Rapid Detection of Rifampicin Susceptibility of Mycobacterium Tuberculosis in Sputum Specimens by Mycobacteriophage Assay

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Abstract

Objective: To evaluate the performance of FASTPlaqueTB-RIF™, a newly introduced bacteriophage assay for rapid detection of rifampicin susceptibility of Mycobacterium tuberculosis in sputum specimens.

Methods: A comparative study of 40 sputum specimens from patients of pulmonary tuberculosis, using FASTPlaqueTB-RIF™ and Bactec 460 TB system carried out at the Armed Forces Institute of Pathology, Rawalpindi between September and November 2001.

Results: Of the 40 clinical isolates of Mycobacterium tuberculosis tested for rifampicin (RIF) susceptibility using the Bactec 460 TB system, 28 isolates were resistant to RIF and 12 isolates were susceptible. FASTPlaqueTB-RIF™ identified 24 specimens as resistant to RIF. Three specimens that revealed susceptible isolates on Bactec 460, were resistant by FASTPlaqueTB-RIF™ while four specimens which revealed resistant isolates on Bactec 460, demonstrated susceptibility to RIF by FASTPlaqueTB-RIF™. The sensitivity and specificity of FASTPlaqueTB-RIF™ were 86% and 73% respectively. The predictive values of positive and negative tests were 0.89 and 0.67 respectively. The overall accuracy of the technique was 82%. The phage assay took 48 hours to perform.

Conclusion: Early detection of rifampicin resistance by the mycobacteriophage technique direct from sputum specimens is a potentially useful new test which would allow decision regarding appropriate therapy to be made early thus having a positive impact on patient care and on prevention of spread of MDR TB (JPMA 54:379;2004).

Introduction

Multidrug-resistant tuberculosis (MDR TB) is a growing worldwide public health problem especially in the developing world.[1] In a survey of 35 countries, 12.6% (range 2.3-42.4%) of Mycobacterium tuberculosis isolates were resistant to at least one anti-tuberculosis drug and 2.2% (range 0 to 22.1%) were resistant to both the primary anti-tuberculosis drugs: isoniazid (INH) and rifampicin (RIF).[2] Early detection of multidrug-resistant (MDR) strains of Mycobacterium tuberculosis is one of the most effective measures for the control of MDR TB.[3-5] However, regions with high prevalence of tuberculosis also lack the resources to institute effective control measures.[5,6]

The conventional methods for testing of anti-tuberculosis drug susceptibility testing as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) are the agar proportion method and the radiometric Bactec 460 TB method.[7] Unfortunately, both the techniques have their limitations; the first requires several weeks of laborious procedures and incubation[8], while the other is expensive and requires disposal of radioactive material. Bactec 460 also lacks a protocol for testing second-line drugs.[9] A cost-effective and rapid drug susceptibility method is required to guide tuberculosis treatment.

FASTPlaqueTB-RIF™ (Biotec Laboratories Ltd., Ipswich, Suffolk UK) is a recently introduced susceptibility test for rapid detection of RIF resistance in Mycobacterium tuberculosis isolates. The test is based upon the principle of phage amplification and utilizes target specific mycobacteriophage as reporter of Mycobacterium tuberculosis presence and viability. The phages infect any tubercle bacilli present in the sample and replicate intracellularly causing the lysis of host cell with release of the progeny phages. These progeny phages are then detected by adding non-pathogenic, rapidly growing Mycobacterium smegmatis also susceptible to the phage, in an agar mixture. Mycobacterium smegmatis rapidly grow in the agar producing a lawn of visible growth. At the same time, they are infected by the phages resulting in their lysis, which appears as visible zones of clearing or "plaques" on the agar plate.

For checking RIF susceptibility, the number of plaques in RIF containing medium is compared to the number of plaques in RIF free medium. Absence of plaques in RIF containing medium indicates susceptibility of the strain to RIF, while presence of plaques indicates resistance.[10,11]

FASTPlaqueTB-RIF™ has been recommended as a test for rapid detection of rifampicin resistance among mycobacterial isolates.[10,11] It however solves only half the problem; while it reduces the time of susceptibility testing, significant time is still required for culture of the mycobacteria. To have a meaningful reduction in detection time, we decided to utilize FASTPlaqueTB-RIF™ to test rifampicin susceptibility of Mycobacterium tuberculosis direct from sputum specimens rather than in isolates obtained after culture, thus reducing the testing time from weeks to days. The

rifampicin susceptibility of Mycobacterium tuberculosis direct from sputum specimens rather than in isolates obtained after culture, thus reducing the testing time from weeks to days. The accuracy of the technique was then evaluated by comparing the results to the susceptibility pattern of isolates from the same specimens by the radiometric Bactec 460 TB system.

