

EVALUATION OF DOT-BLOT AND PHAGE REPLICATION TECHNIQUES FOR DETECTION OF DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS*

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Summary

We have assessed the utility of two new methods, dot-blot and bacteriophage replication techniques, for use in a routine diagnosis laboratory in poor resource settings in the screening of drug resistant *Mycobacteria tuberculosis* by comparing with the conventional proportion method. A total of 145 *M. tuberculosis* clinical isolates were tested for resistance to rifampicin, isoniazid, streptomycin and ethambutol. The dot blot had sensitivities of 91.7%, 100%, 93.5% and 85.7 and specificities of 99.2%, 99.2%, 99.1% and 99.2 for rifampicin, streptomycin, isoniazid and ethambutol, respectively. The phage technique had sensitivities of 92% and 84.6% and specificities of 99.2% and 99.2% for rifampicin and streptomycin, respectively. Both techniques yielded results within 48 hours of receipt of the culture on solid media.

The high sensitivity and specificity coupled with rapidity of results indicate that these methods are potentially useful tools for screening resistance to anti-tuberculosis drugs in our setting. However, the phage replication technique, which is simpler and technically less demanding, seems the most suitable for routine screening of drug resistant mycobacteria in resource deprived countries such as Tanzania. We are recommending further field evaluation of the phage replication method so that it can complement, and possibly replace, the conventional proportion method in drug susceptibility testing.

Key words: *Mycobacterium tuberculosis*, drug susceptibility testing, dot blot, phage replication assays.

Introduction

In areas where both tuberculosis (TB) and Human Immunodeficiency Virus (HIV) infections are common, HIV has exerted a dramatic impact on the course of TB.⁽¹⁻²⁾ The risk of developing active TB is higher in HIV infected individuals than in persons with no known risk factors⁽³⁾. Ten percent, compared with lifetime risk of 5 to 10% for immune competent individuals.⁽⁶⁾ Study reports demonstrate HIV infection as a major factor for reactivation of a latent TB infection and rapid progression of a recent infection to manifest disease.⁽³⁾ The World Health Organization (WHO) global TB control report for 2003 indicated that global incidence rate of TB had decreased to 0.4% per cent per year. However, the report found that TB epidemic is still growing unabated in sub-Saharan Africa and is linked to HIV/AIDS rates and poverty.⁽¹⁾ In Tanzania, the National TB and Leprosy Programme (NTLP) has consistently reported a rise of TB notification rates since 1979.⁽⁴⁾ Coupled with the global rise in the incidence and prevalence of TB is the emergence of drug resistant tuberculosis that threatens the control of the disease. One of the aims of ensuring effective management of tuberculosis is to minimize the development of drug resistance. Surveillance of anti-TB drug resistance is therefore an essential tool for monitoring the effectiveness of TB control programmes and improving national and global control efforts.

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The conventional drug susceptibility testing phenotypic methods requires visible growth (which requires three weeks of incubation) and is associated with significant delays.⁽⁵⁾ In light of the worsening global TB epidemic and the extreme vulnerability of HIV-infected individuals to TB, rapid and reliable antimicrobial susceptibility testing in the laboratory is paramount for proper management of patients, particularly those with multi-drug resistant tuberculosis (MDR-TB).⁽⁶⁾ To facilitate rapid therapeutic decisions for patients, several relatively rapid, growth based, and molecular biological methods are available for antimicrobial susceptibility testing.⁽⁵⁾ Use of liquid media, radiometric and non radiometric methods, yield susceptibility results within five to ten days. However, other techniques such as molecular biology studies targeting mutations on specific genes and phage replication technique can be used for screening drug resistance⁽³⁾ and produce results in a shorter period. Despite this enormous advantage these techniques need further evaluation and improvements before they can be used in clinical diagnostic laboratories.

In Tanzania, anti-tuberculosis drug susceptibility testing relies on the use of the conventional proportion method. In this study we evaluated relatively rapid dot-blot and phage replication techniques as potential tools for screening anti-TB resistance among mycobacteria isolated from TB patients in Tanzania.

Materials and methods

Clinical isolates and reference strains

Isolates of *M. tuberculosis* were obtained from clinical specimens cultured in the National TB Reference Laboratory situated at the Muhimbili National Hospital in Dar es Salaam. They were identified by growth characteristics and conventional biochemical methodology⁽⁷⁾ and for the majority of isolates, identities were also confirmed by DNA probe (AccuProbe; GenProbe, Inc., San Diego, Calif.). The reference strain H37Rv and a well-characterized *Mycobacterium bovis* were obtained from the Medical Research Council (MRC) Pretoria, South Africa. The *Mycobacterium smegmatis* was obtained from the London School of Hygiene and Tropical Medicine, London, UK. The Dot blot hybridization and phage replication assays were performed in the Molecular Biology Unit, Department of Biochemistry of the Muhimbili University College of Health Sciences (MUCHS).

