion:** This test is rapid to perform and will have widespread applications in drug-susceptibility testing of other members of the mycobacterial genus. In addition, the platform could also be used as a tool for high-throughput screening of novel antimycobacterial drugs. The main assets of this assay include its relatively cheap cost, versatility, and quick turnaround time.

Keywords: Drug susceptibility, mycobacteriophages, *Mycobacterium smegmatis*, optimization study

Introduction

The emergence of multi-drug resistant (MDR) bacterial species is a major concern in the modern world. Mycobacterial species, in particular, have developed resistance to a large array of antibiotics which disrupt cell wall biosynthesis. Their ability to resist such antibiotics is caused by the structure of their cell wall, which is composed of approximately 60% lipids making them notoriously difficult to treat. Most mycobacteria are susceptible to the antibiotics clarithromycin and rifamycin, but several antibiotic-resistant strains have emerged.[1]

The delay in identifying drug susceptible isolates is due to the lack of advanced technology and the time involved in sample preparation. Globally, most susceptible patients are not diagnosed in time and do not receive appropriate therapy.[2] They are clearly at risk of developing even more resistant mycobacterial infections and transmitting these resistant mycobacterial strains. With today's knowledge, available diagnostic tools and epidemiological advances, it cannot be considered acceptable to wait for 16 days to know if a clinical isolate is drug susceptible or not. Thus, the traditional practices of identification should be improved on. From the literature, it has been noted that three established methods, in particular, are used to identify drug susceptibilities in mycobacterial species (minimum inhibitory concentrations [MICs], BACTEC 460TB method, and the mycobacteria growth

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indicator tube [MGIT] 960 system).[3] The BACTEC and the MGIT methods have been adopted by many laboratories in the Western world and are based on the automated detection of growth in a liquid medium with anti-tuberculosis drugs. These methods are, therefore, faster than those based on visual detection of growth on solid medium. However, one disadvantage of these systems is the inability to check the colony morphology of the bacterial cultures. Both invisible contamination and overgrowth with atypical mycobacteria affect the reliability of the tests. In addition, these methods have disadvantages in that they involve multiple tubes or bottles and are restricted to discriminating between resistance and susceptibility, rather than determining the exact MICs of the drugs. The three methods mentioned above are, however, reproducible, accurate, and relatively cheap. The materials used in performing each test are cheap, although the sophisticated equipment required for BACTEC and MGIT is relatively expensive.

Laboratories in most developing countries lack this sophisticated and costly equipment required to identify MDR mycobacterial strains. The development of an assay which has the potential to identify MDR-mycobacterial species at a low cost and rapid time would be highly beneficial. One potential way to reduce the cost of drug-susceptibility testing involves the use of mycobacteriophages,[4] which have relatively rapid replication rates (unlike the slow growth of their hosts), are simple to use, and are relatively inexpensive to propagate.

Our study set out to prove the concept of this assay using a fast-growing surrogate strain (*M. smegmatis* mc^2 155), which would determine the viability of mycobacteria following exposure to a range of commonly used drugs. Initially, critical parameters were optimized, after which the efficacy of the assay was evaluated using a blinded study. The principle is based on the fact that the phage will only infect viable cells, and the increase in their numbers over time is consistent with the host strain demonstrating resistance to the drug under investigation.

Methods

Preparation and standardization of *Mycobacterium smegmatis* mc^2 155

M. smegmatis mc^2 155 (CIT culture collection) was utilized in the study to optimize the assay as it is a fast-growing strain and nonpathogenic. Cultures of *M. smegmatis* mc^2 155 (previously stored at −80°C) were standardized using the DEN-1 McFarland Densitometer (Grant-bio) to achieve McFarland Standards of 0.5 (approximately 1.5 × 10^8 cfu/ml) in log phase and suspended in brain heart infusion (BHI) broth (Sigma Aldrich). The mycobacterial cultures of *M. smegmatis* mc^2 155 were then transferred to sterile screw-cap glass tubes-containing glass beads (5 mm) (Sigma Aldrich) (to disrupt the cell wall) in 2 ml of BHI broth. Suspensions were homogenized with a vortex mixer for 15–20 s. Large clumps were allowed to settle by allowing the suspensions to stand at room temperature for 10 min. The supernatants were transferred to sterile tubes and adjusted to 10^1 CFU/ml, 10^2 CFU/ml, 10^3 CFU/ml, 10^4 CFU/ml, and 10^5 CFU/ml with BHI broth (Sigma Aldrich) and standardized using the DEN-1 McFarland Densitometer (Grant-bio) to achieve McFarland standards of 0.5 (1.5 × 10^8 cfu/ml).

