Simple, phage-based (FASTPlaque) technology to determine rifampicin resistance of Mycobacterium tuberculosis directly from sputum

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**SUMMARY**

**SETTING:** Cape Town, South Africa.

**OBJECTIVE:** To evaluate the performance of a simple, manual, phage-based test for determining rifampicin (RMP) resistance of Mycobacterium tuberculosis directly from smear-positive sputum specimens.

**DESIGN:** A comparative study of the performance of the FASTPlaque (phage amplification) technology to determine RMP resistance directly from smear-positive sputum compared with isolation and the conventional indirect Middlebrook 7H11 agar proportion method.

**RESULTS:** The FASTPlaque direct RMP test achieved sensitivity, specificity and overall accuracy of 100% (11/11), 100% (134/134) and 100% (145/145), respectively, compared with the conventional indirect susceptibility test method (resolved data). The FASTPlaque direct RMP test reported results within 2 days from receipt of the specimen, while the conventional method took between 27 and 103 days (mean ± SD 33.2 ± 7.2 days).

**CONCLUSION:** FASTPlaque technology applied directly to smear-positive sputum offers performance comparable to conventional methods, with results available in 2 days instead of weeks to months. The test may form a useful part of DOTS-Plus programmes to combat multidrug-resistant tuberculosis, improving patient prognosis and reducing ongoing transmission of disease. It does not require specialised equipment, making it appropriate for high-burden countries.

**KEY WORDS:** FASTPlaque; phage amplification; multidrug-resistant tuberculosis; rifampicin resistance; susceptibility testing

**There is an urgent need for interventions to combat multidrug-resistant tuberculosis (MDR-TB).**1,2 ‘Hot spots,’ where more than 5% of TB patients have MDR-TB, have been identified in Latvia, India (Delhi state), Estonia, China (Henan Province), Dominican Republic, Argentina, Russia (Ivanovo Oblast) and Ivory Coast.3 Other countries, including Peru, Brazil and Zimbabwe, have also been reported to have a substantial disease burden.6

Rifampicin (RMP) resistance affects the ability to provide effective short-course treatment for TB.5 Effective treatment of patients with resistance to isoniazid (INH), streptomycin or other agents is possible if the strain is susceptible to RMP. However, RMP resistance substantially reduces the likelihood of successful treatment.2,5,7 Unsuccessfully treated patients may remain infectious for extended periods, giving the opportunity for further transmission of disease.8,9

The diagnosis of MDR-TB is based on in vitro drug susceptibility testing (DST). Conventional culture-based methods are still in routine use in most countries.10 Susceptibility testing is typically performed on cultures isolated from clinical specimens. Such methods take 3–4 weeks to produce results and, combined with the time required for primary isolation, may take several months for results. Automated culture methods have reduced this time to several weeks. Timely detection of drug resistance has been identified as an important aspect in the effective management of TB patients.6,11

Phage amplification technology12,13 has been applied to both the diagnosis of TB14–18 and DST from TB cultures19–22 in two commercially available kits, FASTPlaqueTB™ and FASTPlaqueTB™-MDRi, respectively (Biotec Laboratories Ltd, Ipswich, UK). This technology utilises specific mycobacteriophage to detect viable Mycobacterium tuberculosis complex.

The present study evaluates the performance of the FASTPlaque technology to detect RMP resistance directly from smear-positive sputum compared with culture and indirect conventional DST.

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MATERIALS AND METHODS

Sputum specimens

Sputum specimens submitted for routine smear, culture and DST to the National Health Laboratory Service (NHLS), Greenpoint, Cape Town, were used in the study.

Primary isolation of M. tuberculosis complex was performed using a modified N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method23 (5% NaOH solution), and BACTEC MGIT 960 automated liquid culture (Becton Dickinson, Sparks, MD, USA). Following centrifugation, the pellet was suspended in approximately 1 ml of phosphate buffer pH 6.8. Five hundred microlitres of the suspension were inoculated into the MGIT vial and a smear prepared with approximately 0.1 ml suspension and stained using auramine-O stain. Slides were examined under ×450 magnification using a fluorescent microscope, and the results were recorded according to International Union Against Tuberculosis and Lung Disease (IUATLD) guidelines.24 A randomly selected portion of specimens submitted between 31 March and 30 April 2003 were included. Following reporting of smear results (on the same day as sputum processing), all smear-positive specimens (1+ or greater) were collected. The patient category (new case, retreatment or unknown) was recorded.

