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Detection of Rifampicin resistance in Pulmonary Tuberculosis by Molecular methods in a tertiary care hospital

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ABSTRACT

Tuberculosis is a major public health problem and second largest cause of death among infectious diseases. Rapid diagnosis of tuberculosis and detection of drug resistance is essential for effective disease control. WHO has endorsed the use of Line Probe assay (genotype MTBDR plus, Hain Life science) and the Xpert MTB/RIF assay (Cepheid, Sunnywale, USA) for rapid diagnosis of DRTB. The aim of this study is to know the prevalence of TB and its resistance pattern by using CBNAAT and LPA in Govt. General hospital, Kurnool. This is a prospective study done during the period from Jan 2016 to December 2016; all the samples were tested as per the RNTCP guidelines by using CBNAAT and LPA. Out of 1650 sputum samples, 280 (16.97%) were positive for Mycobacterium tuberculosis. Out of these, 18 (6.42%) showed Rifampicin (RIF) resistance by CBNAAT and 22 (7.8%) by LPA. Molecular technologies like LPA and Xpert MTB/RIF are the most promising technologies to detect drug resistance. The LPA test detects RIF due to mutation in *rpoB* gene as well as INH resistance due to mutations in the *inhA* and *katG* genes, while the Xpert MTB/RIF can detect only RIF resistance. In such cases LPA has greater role to play.

Introduction

Tuberculosis (TB) remains as a major global public health problem, affecting millions of people each year. Worldwide incidence of TB was 10.4 million in 2015, among them 5.9 million (56%) were males, 3.5 million (34%) were females and 1.0 million (10%) among children (1). India accounts for one fourth of the global TB burden. An estimated incidence of TB cases occurred was 28,00,000 and 4,80,000 people died due to TB. Over 25% of patients seeking care in India's public sector are neither diagnosed nor started on treatment (2). Tuberculosis (TB) is the second largest killer worldwide, after HIV and is the leading cause of death in HIV patients. Pulmonary TB spreads through aerosols and is highly contagious. Over 80% of TB infections are pulmonary and if left untreated, a pulmonary TB patient can infect up to 10-15 other people through close contact over the course of a year(3). Due to the highly infectious nature of pulmonary TB, it is important to diagnose and treat the disease very early. Despite the availability of highly effective treatment for decades, TB remains a major global health

problem mainly because of poor case detection (4). The most common method for diagnosing pulmonary TB worldwide is sputum smear microscopy. However sensitivity of direct smear microscopy is low and estimates range from 30% to 70%. It is even lower in case of HIV-infected patients. Culture is more sensitive than microscopy and is considered the current gold standard. Culture requires specialized and controlled laboratory facility and highly skilled manpower and takes 2 to 6 weeks to provide the result. Molecular techniques such as polymerase chain reaction (PCR) or Real Time PCR are much more sensitive than microscopy and culture. However these tests have so far been restricted to centralized reference laboratories as they require skilled manpower and elaborate infrastructure. Also the turnaround time for results could take a few days (5,6).

Moreover Drug resistance is a major issue in the treatment of tuberculosis. Drug resistance is because of either mismanagement of TB patients- wrong diagnosis, delay in diagnosis, wrong or interrupted treatment and injudicious use of both first and second line drugs. Multiple approaches to improve diagnosis of TB are in development. Amongst these are CBNAAT (GeneXpert) and LPA, endorsed by WHO to be used in RNTCP for rapid diagnosis of MTB and detection of Rifampicin resistance (7).

Cartridge-based nucleic acid amplification test (CBNAAT)

The CB-NAAT is a semi-quantitative nested real-time PCR which detects both MTB and RIF resistance directly from clinical specimens. It is the WHO-recommended method in 2010 for the diagnosis of both Pulmonary and Extrapulmonary TB and for diagnosing Paediatric TB. Under the current RNTCP guidelines, it is recommended for diagnosis of drug resistant-TB (DR-TB) in presumptive DR-TB and upfront diagnosis of TB in key population like paediatric tuberculosis, extra-pulmonary cases and people living with HIV. The analytical limit is 131 CFU/ml and the TAT is 2–3 h. Results can be ideally available while patient waits in the clinic. Because the cartridges are self-contained, the problem of cross-contamination between samples is eliminated. Sputum is liquefied and inactivated with a sample reagent which kills over 99.9% of TB bacilli in the specimen, and 2 ml of the material is transferred into a cartridge and this is inserted in the MTB-RIF test platform. Inside the cartridge, the sample is automatically filled, washed, filtered by ultrasonic lysis of the filter captured organisms to release the DNA. It uses three specific primers and five unique molecular probes to ensure high degree of specificity. The primers amplify a portion of the *rpoB* gene 81 bp RIF resistance determining region. The probes are capable to differentiate between wild-type (WT) and conserved sequence and mutations in the core region (8).