Confirming the phenotype of *Mycobacterium smegmatis* mc^2 155 through growth profiling, purity streaks, and Ziehl–Neelsen staining

A growth curve of *M. smegmatis* mc^2 155 and BHI broth (Sigma Aldrich) was generated to standardize and confirm the purity and identity of *M. smegmatis* mc^2 155 using established methods.[5] Before the addition of the drug and the mycobacteriophage TM4, *M. smegmatis* mc^2 155 was suspended in 5 ml of BHI broth (Sigma Aldrich) and 0.05% Tween 80 (Sigma Aldrich) and incubated at 37°C for 12 h. The cell suspensions following 12 h incubation were standardized using the DEN-1 McFarland Densitometer (Grant-bio) to achieve a McFarlands standard of 1 × 10^8 cfu/ml (McFarlands standard 0.5).

Each cell suspension grown in broth was checked for purity by performing the four-streak method on BHI agar (Sigma Aldrich) followed by incubation at 37°C for 48 h.

Ziehl–Neelsen staining was also performed as described previously,[6] and visualized using the Olympus CH30 light microscope (Olympus). A 2.5 µl volume of *M. smegmatis* mc^2 155 was added to 100 µl of fresh media (BHI broth) (Sigma Aldrich) and 30 µl of 0.05% Tween 80 contained in three separate wells of a sterile microtiter plate (Sarstedt, Germany). The OD of the three wells was read at 600 nm using the Mutiskan FC (Thermo Scientific) for 40 h at 37°C and average absorbance readings of the three wells were calculated.

Preparation and standardization of the mycobacteriophage TM4

To standardize phage propagation, 100 µl of a TM4 mycobacteriophage stock (Titer 8 × 10^8 PFU/ml) (CIT culture collection) was added to 100 µL of early log phase *M. smegmatis* mc^2 155 cells (log phase was established following repeated standard growth curve analysis) in 5 ml of BHI broth (Sigma Aldrich) and incubated at 37°C for 48 h previously established from other methods.[7] Subsequently after 48 h, 2 ml of the culture was removed and placed in a microfuge tube and centrifuged at room temperature at 2000 × g for 5 min to pellet cellular debris. This was then filter-sterilized using a 0.2 µm filter (Sarstedt, Nümbrecht, Germany).

A volume of 100 µl of the filter sterilized supernatant was added to 500 µL of early log phase *M. smegmatis* mc^2 155 in a microfuge tube and incubated at 37°C for 30 min. The suspension was then added to 5 ml of a BHI broth 0.75% overlay agar tube tempered at 50°C and poured onto the surface of a BHI agar plate. Plates were incubated at 37°C for 48 h and then examined for plaques.

Phage stocks were generated with the addition of 5 ml of phage buffer (50 mM TRIS pH 8, 150 mM NaCl, 10 mM $MgCl_2$, and 2 mM $CaCl_2$) to the plate described in method 3.2.1. Sterile hockey sticks were also aseptically run across the surface of the agar plate to physically recover the phages. The plate was then incubated for 2 h at 37°C with shaking. The buffer was subsequently recovered from the plate, centrifuged to pellet debris and filter-sterilized using a 0.2 μm filter and stored at 4ºC until further use.

Determination of the minimum inhibitory concentrations of isonazid, rifampicin, ampicillin, pyranzamide, erythromycin, ethambutol, amoxicillin, and streptomycin

The MICs of each drug under investigation was performed to establish whether the drug had an antimycobacterial effect and to determine the drug concentration (if any) required to adequately kill *M. smegmatis* mc² 155. The MICs of each drug (isonazid, pyranzamide, ethambutol, and rifampicin) (Sigma Aldrich) were determined as follows. An overnight culture of *M. smegmatis* mc² 155 incubated at 37°C was prepared and standardized using the DEN-1 McFarland Densitometer (Grant-bio) to reach a McFarland standard of 0.5. A stock solution of the four drugs was prepared. Some drugs such as rifampicin were resuspended in methanol (16 mg/ml), pyranzamide was resuspended in ethanol (5.7 mg/ml), and ethambutol and isonazid were resuspended in sterile distilled water (50 mg/ml). Each stock solution was stored at −20°C until needed.