Cultures were incubated according to manufacturer’s instructions for up to 6 weeks. Positive cultures were confirmed as M. tuberculosis complex by Ziehl-Neelsen staining and p-nitrobenzoic acid (PNB) testing.25 Conventional DST was performed using a modified proportion method on Middlebrook 7H11 medium containing 1.0 \( \mu g/ml \) RMP (Sigma Chemical Company, St Louis, MO, USA). MGIT cultures took 12.2 ± 7.2 days (mean ± standard deviation [SD]) to become positive, with times ranging from 6 to 82 days, and conventional DST took 21 days; the total turnaround time for the RIF+ plate was set following preliminary data analysis (not shown).

FASTPlaque direct rifampicin test

FASTPlaque reagents were provided by Biotec Laboratories Ltd. Assay controls were performed according to the manufacturer’s instructions.26

Following inoculation of the MGIT culture and smear preparation, the remainder of the specimen was used to perform the FASTPlaque direct RMP test (approximately 0.5 ml). FPTB Medium Plus (Middlebrook 7H9-based medium) was added to the specimen up to the 15 ml graduation mark on the centrifuge tube. The suspension was mixed, then centrifuged at 3000 g for 20 min. The supernatant was poured off and the pellet suspended in 1 ml FPTB Medium Plus.

A solution of 20.4 \( \mu g/ml \) RMP (Sigma Chemical Company, St Louis, MO, USA) was prepared in FPTB Medium Plus: 0.5 ml of RMP solution was dispensed into a reaction vessel (RIF+); 0.5 ml of FPTB Medium Plus was dispensed into another reaction vessel (RIF−); 0.5 ml of the test suspension was added to both the RIF− and RIF+ reaction vessels (final RMP concentration 10.2 \( \mu g/ml \)). The vessels were gently shaken and incubated for 20–24 h at 37°C. The samples were assayed according to the FASTPlaqueTB procedure.14

Results were considered valid if \( \geq 100 \) plaques were observed on the RIF− plate. A strain was determined to be susceptible to RMP if <50 plaques resulted from the RMP-containing sample (RIF+), and RMP-resistant if \( \geq 50 \) plaques were obtained. The cut-off for the RIF+ plate was set following preliminary data analysis (not shown).

Discrepant results

Identification of mutations in the 81-base pair (bp) region of the \( rpoB \) gene27 and repeat indirect conventional DST were performed on a specimen in which the FASTPlaque and conventional result were discrepant. FASTPlaqueTB™-MDR™ test (indirect RMP susceptibility test) was performed on cultures of all strains in which less than 100 plaques were obtained on the FASTPlaque direct RMP test to determine whether the bacteriophage reagent was able to infect those particular strains of TB.

RESULTS

A total of 195 specimens from 173 patients (115 male and 58 female) were included in the study. Sixteen patients (17 specimens) were new cases, 103 (116 specimens) were retreatment cases and the patient category was unknown in 54 (62 specimens). One hundred and ninety-two specimens were sputum and three specimens were gastric washings (all 1+ smear-positive). Respectively 36, 58 and 101 specimens were 1+, 2+ and 3+ smear-positive.

The FASTPlaque direct RMP test and conventional indirect DST results are shown in Table 1. Overall, 171 specimens (87.7%) were culture-positive for M. tuberculosis complex. MGIT cultures took 12.2 ± 7.2 days (mean ± standard deviation [SD]) to become positive, with times ranging from 6 to 82 days, and conventional DST took 21 days; the total turnaround time therefore ranged from 27 to 103 days, with a mean ± SD of 33.2 ± 7.2 days. FASTPlaque results were available in 2 days from receipt of the specimen.

Fourteen strains (9.0%) were RMP-resistant and 155 (91.0%) were RMP-susceptible according to the conventional method. All RMP-resistant strains were also resistant to INH (MDR). In addition, six strains were INH-resistant but RMP-susceptible. Of the RMP-resistant strains, four were from retreatment patients, two from new cases and eight from patients of unknown category.