The sensitivity was 99.8% for smear- and culture-positive cases and 90.2% for smear-negative, culture-positive cases.[29]. The estimated specificity was 99.2% for a single direct MTB/RIF test, 98.6% for two MTB/RIF tests and 98.1%. The MTB/RIF test correctly detects RIF resistance with a sensitivity of 99.1% and 100% specificity. Thus, the test detects TB in essentially all smear-positive samples and the majority of smear-negative samples. The presence of non-tuberculous *Mycobacteria* does not confound testing. The cartridges are stable at room temperature. Issue to be considered while using CB-NAAT is the presence of mono-resistance to INH which is not detected in this test. INH mono-resistance is documented to be 7%–11% in the first-line treatment failures and newly diagnosed and previously untreated patients, respectively (9). Loss of therapeutic

efficacy of this important anti-TB drug has considerable implications for treatment and control strategies. Both live and dead bacilli are picked up by the CB-NAAT thus making this test in the current format useless to assess post-therapy efficacy. Concerns exist regarding false-positive RIF resistance results; hence, samples found to be resistant must be confirmed by a second Xpert MTB/RIF test or an LPA and phenotypic culture testing. In case an indeterminate result is obtained on the first specimen, a repeat testing of a new specimen by CBNAAT is required, if the result of this is also indeterminate, testing by culture and DST or Line Probe assay is mandated. Each cartridge has its internal quality control viz. sample processing control and Probe Check control. If Probe check control fails the test is stopped and an error is generated (>5% errors need to be investigated). The sample processing control must be positive when MTB is NOT detected but may be positive or negative is MTB is detected. The test requires a trained and computer-literate operator, a stable supply of electricity and air-conditioned settings (10).

Line probe assay (LPA)

This strip test detects TB DNA and genetic mutations associated with drug resistance from sputum specimens or culture isolates after DNA extraction and PCR amplification. This is a hybridisation assay that allows differentiation between *Mycobacterium* species. Each strip consists of 27 reaction zones (bands), including six controls (conjugate, amplification, *M. tuberculosis* complex, *rpoB*, *katG* and *inhA* controls), eight *rpoB* WT and four mutants (MUT) probes, one *katG* WT and two MUT probes and two *inhA* WT and four MUT probes. Theoretically the TAT is 5–6 h but the entire procedure usually takes upto 72 hours. It has a good sensitivity and specificity when performed on smear-positive and on culture isolates. WHO has endorsed LPA for MDR-TB in 2009. Two commercially available products are (1) InnoLiPA assay-Innogenetics, Belgium, and (2) Hain Lifescience GenoType® MTBDRplus. LPAs are as complex to perform as conventional culture and DST and require skilled and well-trained laboratory personnel, as well as adequate laboratory space and design (BSL-2/3 level laboratory with Class II Biological Safety Cabinet) to reduce the risk of false-positive results.

Hence, this study was taken up to show importance of bacteriological confirmation for accurate and early treatment. The aim of this study is to know the prevalence of Pulmonary tuberculosis and its resistance pattern, by using CBNAAT and LPA and to stratify the patients based on the variables like age, sex, New or Previously treated case and drug resistance.

Materials and Methods

This is a prospective study done during the period from Jan 2016 to December 2016; all the samples were tested as per the RNTCP guidelines by using CBNAAT and LPA. Samples from Presumptive TB and Presumptive DR-TB were collected in the DTCO office, Kurnool and processed by CBNAAT at department of Microbiology, Government Medical College, Kurnool.

Samples which were positive in CBNAAT were transported in cold chain to Damien Foundation Urban Leprosy and TB centre, Nellore which is operated by DFIT for I line and II line LPA. The lab is accredited as an IRL for LPA testing. Since the observations were made as a part of National TB control program, a separate ethical clearance was not required.

The samples were processed using NALC-NaOH method. Samples were decanted following centrifugation and the sediments were resuspended in phosphate buffer solution. The LPA was performed according to the manufacturer's protocol. Results were obtained by e-mail to the DTCO office within a week.