Choice of drugs

Four of the six anti-TB drugs used in first-line treatment; isoniazid, rifampicin, streptomycin and ethambutol were used in this study. These drugs were chosen because they have been, and continue to be, widely used throughout the world, and resistance can reliably be measured by standardized techniques and have been studied for many years.

Proportion method

Susceptibility testing of the clinical isolates to isoniazid, ethambutol, streptomycin, and rifampicin was performed by the proportion method using the Lowenstein Jensen medium⁸. Resistance was expressed as the percentage of colonies that grew on critical concentrations of the anti-TB drugs, i.e. 0.2 mg/l for isoniazid, 2 mg/ml for ethambutol, 4 mg/l for dihydrostreptomycin sulphate, and 40 mg/l for rifampicin. The interpretation was based on the usual criteria for resistance, i.e. 1% for all drugs. Susceptibility test results of the proportion method were used as the "gold standard" to evaluate intrinsic characteristics, such as the sensitivity and specificity of both dot blot and phage replication drug resistance testing.

Dot-Blot hybridization

Genotypic drug resistance testing was performed by mutation analysis according to a recently described PCR-based dot blot method.⁽⁹⁾ Specially designed primers (for regions in genes known to confer resistance in *M. tuberculosis*) were used to amplify genomic DNA extracted from clinical isolates of *M. tuberculosis*. Efficient PCR amplification was confirmed by gel electrophoresis. An aliquot of each PCR product was denatured and fixed on a Hybond-N⁺ membrane by use of a dot blot apparatus (Bio-Rad). Discrimination between wild-type and mutant sequences was obtained under stringent hybridization conditions with labeled wild-type and mutant probes, respectively. All samples were tested for mutations at the following codons: *katG315*, *kasA269*, *inhA10* (putative promoter), *inhA34* (putative promoter), *rpoB531*, *rpoB526*, *rpoB516*, *rpsL 43*, *rpsL88*, *rrs-513*, *rrs-491*, and *embB306*. When resistance could not be explained by the identification of mutations in the above gene codons, samples were also tested for mutations in additional codons (*katG 275*, *katG409*, *kasA66*, *kasA312*, *kasA413*, *inhA15* (putative promoter), *rpoB533*, *rpoB513*, and *rrs-904*).

When discrepancy between the results of the phenotypic and genotypic drug resistance tests was encountered the isolate was retested by both methods.

Phage replication assay

Preparation of isolates and exposure to drug

The micro-well susceptibility assay was performed in sterile flat bottom 96 well microtitre plates with lids (Greiner Labortechnik, Stonehouse, UK).¹⁽¹⁰⁾ Drugs were prepared at double test concentration and 75- μ l aliquots placed in the wells. A 1- μ l plastic loopful of bacteria was

transferred from growth on Lowenstein-Jensen slopes to a 7ml plastic screw-cap bijoux bottle containing 4-10 sterile glass beads (1 to 4 mm in diameter) in 2 ml of 7H9 broth (Becton Dickinson, Oxford, United Kingdom) with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco Laboratories, Detroit, Mich.) and 1-mM CaCl₂. Organisms were vortexed for 20 seconds on the maximum speed setting to disperse the bacteria and left to stand for approximately three minutes to allow the aerosols to settle. Aliquots of 75 μ l of bacteria were placed in each well of the microtitre plate containing the appropriate concentration of drug. Control samples consisting of the same suspension without antibiotic were included. The plate was covered and sealed in a plastic bag before incubating at 37°C for 24 hours.

Preparation of phage D29 suspension

A plate lysate of D29 was prepared by the standard plate lysate method. The titers of the phage stock were determined by pipetting 10- μ l aliquots of 10-fold dilutions onto a lawn of *M. smegmatis*. Phage stock, containing approximately 10⁹ PFU per ml, was stored at 4°C in 7H9-glycerol-CaCl₂ and 10% (vol/vol) OADC with 0.05% (wt/vol) sodium azide. Prior to the phage assay, the phage suspension was diluted 100-fold in 7H9-glycerol-CaCl₂ and 10% (vol/vol) OADC¹⁰.

Phage assay

The Phage assay was performed as described.⁽¹⁰⁾ After samples had been incubated at 37°C, with or without antibiotic, 50 μ l of phage D29 suspension was added to each well. The test and control tubes were incubated for 2 h at 37°C, which corresponds to the absorption and uptake time for D29 with *M. tuberculosis*. Extracellular phage was neutralized by adding 100 μ l of 4% (wt/vol) ferrous ammonium sulfate (FAS) (Sigma-Aldrich, Ltd., St. Louis, Mo.) to each sample. Each sample was mixed by pipetting, prior to spotting a 10 μ l drop onto the surface of a *M. smegmatis* indicator plate. Once the drops had been absorbed the plates were sealed in plastic bags and placed in the incubator. Indicator plates had been prepared previously and stored at 4°C until required. Molten 1.5% bacto agar (Difco Laboratories, Detroit, USA) was prepared in LB broth and allowed to cool to approximately 45°C before the addition of 10-15% vol/vol of *M. smegmatis* stock. Following mixing by inversion the indicator agar was poured into 90 mm triple vented plastic petri dishes. (Greiner Labortechnik, Stonehouse, UK).