Specimens for which both the FASTPlaque direct RMP result and the conventional result were available (n = 145) were used to determine performance parameters. Results of the FASTPlaque direct RMP test agreed with the conventional result in 99.3% (144/145) of the specimens. All strains resistant by
the proportion method (10/10) were correctly identified by the \textit{FASTPlaque} direct RMP test. The \textit{FASTPlaque} test also agreed with the conventional susceptible result in 99.3\% (134/135) of the strains. The sensitivity (ability to detect resistance), specificity (ability to detect susceptibility) and overall accuracy of the \textit{FASTPlaque} direct RMP test compared with the indirect susceptibility test were 100\% (10/10), 99.3\% (134/135) and 99.3\% (144/145), respectively.

One discrepant result was obtained in which the \textit{FASTPlaque} test identified a strain as being resistant, whereas the indirect susceptibility test initially determined it as susceptible. \textit{RpoB} mutation analysis determined the strain to be wild type (i.e., no mutation in the 81-bp region of the \textit{rpoB} gene). The indirect susceptibility test was repeated and the strain was found to be resistant to 1 $\mu$g/ml RMP. Resolved performance parameters of the \textit{FASTPlaque} direct RMP test were therefore 100\% (11/11), 100\% (134/134) and 100\% (145/145) for sensitivity, specificity and overall accuracy, respectively. Calculation of the kappa statistic showed almost perfect agreement ($\kappa = 0.95$) of the two tests.28

A similar proportion of specimens gave interpretable results with each method: 82.6\% (161/195) of the results were interpretable with the \textit{FASTPlaque} direct RMP test, compared to 86.7\% (169/195) for the conventional proportion method. Multiple specimens from the same patient gave the same results for both tests in all cases (data not shown). The 20 culture-positive specimens that had <100 plaques on the RIF2 plate took longer than average to become positive on culture (mean ± SD 6 ± 6.1 days), suggesting poor viability. The 13 available cultures from these specimens tested by the \textit{FASTPlaque} TB™-MDR test26 were all successfully infected and resulted in >100 plaques on the RIF2 plate.

Table 2 shows the \textit{FASTPlaque} direct RMP results

\begin{table}[h]
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\begin{tabular}{|l|l|l|l|l|}
\hline
\textbf{MGIT + indirect 7H11 proportion method} & & & & \\
\hline
\textbf{FASTPlaque} & Resistant & Susceptible & MGIT culture negative & Contaminated* & Total \\
\hline
Resistant & 10 & 1 & 0 & 1 & 12 \\
Susceptible & 0 & 134 & 1 & 14 & 149 \\
RIF – <100 plaques† & 4 & 16 & 5 & 2 & 27 \\
Contaminated & 0 & 4 & 0 & 3 & 7 \\
Total & 14 & 155 & 6 & 20 & 195 \\
\hline
\end{tabular}
\end{table}

* Contaminated on either MGIT culture or 7H11 susceptibility test.
† This specimen was found to be rifampicin-resistant upon repeat testing by the proportion method.
‡ Less than 100 plaques obtained on the RIF2 plate.
MGIT = Mycobacteria Growth Indicator Tube; RIF = rifampicin-free sample.

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\hline
\textbf{MGIT + indirect 7H11 proportion method} & & & & \\
\hline
\textbf{FASTPlaque} & Resistant & Susceptible & MGIT culture negative & Contaminated* & Total \\
\hline
3+ smear-positive (n = 101) & & & & \\
Resistant & 5 & 1 & 0 & 1 & 7 \\
Susceptible & 0 & 70 & 0 & 9 & 79 \\
RIF – <100 plaques§ & 2 & 7 & 0 & 0 & 9 \\
Contaminated & 0 & 4 & 0 & 2 & 6 \\
2+ smear-positive (n = 58) & & & & \\
Resistant & 5 & 0 & 0 & 0 & 5 \\
Susceptible & 0 & 41 & 1 & 3 & 45 \\
RIF – <100 plaques§ & 0 & 4 & 1 & 2 & 7 \\
Contaminated & 0 & 0 & 0 & 1 & 1 \\
1+ smear-positive (n = 36) & & & & \\
Resistant & 0 & 0 & 0 & 0 & 0 \\
Susceptible & 0 & 23 & 0 & 2 & 25 \\
RIF – <100 plaques§ & 2 & 5 & 4 & 0 & 11 \\
Contaminated & 0 & 0 & 0 & 0 & 0 \\
\hline
\end{tabular}
\end{table}

