Original Research Article

A study on mutation in genes associated with rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* complex using line probe assay

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ABSTRACT

**Background:** The term tuberculosis describe a clinical illness, which is predominantly caused by *Mycobacterium tuberculosis* and less common by other species. Infection is transmitted by infected droplets through respiratory route. Early diagnosis and appropriate management is the only way to control the spread of infection. The available diagnostic tools include, smear microscopy, culture and molecular methods. Culture is the gold standard, but it takes around 2-8 weeks to get the result and smear microscopy having less sensitivity. Molecular technique especially Line probe assay can be better option because of high sensitivity and specificity, and directly clinical sample can be used, and result will be made available within same day with sensitivity pattern. Present study was designed to use of LPA for early diagnosis.

**Methods:** Laboratory based observational study conducted in department of microbiology, IGIMS Patna and TBDC, Patna. Sputum specimens were collected from clinically suspected cases of pulmonary tuberculosis, and subjected to smear microscopy, culture and LPA.

**Results:** During the study period, 2841 patients were diagnosed as pulmonary tuberculosis. Strain of *Mycobacterium tuberculosis* complex in, 12% (347) patients were rifampicin and isoniazid resistant, 4% (117) and 3% (86) patients were rifampicin and isoniazid mono-resistant respectively. We found that *rpoB* MUT3 was the most common mutation in gene associated with rifampicin resistant and *katG* MUT1 gene associated with isoniazid resistant.

**Conclusions:** Present study support the use of LPA for early diagnosis of smear positive as well as smear negative pulmonary tuberculosis cases. Resulting early diagnosis and appropriate management of patients.

**Keywords:** Culture, Drug resistant, Line probe assay, Pulmonary tuberculosis, Smear microscopy

INTRODUCTION

In 1720, for the first time, the infectious origin of tuberculosis (TB) was conjectured by the English physician Benjamin Marten, in his publication “A new theory of Consumption”. The famous scientist Robert Koch was able to isolate the tubercle bacillus and presented this extraordinary result to the society of Physiology in Berlin on 24 March 1882.¹ Despite of that still TB is a major public health concern worldwide. The emergence of multidrug and extensively drug-resistant tuberculosis is a major threat to global tuberculosis control. According to Indian TB report 2018, the estimated incidence of TB in India was approximately 280000, whereas incidence of MDR-TB/RR was 147000, which accounting for about a one fourth of the
world’s TB cases. It is estimated that nearly 50% of the world’s burden of MDR-TB cases is in India and China. In India 4% and 21% of the new and re-treatment cases being caused by multi drug resistant strains of Mycobacterium tuberculosis respectively.3 It has been seen that most of the developing countries depend on sputum smear microscopy for TB diagnosis. Which play a major role in failure of TB control program in any country. TB is a communicable disease which can be easily transmitted from person to person by infected droplets, means if not diagnosed early one infected individual can transmit infection to most of the surrounding population. The sensitivity of sputum smear microscopy has been reported between 20% and 80%. It has been reported that one fifth of TB transmission occurs due to smear negative pulmonary TB.4 Culture is gold standard technique having high sensitivity and specificity, but it require 2-8 weeks for result. The delay associated with identification and DST lead to prolonged periods of ineffective therapy and ongoing tuberculosis transmission. The development of rapid molecular diagnostic tests for the identification of Mycobacterium tuberculosis and drug resistance has consequently become a research and implementation priority. Line probe assay (LPA) is a rapid molecular diagnostic technique that can detect M. tuberculosis complex in addition to rifampicin and isoniazid resistant directly from clinical samples. LPA detect RIF and INH resistance by identifying mutations in the rpoB, katG, and inhA genes. More than 95% of all rifampicin resistant strains can be detected by targeting mutations in the 81-base pair “core region” of the rpoB gene.5 And approximately 80-90% of INH-resistant strains can be detected by targeting mutations in katG and inhA. However, approximately 5-10% of INH-resistant strains have mutations in the ahpC, oxyR intergenic region.6,7 The World Health Organization in 2008 recommended a new policy to use LPA for rapid screening of patients at risk of MDR-TB.8 The LPA is a strip based reverse hybridization technique wherein DNA extraction and amplification, is subjected to hybridization with membrane strips coated with complementary probes targeted against specific genes. The LPA strip contains 27 reaction zones, which include 21 wild type and mutations probes and 6 control probes include a conjugate control, amplification control, M. tuberculosis complex specific control (TUB), rpoB locus control, katG locus control, and an inhA locus control. The present study was designed to identification and detection of drug resistant MTB directly from clinical specimens using line probe assay.