Two-fold dilutions of each drug under investigation were prepared using the stock solutions as outlined above to achieve final assay concentrations of 16 μg/ml, 8 μg/ml, 4 μg/ml, 2 μg/ml, 1 μg/ml, 0.5 μg/ml, 0.25 μg/ml, and 0 μg/ml. These dilutions were added to an appropriate volume of log phase *M. smegmatis* mc² 155 cells which was previously established in section 2.2 (contained in BHI broth (Sigma Aldrich), resulting in a cell density of 5×10^5 cfu/ml. The tubes were incubated at 37°C for 24 h. The concentration of antimicrobial in the first clear tube (no growth) after 24 h was indicative of the MIC for each drug.[8]

Determination of the minimum exposure time for *Mycobacterium smegmatis* mc² 155 and the drugs under investigation to be in contact

To prove the efficacy of the four drugs that have an effect on *M. smegmatis* mc² 155 (proven by the MIC values) and the other four drugs that do not have an effect on *M. smegmatis* mc² 155, an optimization assay was conducted to determine the minimum exposure time for the drug and *M. smegmatis* mc² 155 to be in contact.

For isonazid (0.25 μg/ml), 100 μl of *M. smegmatis* mc 2 155 suspension (Neat, 10^1, 10^2, 10^3, 10^4, and 10^5 CFU/ml) was placed in six allocated wells of five sterile microtiter plates (Sarstedt, Germany) (one for each different temperature was used). The first microtiter plate was tested directly after the addition of the drug and bacteria (i.e., 0 h) (second plate: 2 h, third plate: 4 h, fourth plate: 6 h, and fifth plate: 8 h).

Each sterile microtiter plate was removed from the 37°C incubator following its allocated time period. BHI (Sigma Aldrich) agar plates were seeded with a 0.5 McFarland standard of *M. smegmatis* mc² 155. A three microliter volume of the drug from the appropriate microtiter plate was added to the appropriate seeded agar plates. Plates were incubated at 37°C for 24 h and plates were subsequently examined for the presence of zones of inhibition (mm) after each time according to the CLSI guidelines.

Determination of the minimum exposure time for *Mycobacterium smegmatis* mc² 155, ampicillin/rifampicin, and TM4 mycobacteriophage to be in contact

The first part of this procedure was conducted using the optimized conditions as outlined in Section 2.5, utilizing an incubation period of 2 h for *M. smegmatis* mc² 155 and ampicillin and rifampicin in the microtiter plates.

Following the 2 h incubation period, 48 μl of mycobacteriophage TM4 (Neat, 10^1, 10^2, 10^3, 10^4, and 10^5 PFU/ml) was added to the six allocated wells of the sterile microtiter plates-containing ampicillin (2 μg/ml) (Sarstedt, Germany) for a specified time of 0, 30, 60, 90, and 120 min and incubated at 37°C to establish the necessary time for the drug, the phage, and the bacteria to be in contact. The ten sterile microtiter plates (10 plates for rifampicin in duplicate) (plate one and six were incubated for 0 min, plate two and seven were incubated for 30 min, plate three and eight were incubated for 60 min, plate four and nine were incubated for 90, and plate five and ten were incubated for 120 min) were removed from the 37°C incubator following their allocated incubation periods and standard plaque assays were performed to determine the effect on plaque numbers.

For ampicillin and rifampicin, phage numbers were determined from the wells by adding 100 μL of the bacterial/drug/phage suspension from separate wells to 100 μL of early log phase *M. smegmatis* mc² 155 cells in a five microliter BHI 0.75% w/v overlay agar tube which was tempered to 50°C. The overlay was then poured onto the surface of a BHI agar plate (Sigma Aldrich). Plates were incubated at 37°C overnight and then examined for phage plaques. The same procedure was performed for rifampicin.

Application of the optimized mycobacteriophage-based microtiter plate assay to identify drug susceptibilities of six additional drugs

Following the optimization assays, the efficacy of six commonly used drugs was evaluated (streptomycin, erythromycin, ethambutol, pyranzaimide, amoxicillin, and isonazid). The same procedure was performed as outlined in Section 2.5 and Section 2.6; however, the optimized time periods previously established for the drug, the mycobacteriophage TM4 and *M. smegmatis* mc² 155 were utilized (i.e., 2 h) throughout as outlined in Figure 1.

Evaluation of the efficacy of the mycobacteriophage-based drug-susceptibility assay by undertaking a blinded study

Following the assay, a blinded study was conducted to ensure that the results obtained were not affected by bias. For the