After overnight incubation the degree of lysis observed on the indicator plate was recorded. Strains that produced lysis at concentrations of drug that inhibited plaque formation in wild type or reference strain were classed as resistant. When the *M. smegmatis* growth was insufficiently dense to allow the visualization of the plaques, the incubation period was extended to 24 h.

Antibiotic solutions

Antibiotic stock solutions were made up as follows ¹⁰. Isoniazid (Sigma-Aldrich, Poole, United Kingdom), streptomycin (Sigma-Aldrich), rifampicin (Sigma-Aldrich) and ethambutol (Sigma-Aldrich) were all made up as 1-mg/ml stock solutions in sterile distilled water and stored at 4°C.

Interpretation of the phage assay

The assay is simple and is based on specific mycobacteriophages, which reflect presence of viable TB bacilli in drug containing sample compared with that in drug free control. On addition of these phages rapidly infect target bacteria and on treatment with virucidal solution, all unadsorbed phages are killed. The only phages that remain are those, which are protected within viable TB bacilli. These phages will replicate and lyse the cells to release new progeny phages that then undergo cycles of infection, replication and lysis within the *M. smegmatis* of the indicator plate forming clear areas (plaques) in the lawn of rapid growing mycobacteria.

Results

A total of 145 *M. tuberculosis* clinical isolates were tested for resistance to rifampicin, isoniazid, streptomycin and ethambutol using the dot blot technique. The phage replication assay was used for rifampicin and streptomycin only. For the dot blot technique mutations in genes conferring resistance to RIF were detected in 11 isolates (91.7%) of the 12 clinical samples identified resistant to rifampicin. One sample (8%) had mutation detected but was characterised as sensitive to RIF by the proportion method. The most frequent mutations were at codon 531 of the *rpoB* gene where 4 samples (33%) were identified (Fig 1). Other mutations were detected in codon 526 and codon 516 in (33%) samples and 1 sample (8%) respectively. One sample (8%) classified resistant to RIF had no detectable mutation. Of the 133 samples identified as susceptible to RIF only 1 sample (0.8%) had mutation in codon 516 of the *rpoB* gene. Similar results were obtained for other genes including *katG*, *inhA*, *kasA* gene in which mutations were associated with resistance to INH (Fig 2). Mutations were detected in 29 samples (93%) of the 31 clinical samples that were identified to be resistant to INH. Two samples had no mutations but were classified to be phenotypically resistant to INH. Mutation in *inhA* gene was identified in one sample. No mutation was detected in *kasA* gene. One sample characterized sensitive to INH had mutation identified in *katG* gene.

1 2 3 4 5 6 7 8

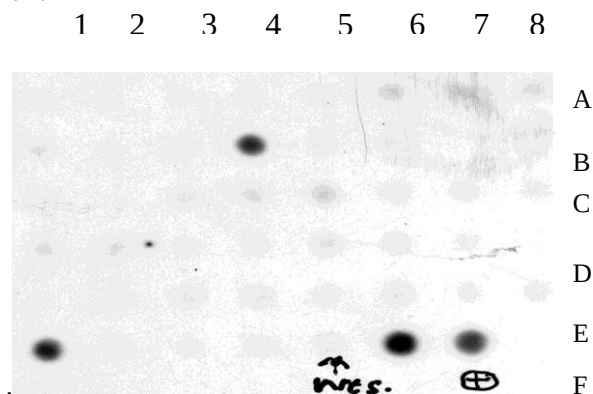


Figure 1. Mutation detected in codon 531 of the *rpoB* gene using *rpoB* 531mutant probe.

Mutations detected in *rpoB* gene codon 531 using γ 32pATP radioactivelylabelled probe *rpoB* 531 mutant. Final wash was done at 68°C for 10 minutes in 0.75XSSPE+0.1%SDS. Of the 55 isolates, mutant samples are lane 4B, 1F and 6F.

Lane 5F is a wild type H37Rv and 7F is a mutant control. Mutations detected in *katG* gene codon 315. Radioactive γ 32p ATP labelled probe *kaG315wt* was used. Final wash was at 70°C for 10 minutes in 1 x SSPE + 0.1%SDS. Of the 26 isolates, mutant samples are 2A, 6A, 7A and 8B. Lane 1C is a mutant control and 2D is a susceptible H37Rv.