* Contaminated on either MGIT culture or 7H11 susceptibility test.
† This specimen was found to be rifampicin-resistant upon repeat testing by the proportion method.
§ Less than 100 plaques obtained on the RIF2 plate.
MGIT = Mycobacteria Growth Indicator Tube; RIF = rifampicin-free sample.
compared with the conventional method, sub-divided according to smear grading (1+, 2+ or 3+). Of the specimens that were culture-positive and had a conventional susceptibility test result, 85.8% (143/169) specimens gave interpretable result, by the FAST-Plaque direct RMP test; 76.7% (23/30) of 1+ smear-positive specimens, 92.0% (46/50) of 2+ smear-positives and 85.4% (76/89) of 3+ smear-positive specimens gave interpretable results. In addition, the FASTPlaque direct RMP results of the two culture-positive gastric wash specimens (1+ smear-positive) agreed with the conventional method (RMP susceptible). The third gastric wash specimen was culture-negative, and gave <100 plaques on the RIF-plate.

DISCUSSION

DST is a critical component of programmes aimed at combating MDR-TB. While effective implementation of the DOTS strategy will prevent the emergence of resistance in areas with a low level of drug resistance, DOTS alone will not be effective in areas in which there is already a significant level of MDR-TB. DOTS-Plus pilot programmes have been introduced in several locations, aimed at incorporation of treatment with second-line regimens for patients who are unsuccessfully treated with standard short-course chemotherapy.

Conventional DST methods for M. tuberculosis have been criticised for their slow turnaround time. MDR-TB patients who remain undetected and untreated with appropriate therapy will have a prolonged infectious period, leading to further disease transmission.

In this study, the FASTPlaque direct RMP test was compared with conventional culture and indirect DST using the ‘gold standard’ proportion method recommended by the World Health Organization (WHO) and the IUATLD. The FASTPlaque test showed excellent correlation with the indirect proportion method. A single specimen gave a discrepant result, with the FASTPlaque direct RMP test detecting resistance while the proportion method reported the strain as susceptible. However, repeat testing of the strain by the proportion method determined the strain to be resistant. No mutation was detected in the 81-bp region of the rpoB gene. However, although 95–98% of RMP-resistant clinical strains have a mutation in that region, mutations have also been found outside this region in RMP-resistant strains. As low numbers of RMP-resistant isolates were included in this study, further studies are required to confirm these findings.

The proportion of interpretable results seen with each method was similar and compared favourably with published results using other DST methods. Three gastric wash specimens also gave results in agreement with the conventional method, suggesting that the test may be suitable for other clinical specimens. Further testing is required to confirm this.

Of the 27 specimens that had fewer than 100 plaques on the RIF-plate, 20 specimens were culture-positive, suggesting that successful phage attachment or replication had not occurred in these cases. This is likely to be due to the poor metabolic activity of the TB and reduced viability and/or phage receptor expression due to damage caused by TB therapy, and the decontamination process. It was shown that Actiphae was able to infect cultures of these strains, confirming that phage host range was unlikely to be a factor. This study used specimens that had been decontaminated with a slightly higher NaOH concentration than is recommended for use with the FASTPlaque technology (2.5% compared to 2%). It is known that higher NaOH concentrations have a detrimental effect on sensitivity of the assay (unpublished data). A reduction in the NaOH concentration may therefore lead to an increase in the proportion of interpretable results.

In this study, 100% (14/14) of the RMP-resistant strains were multidrug-resistant. This is in agreement with other data showing that RMP resistance is often a good marker for multidrug resistance. In accordance with National TB Programme guidelines, most specimens submitted for culture and DST in this study were from retreatment cases, who are at increased risk of drug resistance.

Results of the FASTPlaque direct RMP test were available in 2 days from receipt of the specimen, a reduction of at least 1 month, and often substantially longer, compared with conventional indirect DST. The conventional indirect susceptibility test took between 27 and 103 days to report a result, even though an automated culture method was used for the primary isolation. The total turnaround time would be substantially longer if conventional solid culture were used for both primary isolation and DST, as is the case in many countries.

The FASTPlaque direct RMP test is being developed into a commercial kit, named FASTPlaque (Biotec Laboratories Ltd). No specialised equipment is required to perform the test, an important consideration in high-burden countries where acquisition and maintenance of capital equipment can be a significant problem. It has been reported that a substantial number of countries, including all countries identified by the WHO as MDR-TB ‘hot spots’, have sufficient infrastructure and laboratory experience to be able to perform conventional DST. Labour time to perform the FASTPlaque test is relatively short (approximately 2½ hours for 10–20 specimens, excluding the decontamination process) and requires only simple pipetting steps using standardised reagents. Only basic additional consumable items, such as Petri dishes and pipettes, are needed. Results are read by
eye and are simple to interpret. The commercial test kit must be reasonably priced to enable its widespread implementation in high-burden countries.