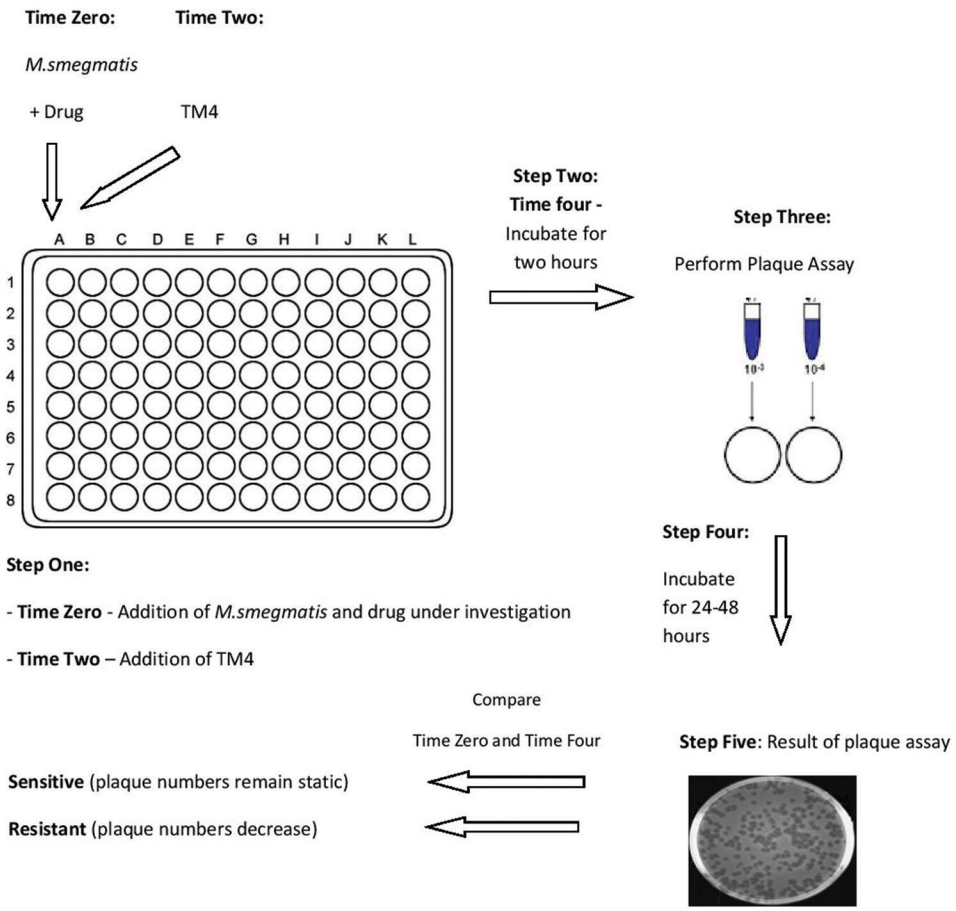


Figure 1: The procedure involved in the development of a mycobacteriophage-based drug-susceptibility assay by incubating *Mycobacterium smegmatis*, a drug under investigation and the mycobacteriophage TM4 to identify drug susceptibilities through plaque assays

blinded study the same eight drugs, isonazid, rifampicin, ampicillin, pyranzamide, erythromycin, ethambutol, amoxicillin, and streptomycin (Sigma Aldrich) were retested. The drugs were randomly coded. The primary objective was to identify drug susceptibilities of *M. smegmatis*[255] to eight randomized unknown drugs based on the formation of plaques.

Data analysis

Statistical analysis was performed with IBM SPSS Software v. 24, (Armonk, NY, USA). To determine if the data were statistically significant, the normality of the data was first assessed with the Shapiro–Wilks test at a significance level of 0.05. A value of $P < 0.05$ was interpreted as indicating significant correlation at a confidence interval of 95%. Nonparametric tests were chosen for data not normally distributed.

Results

Phenotype of *Mycobacterium smegmatis* mc² 155 by using growth profiling, purity streaks, and Ziehl–Neelsen staining

Ziehl–Neelson staining, the culture appeared as red bacilli in clusters under the × 100 objective lens as expected as outlined in Figure 2a. Tween Eighty (0.05%) prevented clumping of *M. smegmatis* mc² 155 throughout its growth and led to an efficient growth curve. The doubling rate of *M. smegmatis* mc² 155 is typically one to 2 h; therefore, readings were taken every hour to observe this effect. Following 12 h of incubation *M. smegmatis* mc² 155 entered its log phage for approximately 12 h until it reached stationary phase at 24 h. Following. The cultures of the *M. smegmatis* mc² 155 culture on BHI agar (Sigma Aldrich), appeared as filamentous, irregulated, undulated margin, flat in shape, and cream-white in color indicating that pure colonies were obtained when a purity streak was performed as described previously[9] and as outlined in Figure 2b. The presence of red bacilli following Ziehl–Neelsen staining and the colony morphology described above confirms the identity of *M. smegmatis* mc² 155. The growth rate of *M. smegmatis* mc² 155 was measured spectrophotometrically at an OD of 600 nm as demonstrated in Figure 2c.