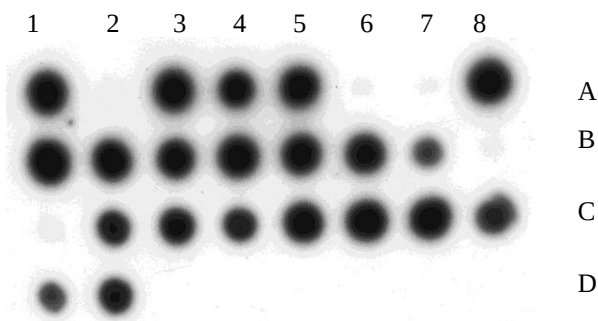


Figure 2. Dot-blot hybridization of codon 315 of the *katG* gene

Table 1. Specificity, sensitivity and positive and negative predictive values of the dot-blot and phage replication techniques

Drugs	Dot-Blot				Phage replication			
	IN	RIF	EM	ST	IN	RIF	EM	ST
Sensitivity	93.	91.	85.7	100	100	91.	ND	84.6
Specificity	99.	99.	99.2	99.2	ND	99.	ND	99.2
PPV	96.	91	86.7	90.2	ND	92	ND	92
NPV	98.	99.	99.2	100	ND	99.	ND	98

Key: ND = Not Done, PPV = Positive Predictive Value, NPV = Negative Predictive Value

Mutations in genes conferring resistance to SM were detected in 13 clinical isolates where 12 isolates were

phenotypically identified to be resistant to streptomycin. Twelve samples had mutations detected in codon 43 of the *rpsL* gene and 1 sample had in codon 491 of the *rrs* gene. One isolate was detected with mutation in *rpsL* gene, but was sensitive to SM by the proportion method. Mutations were detected codon 306 of the *embB* in 6 (85%) of the 7 isolates phenotypically resistant to EMB. One sample which was phenotypically resistant to EMB had no detectable mutation. Some of the isolates were found to have mutations in more than one gene. Five isolates were found to have mutations in both the *katG* and the *rpoB* genes and these were MDR with the proportion technique. However, mutations to both INH and RIF were absent in 2 of the 7 isolates phenotypic classified as MDR.

Regarding phage replication results, of the 12 rifampicin resistant isolates, eleven (92%) were characterized by phage replication as resistant by having plaques more at 4µg/ml of rifampicin. One isolate had plaques below the 4µg/ml RIF concentration. Out of the 133 isolates which were classified as being susceptible to rifampicin by the conventional method, one had plaques in more than the concentration of the cut off. For streptomycin, eleven isolates (84%) of the thirteen defined as resistant to streptomycin by the proportion method were also resistant by the phage technique. One isolates had plaques and was characterized as being resistant by phage technique but was sensitive to SM by the proportion method. Two samples characterized by the proportion method as being resistant to SM were sensitive by phage technique.

Discussion

The objective of this study was to evaluate dot blot and phage based methods for detection of drug resistant tuberculosis. Both techniques yielded results for susceptibility testing within 48 hours of receipt of the culture on solid media. The use of these techniques will, thus reduce the time to define susceptibility results from radiometric BACTEC 460 TB and conventional LJ proportion method which take 5-7 days and 6-8 weeks, respectively.

The dot blot method achieved sensitivities of 91.7%, 93.4%, 100%, and 85.7% and specificities of 99.2%, 88.9%, 99%, and 97% respectively for RIF, INH, SM, and ethambutol, respectively. These results are in keeping with a previous study that demonstrated that mutations on three codons (*rpoB*531, *rpoB* 526 and *katG*315) can be identified in up to 90% of MDR-TB cases.

The phage technique had sensitivities of 92% and 84.6% and specificities of 99.2% and 99.2% for rifampicin and streptomycin, respectively. The high specificity and sensitivity of the dot blot and phage replication techniques (table 1), coupled with the rapid availability of the results, suggests a beneficial role for these methods in the early detection of drug resistance mycobacteria.

When the two methods are compared, the phage replication method is cheaper and technically less

demanding and could easily be adopted for quick screening of *M tuberculosis* isolates at the National TB Reference laboratory. The technique will provide the National Tuberculosis and Leprosy Programme with a simple rapid tool for screening of drug resistant strains and provide patient harbouring such strains with prompt alternative effective treatment needed to lower morbidity and mortality and curb the spread of drug resistant mycobacterial strains.

In conclusion, the dot blot and phage replication techniques are good tools for rapid screening of drug resistant mycobacteria. The Phage technique is the simpler of the two techniques and seems to be the more suitable especially for developing countries like Tanzania. We are recommending further field evaluation of the phage replication method so that it can complement, and possibly replace, the conventional proportion method in drug susceptibility testing.

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