Results and Discussion

CBNAAT Results

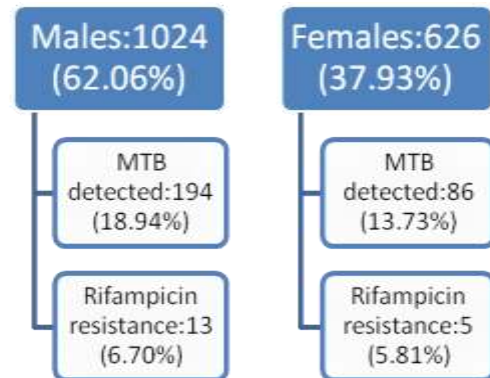
Total number of sputum samples included in this study was 1650, which are tested by CBNAAT. Among these, 280 (16.97%) were positive to Mycobacterium tuberculosis (MTB). Out of these, 18 (6.42%) showed rifampicin (RIF) resistance. Out of these MTB positive cases, presumptive TB cases were 112 and presumptive DRTB were 168. Rifampicin resistance was more among Presumptive DRTB cases 7.14% as compared to presumptive TB cases 5.35%.

MTB detected : 280 (16.97%)			
Presumptive TB : 112		Presumptive DR-TB : 168	
R sensitive	R resistant	R sensitive	R resistant
105	6(5.35%)	157	12(7.14%)

Most of the samples were from age group 40-60 (46.67%) followed by 20-40 (36.54%)

Age in years	No. of samples
0-20	121 (7.33%)
20-40	603 (36.54%)
40-60	770 (46.67%)
>60	156 (14.02%)
Total	1650

Out of the total 1650 cases, Males were 1024 (62.06%); amongst them, MTB was detected in 194 (18.94%) and Rifampicin resistance seen in 13 (6.70%). Females contributed to 626 (37.93%); amongst them MTB was detected in 86 (13.73%) and Rifampicin resistance seen in 5 (5.81%).



LPA results

All the RIF resistant cases by CBNAAT were RIF resistant by LPA also. In addition 4 cases were detected as RIF resistant contributing to total of 22 RIF resistant cases (7.85%). 8 cases (2.85%) were detected resistant to only INH i.e. H Mono resistance. 45 cases (16.07%) showed resistance to both INH and RIF. Treatment was started for MDR-TB and H Mono resistance depending on the LPA results.

There is an urgent need for rapid diagnostic methods for early diagnosis and initiation of treatment of Tuberculosis. Over the last years, efforts have been made to improve and develop rapid diagnostic tools and drug susceptibility testing (DST) for TB. During this period, the World Health Organization (WHO) had issued 10 policy statements for improving diagnosis of TB, including the use of commercial and noncommercial DST methods and implementation of molecular methods such as the line probe assay (LPA) and Xpert MTB/RIF (or GeneXpert) assay (11). These molecular methods are developed to target the *rpoB* gene, which consists of a 81-bp hot-spot region from codons 507 to 533, called the rifampin resistance-determining region (RRDR) (12). So far more

than 50 mutations have been characterized within this region by DNA sequencing but only point mutations at codons 526 or 531 are known to cause high levels of RIF resistance (13). In contrast, mutations in codons 511, 516, 518, 522, and 533 cause low-level resistance to RIF. Mutations conferring RIF resistance occur rarely in other regions of the *rpoB* gene (14).

In the present study males were predominantly affected. Age group mostly affected was 40-60 years followed by 20-40 years. Presumptive DRTB (Previously treated) cases showed more positivity rate. Rifampicin resistance was more in males and among previously treated cases (7.14%). Similar results were observed in study by Syed Beenish Rufai et al.(8) In the study by R.Tripathi et al(20) RIF resistance was very high 54.4% due to selection bias of patients. Kumar *et al*.(15) and Sharma *et al*.(16) reported 25.8% and 22% of MDR, respectively.

In our study LPA, it was observed that LPA was more sensitive in detection of RIF resistance than CBNAAT. Sequencing analysis of samples done by Syed Beenish Rufai et al showed 91.3% concordance with LPA but only 8.7% concordance with the Xpert MTB/RIF assay (8).

H Mono resistance was detected in 8 cases in our study. Kumar P et al (15) stated that Isoniazid resistance is more common in high TB burden countries and those isolates may not be resistant to Rifampicin. In contrast this statement, Somoskovi A et al (17) noted if the isolate is RIF resistant, it is more likely that it is also INH resistant, thus making RIF resistance a surrogate marker for the identification of MDR-TB. In High TB burden countries like India, higher rifampicin mono resistance was observed. Whereas, in South Africa lower rifampicin mono resistance was reported (13.5%) (18). In United States with low TB burden, low rifampicin resistance levels were observed by Ridzon R et al (19).

CBNAAT and LPA assays have been extremely useful in the diagnosis of DR -TB. Though CBNAAT is very user friendly and is considered the method of choice in identification of MTB and detection of RIF resistance, it should always be kept in mind that H Mono resistance and resistance to second line drugs is also very common. In such cases LPA has greater role to play.

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