Use of a simple, rapid test such as the FASTPlaque direct RMP test could play an important role in DOTS-Plus programmes enabling the rapid detection of MDR-TB cases, allowing use of appropriate therapy, improving patient prognosis and reducing the period of infectiousness of these patients. Patients who are RMP-susceptible can be successfully treated using standard first-line regimens, while RMP-resistant cases can be treated rapidly with an appropriate standardised second-line regimen. Further susceptibility testing against second-line drugs may then be carried out to individualise therapy, if available. This could contribute to reducing the ongoing transmission of MDR-TB strains in the community and ultimately the overall burden of disease.

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Rapid rifampicin resistance test


Résumé

Contexte : Cape Town, Afrique du Sud.
Objectif : Évaluer les performances d’un test simple, manuel et basé sur les phages pour déterminer la résistance de Mycobacterium tuberculosis à la rifampicine (RMP), directement à partir d’échantillons d’expectoration positifs à l’examen direct.
Schéma : Étude comparative des performances de la technologie FASTPlaque (amplification par phage) pour déterminer la résistance à la RMP directement à partir d’expectorations à bacilloscopie positive par comparaison avec l’isolement et la méthode indirecte conventionnelle des proportions Middlebrook 7H11 sur agar.
Résultats : Le test direct FASTPlaque pour la rifampicine obtient une sensibilité, une spécificité et une précision globale respectivement de 100% (11/11), de 100% (134/134) et de 100% (145/145) par comparaison avec la méthode conventionnelle indirecte du test de sensibilité (données résolues). Le test direct FASTPlaque pour la rifampicine obtient les résultats en 2 jours après réception des échantillons, alors que la méthode conventionnelle prend au total entre 27 et 103 jours (moyenne ± DS de 33,2 ± 7,2 jours).
Conclusion : La technologie FASTPlaque appliquée directement aux expectorations à bacilloscopie positive offre des performances comparables à celles des méthodes conventionnelles, mais ses résultats sont disponibles en 2 jours au lieu de quelques semaines à quelques mois. Le test peut constituer un élément utile d’un programme DOTS-Plus pour combattre la tuberculose multirésistante en améliorant le pronostic des patients et en réduisant la poursuite de la transmission de la maladie. Il n’exige pas d’équipement spécialisé, ce qui le rend approprié dans les pays à lourd fardeau de tuberculose.

Resumen

Contexto : Ciudad del Cabo, Sudáfrica.
Objetivo : Evaluar el rendimiento de un test simple, manual, basado en fagos, para la determinación de la resistencia de Mycobacterium tuberculosis a la rifampicina (RMP), directamente, a partir de las muestras de esputo con baciloscopia positiva.
Diseño : Estudio comparativo del rendimiento de la tecnología FASTPlaque (amplificación de fago) para determinar la resistencia a la RMP directamente a partir de la expectoración con baciloscopia positiva, comparada con el aislamiento y el método indirecto convencional Middlebrook 7H11 de proporciones en agar.
Resultados : El test FASTPlaque directo para la RMP mostró una sensibilidad, especificidad y precisión global de 100% (11/11), 100% (134/134) y 100% (145/145), respectivamente, comparado con el método indirecto convencional de sensibilidad (datos resueltos). El test directo FASTPlaque para la rifampicina entregó resultados en 2 días a partir de la recepción de la muestra, mientras que el método convencional demoró entre 27 y 103 días (promedio ± DS de 33,2 ± 7,2 días).
Conclusión : La tecnología FASTPlaque aplicada directamente a la expectoración con baciloscopia positiva ofrece un rendimiento comparable al de los métodos convencionales, con resultados disponibles en 2 días en vez de semanas o meses. El test puede ser un instrumento útil para los programas DOTS-Plus para combatir la tuberculosis multirresistente, mejorando el pronóstico del paciente y disminuyendo la posibilidad que la enfermedad siga transmitiéndose. No requiere equipamiento especializado, lo que es apropiado para los países de alta prevalencia.