The determination of the minimum inhibitory concentrations and determination of the minimum exposure time of *Mycobacterium smegmatis* mc² 155 and the drugs under investigation to be in contact

The MIC of each drug under investigation was determined using *M. smegmatis* mc² 155 to record the concentration

required to kill viable *M. smegmatis* mc^2 155 cells and to establish an effective drug concentration for the assay. The four drugs in Table 1 exhibited growth-inhibitory effects against *M. smegmatis* mc^2 155, although some drugs had a better inhibitory effect than others. The MICs of each drug varied as outlined in Table 1. The highest MIC obtained was for ethambutol at 5 μg/ml, whereas the lowest MIC value obtained was for isonazid at 0.25 μg/ml. The highest MIC values (zones of inhibition cm) for each drug were obtained after 2 h. From 4 to 8 h, the zones of inhibition began to decrease.

Optimization assays were conducted according to the CLSI guidelines to determine the minimum contact period for *M. smegmatis* mc^2 155, and the drug under investigation. All eight drugs were analyzed for this optimization assay.

The optimization assay was undertaken to determine the minimum contact time period for *M. smegmatis* mc^2 155, and the drug of choice (in this case the eight drugs were utilized). The determination of the minimum contact time was based on MIC values as outlined in Table 1. Comparison of contact periods was determined over an 8 h time frame.

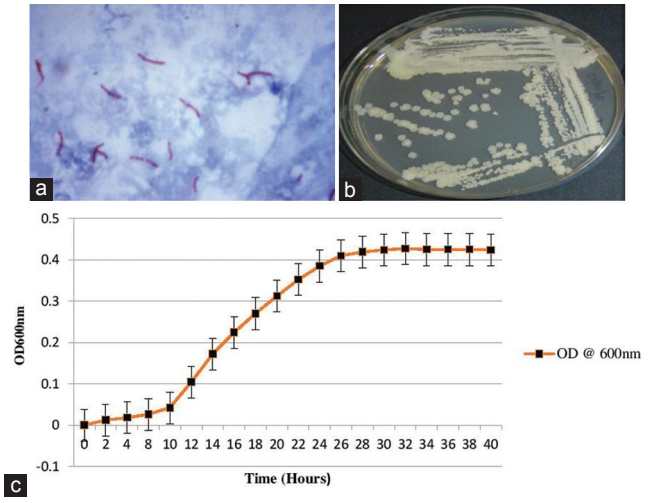


Figure 2: (a) Ziehl Neelsen staining of *Mycobacterium smegmatis* mc2 155 as described previously (Cappucino and Sherman, 2014) (b) Purity streak of *Mycobacterium smegmatis* mc2 155 on brain heart infusion agar (Sigma Aldrich) after incubation for 48 h at 37°C (c) Growth curve of *Mycobacterium smegmatis* mc2 155 at OD600nm

Table 1 provides an accurate determination of the contact time required. It is evident that a contact time of 2 h is sufficient to achieve an efficient response.

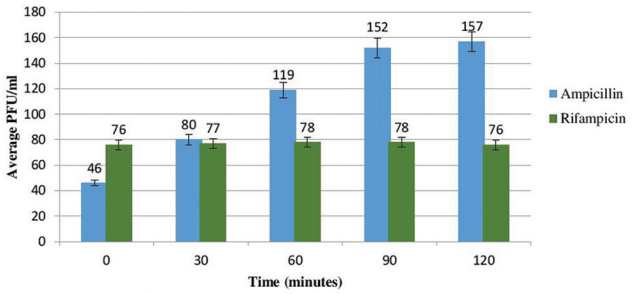
Determination of the minimum exposure time of *Mycobacterium smegmatis* mc^{2}155, ampicillin/rifampicin and TM4 mycobacteriophage to be in contact

Optimization assays were conducted to determine the optimum contact period for *M. smegmatis* mc^2 155, the drug and the mycobacteriophage TM4. Ampicillin (2 μg/ml) (Sigma Aldrich) and rifampicin (4 μg/ml) (Sigma Aldrich) were used for the purpose of the optimization assays.

The optimization assay was undertaken at time intervals of 30 min in duplicate and was based on the changes in plaque numbers (pfu/ml) as outlined in Figure 3. An increase in phage numbers was seen to be indicative of drug resistance as TM4 can lyse viable mycobacterial cells which have not been inactivated by the drug.

For ampicillin (2 μg/ml) (Sigma Aldrich), at time zero, the average plaque numbers obtained were 46 pfu/ml and after 120 min, the average plaque numbers obtained were 152 pfu/ml. For rifampicin (4 μg/ml) (Sigma Aldrich), the plaque numbers (pfu/ml) obtained at 0 min were 76 pfu/ml and following 120 min the plaque numbers obtained were 76 pfu/ml.