Materials and Methods

A comparative study of rifampicin-susceptibility testing by FASTPlaqueTB-RIF™ and Bactec 460 TB system, was carried out at the Armed Forces Institute of Pathology, Rawalpindi between September and November 2001. Sputum specimens were collected from patients of pulmonary tuberculosis referred from various civil and military hospitals in Rawalpindi-Islamabad for culture and antimicrobial susceptibility testing.

Forty patients who were diagnosed as cases of pulmonary tuberculosis by culture were selected for the study. The culture isolates from the specimens of the same patients were also tested for RIF susceptibility on Bactec 460. Patients on anti-tuberculosis treatment were excluded from the study. No discrimination was made on the basis of age or gender.

Specimens were processed by the standard N-acetyl-L-cysteine-sodium hydroxide digestion-decontamination method[12] and concentrated by centrifugation at 3000g for 15 minutes. Part of this sediment was used for smear preparation (Ziehl Neelsen staining) and for culture isolation. The remaining sediment was re-suspended in 15 ml of FASTPlaqueTB (FPTB) Medium Plus™ (Biotec Laboratories). The antimicrobial agent Microclens, 100 ml was added to 270 ml of FPTB Medium Plus™ after autoclaving to prevent contamination. The specimen was centrifuged again at 3000g for 15 minutes. The supernatant was discarded and the pellet was re-suspended in 1ml of FPTB Medium Plus™.

Culture

Primary isolation of Mycobacterium tuberculosis was done by the radiometric Bactec 460 TB mycobacterial broth culture system (Becton Dickinson Instrument Systems, Towson, Md. USA). The isolates were identified as Mycobacterium tuberculosis by Ziehl Neelsen staining, radiometric p-nitro-a-acetylamino-β-hydroxy propiophenone (NAP) test (Becton Dickinson)[13,14] and the niacin test.[15]

RIF susceptibility testing by Bactec 460

RIF susceptibility testing was performed at the concentration of 2 µg/ml on Bactec 460 cultures by single concentration break point method. Bactec growth index (GI) of 20 or more was considered positive and identification of M. tuberculosis complex was performed once the GI of the culture had reached 300 or more. Susceptibility testing by Bactec method was performed at a GI of 500 or more. If the cultures had a GI of more than 800, 1:2 dilution was used for susceptibility testing. The susceptibility was determined when the GI in control vial was 30 or more.[13]

RIF susceptibility testing by FASTPlaqueTB-RIF™

RIF susceptibility testing by FASTPlaqueTB-RIF™ was performed as described previously.[10,11] However, in our study, we used sputum specimens instead of Mycobacterium tuberculosis isolates. The final concentration of 5 µg/ml RIF solution was prepared by dissolving 50 µg RIF tablet in 5 ml of FPTB Medium PlusTM. Two reaction vessels were taken and labeled as "RIF+" and "RIF-". In "RIF+" reaction vessel 0.5 ml of RIF solution and in "RIF-" reaction vessel 0.5 ml of FPTB Medium PlusTM were added. Then 0.5 ml of re-suspended specimen suspension was added in both the reaction vessels. After mixing gently, both were incubated for 24 hours at 37° C. Next morning 100 ml of target-specific reconstituted lyophilized bacteriophages (Biotec Laboratories) was added in both the reaction vessels, followed by incubation at 37° C for 90 minutes to allow the mycobacteriophages to infect the tubercle bacilli. After incubation, 0.1 ml of reconstituted virucide tablet (Biotec Laboratories) was added to destroy any bacteriophages present outside the target cells. The virucide was neutralized by adding 5 ml of FPTB Medium PlusTM. A suspension of 1 ml reconstituted lyophilized non-pathogenic, rapidly growing Mycobacterium smegmatis (Biotec Laboratories), also susceptible to the mycobacteriophage, was added to each reaction vessel. The mixtures were then added to a molten agar mixture (Biotec Laboratories) kept in a water bath at 55° C, poured into two Petri dishes and allowed to settle at room temperature. After solidification at room temperature the plates were incubated at 37° C for 18-24 hours and the number of plaques (zones of clearing in the lawn of M. smegmatis growth) in both "RIF+" and "RIF-" plates were recorded by eye.

A strain was considered susceptible to RIF if 10 or fewer plaques were seen on "RIF+" plates. A strain was considered resistant to RIF if 20 or more plaques were seen on "RIF+" plates. If plaques on "RIF+" plates were between 11 and 19, it was labeled as doubtful susceptibility (intermediate).