M. smegmatis mc^2 155 is resistant to ampicillin as outlined in Figure 3 given the large increase in plaque numbers (pfu/ml)



Blue bar – Ampicillin, Orange bar - Rifampicin

Figure 3: Optimum time (minutes) required for ampicillin and rifampicin under investigation to be in contact with *Mycobacterium smegmatis* mc2 155 based on the change/no change in PFU/ml for the mycobacteriophage TM4

Table 1: Minimum inhibitory concentrations and zones of inhibition (cm) of four drugs used to determine the minimum contact time for *Mycobacterium smegmatis* mc^2 155 and the drug under investigation to be in contact

Drug	MIC (μg/ml)	Zones of inhibition (cm) after two hours	Zones of inhibition (cm) after four hours	Zones of inhibition (cm) after six hours	Zones of inhibition (cm) after eight hours
Isonazid	0.25	1.3	1.1	1	0.7
Rifampicin	2	2.6	2.4	1.9	1.9
Pyrazinamide	0.5	1.6	1	0.9	0.9
Ethambutol	5	2.9	2.7	2.3	2.2

from time 0 min to time 120 min. Phage numbers (pfu/ml) that remain static from time 0 to time 120 min suggest that *M. smegmatis* mc^2 155 is sensitive to the drug as the drug has inactivated viable cells.

A graphical representation of the optimum time period for the mycobacteriophage TM4, *M. smegmatis* mc^2 155 and the drug to be in contact was constructed. As outlined in Figure 3, the average pfu/ml either increased with respect to time (minutes) or remained static throughout the indicated time periods for ampicillin and rifampicin according to their efficacy against the host. A time period of 2 h was chosen based on Figure 3.

Application of the optimized mycobacteriophage-based microtiter plate assay to identify drug susceptibilities of six drugs

To ensure consistency of results and to test the efficacy of the optimized assay, various other drugs were used to indicate drug susceptibilities. Each assay was performed in triplicate.

The drugs ampicillin (4 μg/ml) (Sigma Aldrich) and rifampicin (2 μg/ml) (Sigma Aldrich) were tested for drug susceptibilities as outlined in Figure 4. To allow for comparison in identifying drug susceptibilities two time points (t = 0 h and t = 2 h) were utilized based on the experiment above. As already discussed, an increase in phage numbers is indicative of drug resistance. From Figure 4 it can be said that *M. smegmatis* mc^2 155 is resistant to Ampicillin (P = 0.028) as a result of the large increase in plaque numbers (pfu/ml) from time 0 min to time 120 min. *M. smegmatis* mc^2 155 appears to be sensitive to rifampicin (Sigma Aldrich) (P = 0.018) as phage numbers (pfu/ml) remain static from time 0 to time 4 h.

The drugs streptomycin (2.5 μg/ml) (Sigma Aldrich) and isonazid (0.25 μg/ml) (Sigma Aldrich) were also tested against *M. smegmatis* mc^2 155 as outlined in Figure 4. Similarly, two time points (t = 0 h and t = 4 h) were utilized to contrast the difference in plaque numbers between the two points. As already discussed, an increase in phage numbers is indicative of drug resistance. From Figure 4, it can be said that *M. smegmatis* mc^2 155 is resistant to streptomycin (P = 0.049) as a result of the large increase in plaque numbers (pfu/ml) from time 0 min and time 120 min. *M. smegmatis* mc^2 155 appears to be sensitive to isonazid (P = 0.015) as phage numbers (pfu/ml) remain static from time 0 to time 120 min.

Pyranzamide (0.5 μg/ml) (Sigma Aldrich), erythromycin (0.15 μg/ml) (Sigma Aldrich), ethambutol (5 μg/ml) (Sigma Aldrich), and amoxicillin (4 μg/ml) (Sigma Aldrich) were also used in this assay to demonstrate drug susceptibilities based on differences in plaque numbers (pfu/ml) as outlined in Figure 5. *M. smegmatis* mc^2 155 appears to be resistant to erythromycin (Sigma Aldrich) (P = 0.048) and amoxicillin (Sigma Aldrich) (P = 0.049) as plaque numbers (pfu/ml) obtained in Figure 5 increase from time 0 to time 4 h indicating that *M. smegmatis* mc^2 155 is resistant to the three drugs. *M. smegmatis* mc^2 155 is sensitive to the drugs Pyranzamide (Sigma Aldrich) (P = 0.014) and Ethambutol (Sigma Aldrich) (P = 0.02) as indicated in Figure 5. The plaque numbers (pfu/ml) from time 0 to time 4 h remain relatively static indicating that the cells are still viable.