Quality control of the assay was assured by including positive and negative controls with every batch of specimens tested as described in the assay protocols. Positive control consisted of M. smegmatis infected with mycobacteriophages and treated with virucide. Negative control consisted of FPTB Medium Plus™ and mycobacteriophages, treated with the virucide. Results were considered valid

mycobacteriophages, treated with the virucide. Results were considered valid only if the positive control had 20-300 plaques, the negative control had less than 10 plaques and 20 or more plaques were counted on "RIF-" plates. The manufacturer recommends that the number of plaques should be 100 or more on the "RIF-" plates when M. tuberculosis isolates are used. But as our tests were performed directly on sputum specimens, the number of 20 plaques was taken as valid keeping in view the recommendations of the manufacturer for mycobacteriophage assay for detection of M. tuberculosis from sputum specimens.[10]

Results

Of the 40 clinical isolates of Mycobacterium tuberculosis tested for RIF susceptibility using the Bactec 460, 28 isolates were resistant to RIF and 12 isolates were susceptible. FASTPlaqueTB-RIF™ identified 24 specimens exhibiting resistance against RIF. Three specimens revealed susceptible isolates on Bactec 460, but the same specimens demonstrated resistance against RIF by FASTPlaqueTB-RIF™ while four specimens which revealed resistant isolates on Bactec 460, demonstrated susceptibility to RIF by FASTPlaqueTB-RIF™. There was one specimen which showed intermediate susceptibility to RIF by FASTPlaqueTB-RIF™ (number of plaques was 15) whereas the isolate from the same specimen was susceptible to RIF by the Bactec 460 technique (Table).

The sensitivity and specificity of the assay were 86% and 73% respectively while the positive and negative predictive values were 0.89 and 0.67 respectively. The overall accuracy of the test was 82%.

The phage assay took 48 hours to perform while Bactec 460 took 12.7$\pm$1.7 days (mean+standard deviation) to become positive. A further 5-8 days were required for susceptibility testing on Bactec 460.

Discussion

FASTPlaqueTB-RIFTM is a rapid screening assay for testing of rifampicin susceptibility in clinical isolates of Mycobacterium tuberculosis using the principle of phage amplification. Early evaluation studies have shown excellent correlation with the standard drug susceptibility techniques.[10,11] However, as Takiff and Heifets[16] have observed, it is neither rapid nor cost-effective as Mycobacterium tuberculosis have to be first isolated by culture. We performed the assay directly on sputum specimens instead of mycobacterial isolates to save both time and the costs of culture.

Although our study has shown good correlation of the phage technique with the Bactec 460 method in determining RIF susceptibility, there were three strains which were RIF resistant by FAST PlaqueTB-RIF™, but were susceptible by the Bactec method. This may be due to the low level of resistance due to mutation in rpoB533 rather than the more common mutation site of resistance, rpoB331.[17]

Multidrug resistant tuberculosis (MDR TB) is defined as simultaneous resistance of M. tuberculosis against RIF and INH.[5] Resistance against RIF is considered a good predictor of MDR Mycobacterium tuberculosis, as was observed in our study which showed that out of ~28 RIF resistant isolates, ~26 were also resistant to INH by the Bactec 460 technique ($p>0.5$). This is also in agreement with other data reported in the literature showing that RIF resistance is often a good marker of multidrug-resistance and would identify the patients most likely to fail the standard treatment regimen.[5,10]

The Mycobacteriophage assay does not require sophisticated equipment and is relatively simple and inexpensive. Results of the test were available within two days. Cultures (with GI >500) and susceptibility testing (with GI in control of 30 or more) done on Bactec 460 took 2-3 weeks. The susceptibility results from sputum specimen with FASTPlaqueTB-RIF™ were available much earlier than by the Bactec 460. This benefit of early results is quite significant and becomes even more so when compared to the much longer time required by the conventional culture techniques.

Although our study has shown encouraging results, its scope and size was limited; further trials are needed to establish the validity of the technique before it can be recommended for wider use. There is also a need to introduce susceptibility testing against other anti-tuberculosis drugs particularly the first line drugs by this technique.

The mycobacteriophage technique cannot replace the standard culture techniques. However, in our set-up, where facilities for culture and susceptibility testing are rarely available and the diagnosis of tuberculosis is mostly clinical, the phage technique holds the promise of an excellent method for rapid detection of RIF resistance. Early detection of drug resistance in Mycobacterium tuberculosis would benefit the patients with early institution of appropriate treatment and prevent the spread of multidrug-resistant strains of Mycobacterium tuberculosis.

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