The data obtained in relation to the drug susceptibilities appears to be statistically significant as $P < 0.05$ at a confidence interval of 95% were obtained as outlined above.

Two-time points (Time zero and Time 4 h) were utilized to assess the drug susceptibility of four drugs (in this case streptomycin, isonazid, rifampicin, and ampicillin) as before. The average pfu/ml versus time (hours) was measured in this assay. An increase in phage numbers from time point 0 to time point 4 h indicates that *M. smegmatis* mc^2 155 is resistant to the drug. If the phage numbers (pfu/ml) remain static then *M. smegmatis* mc^2 155 is sensitive to the drug under investigation.

Evaluation of the efficacy of the mycobacteriophage-based drug-susceptibility assay by undertaking a blinded study

To rule out the chance of bias, a blinded study was conducted. At the end of the blinded study, the identity of each drug was confirmed. The bars indicate drugs to which *M. smegmatis* mc^2 155 is sensitive as phage numbers remain static from time 0 to time 4 h. The bars which increase significantly from time 0 h to time 4 h are drugs to which *M. smegmatis* mc^2 155 are resistant to as outlined in Figure 6. The identity of the A was confirmed as isonazid (P = 0.016), B was

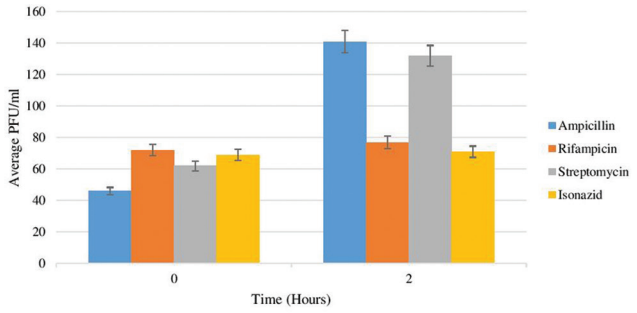


Figure 4: Drug-susceptibility profiles of four drugs (streptomycin, isoniazid, rifampicin and ampicillin) based on changes in average pfu/ml of the mycobacteriophage TM4 versus time (hours T0–T2)

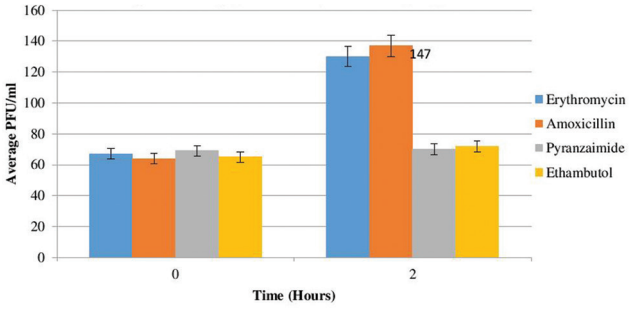


Figure 5: Drug-susceptibility profiles of four drugs (erythromycin, amoxicillin, pyranzaimide and ethambutol) using changes in average pfu/ml of the mycobacteriophage TM4 versus time (hours T0–T2), utilizing optimized conditions established previously

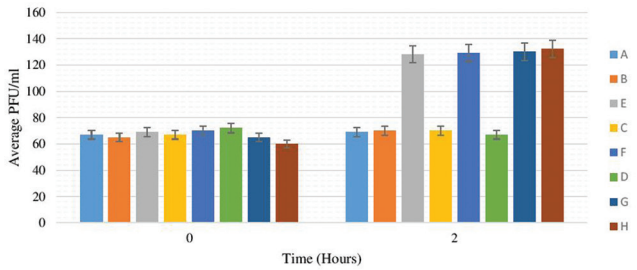


Figure 6: Blinded study to determine the resistance profiles of eight drugs utilized above using the mycobacteriophage TM4 based on the changes in average PFU/ml versus time (hours T0–T2), utilizing the optimized conditions established previously

confirmed as rifampicin (P = 0.012), C was confirmed as pyranzaimide (P = 0.019), and D was confirmed as ethambutol (P = 0.011). *M. smegmatis* mc^2 155 is, in fact, sensitive to these four drugs as outlined in the literature,[10,11] thereby validating this method. The identity of E was confirmed as ampicillin (P = 0.049), F was confirmed as erythromycin (P = 0.041), G was confirmed as amoxicillin (P = 0.044), and H was confirmed as streptomycin (P = 0.047). Various studies have outlined that *M. smegmatis* mc^2 155 is resistant to these four drugs.[12,13]

Universally, the data obtained in relation to the drug susceptibilities is statistically significant as $P < 0.05$ at a confidence interval of 95% were obtained as outlined above.

Discussion

Over the past years, the use and misuse of antimicrobials has increased the numbers and types of resistant organisms including mycobacterial species. As a large majority of mycobacterial species have relatively slow-growth rates, it would be of importance to detect these resistant strains within an adequate period of time to provide appropriate treatment options. Advanced drug-susceptibility assays have been developed within recent years such as the BACTEC TB480 method and the MGIT method particularly in hospital settings, however these assays can take days or weeks to generate results.[14,15] Based on this knowledge, it would be of interest to be able to construct an assay which can identify drug susceptibilities within a shorter period, to expedite clinical treatment. The major applications of such a rapid viability assay would include (1) determining drug sensitivities of clinical mycobacterial isolates and (2) identifying novel antimycobacterial drugs using high-throughput screening (HTS).

The assay was a preliminary study to provide proof of concept that mycobacteriophages can be used to detect drug resistance or sensitivity using a fast-growing mycobacterial strain before the assay can be translated to more complex mycobacterial species. For this purpose, *M. smegmatis* mc^2 155 was employed in this study as it is a very useful surrogate for the research of other species in the genus mycobacteria (slow growers) in laboratories, due to its rapid replication (reaches stationary phase within 48 h) and its nonpathogenic nature (i.e., model organism). The rationale for utilizing mycobacteriophages in this study is that they are widely applicable, cheap to propagate, and have a rapid replication rate.

To assess drug susceptibilities of *M. smegmatis* mc^2 155, eight drugs typically used to treat mycobacterial infections were chosen, four drugs, in which *M. smegmatis* mc^2 155 has been shown to be resistant and four drugs, in which *M. smegmatis* mc^2 155 has exhibited sensitivity. Before conducting the assay, optimization steps had to be performed. The length of time the drug, bacteria, and mycobacteriophage were in contact was an important optimization step, as the less time required for contact implied that the drug-susceptibility results could be obtained rapidly, which was a main focus of the study.

The following completion of the optimization steps and the assay, a blinded study was conducted, to authenticate that the results generated were not affected by bias. The blinded study was another crucial part of the assay to provide proof of concept that the assay can work efficiently. The blinded study had the ability to validate that the utilization of mycobacteriophage has the potential to identify drug-susceptible isolates within a shorter period compared to the BACTEC and MGIT methods. In comparison to the cost of the BACTEC 460TB method (One US dollar and three dimes) and MGIT method (One US dollar and 2 dimes), drug susceptibilities can be detected within 1½ days (albeit for a rapid mycobacterial host) with an average cost of approximately one euro and fifteen cent.[16,17]

Although initially promising, there is uncertainty about the applicability of this assay to other mycobacterial species. Incubation periods for slow-growing mycobacterial species are approximately 4–16 weeks, in comparison to a fast-growing mycobacterial host (in this case *M. smegmatis* mc^2 155) which requires an incubation period of 24–48 h. Future work will focus on developing this assay to generate rapid results with slow-growing pathogenic Mycobacterial strains.

As the assay has demonstrated the ability of one mycobacteriophage strain to identify drug-susceptible/resistant isolates against *M. smegmatis* mc^2 155 it may be possible to utilize this assay as an HTS tool for novel anti-Mycobacterial drugs based plaque numbers. The assay would also increase the speed in which novel drugs or compounds are detected. As antibiotic resistance is becoming a major global threat, the development of novel drug compounds is of major importance.

Conclusion

Rapidly, emerging resistant bacteria place substantial health and economic burden globally. Till date, there has been a lack of research in the development of a novel method to identify drug susceptibilities for mycobacteria. Established drug-susceptibility methods such as the MIC assay, Kirby Baeur method are currently in use but are extremely time-consuming (8–15 days). To reduce the time involved, it is quite evident that progress is required in this area of research.

The utilization of mycobacteriophage as a novel indicator of drug susceptibilities has been explored in this study. The addition of mycobacteriophage to the study has a significant reduction in the time required to obtain drug-susceptibility results for *M. smegmatis* mc^2 155. As mycobacteria are a slow-growing bacterial species, this assay may, therefore, have the potential to be applied to other slow-growing bacterial species. The cost of performing the assay is relatively cheap allowing more samples to be analyzed in one test. This assay, therefore, has multiple applications such as the potential identification of slow-growing bacteria drug-susceptible isolates, its use in HTS to identify novel compounds and drugs and the speed and cost involved in identifying drug-susceptible isolates.

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Conflicts of interest

There are no conflicts of interest.

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