METHODS

This was a laboratory based prospective study, which were conducted in the Department of Microbiology, IGIMS Patna and TBDC, Patna from January 2016 to December 2017. Study was approved by Institute’s ethics committee. Authors included only respiratory specimens, all age groups and both sexes. Patients were provided a wide mouth, sterile leak-proof plastic container for sample collection, which were subjected to smear microscopy, solid culture using LJ media and Line probe assay. Standard RNTCP protocol were followed for sample collection and laboratory procedures.

Processing of sputum samples

All clinical material was handled and processed in class II biosafety cabinet in a bio-safety level (BSL)-3 laboratory. Sputum specimens were subjected to direct smear microscopy using ZN staining and it was examined in bright field binocular microscope. Then it was subjected to culture and LPA after concentration and decontamination techniques using NALC-NaOH (N- acetyl L-cysteine-Sodium hydroxide).9

Solid culture

Around 100µL of decontaminated specimen were inoculated on two slopes of LJ medium which were incubated at 37°C. Monday of every week were designated as culture reading day. On the basis of colony morphology and which were subjected to ZN staining, presence of AFBs in culture were reported as positive.10

Line probe assay

The test was performed as per manufacturer’s instruction and direct sputum specimen after decontamination were used.11 Test is based on DNA strip technology which includes three steps: DNA extraction, multiplex PCR amplification, and reverse hybridization. DNA were extracted using DNA extraction kit (Genolyse®- Hain Lifescience), after DNA extraction 5µl of the supernatant were mixed with 10 µL of amplification mix-A and 35µl of amplification mix-B (provided with the kit) to make a final volume of 50µl for amplification. Amplification consisting of an initial step of denaturation, annealing and a final extension. Finally, amplified products were analyzed by ‘Reverse Hybridization’ technique using DNA strip technology. Results were analyzed using reading scale which were provided by the manufacturer. Test were considered valid when there was appearance of respective controlled bands. The presence of TUB band indicated M. tuberculosis complex, whereas presence of wild type bands without a mutant band is interpreted as sensitive and the presence of a mutant band with or without the simultaneous absence of the corresponding wild type band is interpreted as resistant pattern.

RESULTS

During the study period we have collected 3729 sputum specimen, a total of 2841 patients were diagnosed as Pulmonary tuberculosis based on LPA test positive. Out of 3729 patients, 70% were male and 30% were female. It was seen that among the diagnosed cases of pulmonary tuberculosis cough >2weeks was the most common complain, which were reported by 87% of patients.
followed by abnormal chest X-ray, chest pain and fever, which were seen in 68%, 61% and 52% of cases respectively. Whereas haemoptysis, loss of appetite, night sweating and weight loss were complained by 26%, 32%, 59% and 27% of patients respectively. Among the 2841 diagnosed cases, 1919 (67.54%) sample were smear microscopy and culture positive, 169 (5.94%) samples were smear microscopy positive and culture negative, 673 (23.68%) samples were smear microscopy negative and culture positive, 80 (2.81%) samples were smear microscopy and culture negative (Table 1).

Table 1: Diagnosis of pulmonary tuberculosis based on LPA Vs smear microscopy Vs culture.

| Diagnostic tests | Number (N=2841) | % |
|------------------|-----------------|---|
| LPA (+ve), Smear microscopy (+ve), Culture (+ ve) | 1919 | 67.54% |
| LPA (+ve), Smear microscopy (+ve), Culture (-ve) | 169 | 5.94% |
| LPA (+ve), Smear microscopy (-ve), Culture (+ve) | 673 | 23.68% |
| LPA (+ve), Smear microscopy (-ve), Culture (-ve) | 80 | 2.81% |

Whereas among the diagnosed cases it has been seen that, 736 sample were 3+, 591 sample were 2+, 479 sample were 1+, 282 sample were scanty, and 753 sample were smear microscopy negative (Table 2).

Table 2: Comparative evaluation of LPA vs smear microscopy.

| LPA test | Smear grading system | Total |
|----------|----------------------|-------|
|          | 3+ | 2+ | 1+ | Scanty | Negative |
| Positive | 736 | 591 | 479 | 282 | 753 | 2841 |

Strain of *Mycobacterium tuberculosis* complex in, 81% (2291) patients were rifampicin and isoniazid sensitive, 12% (347) patients were rifampicin and isoniazid resistant, 4% (117) and 3% (86) patients were rifampicin and isoniazid mono-resistant respectively (Table 3).

Table 3: Interpretation of LPA result.

| Resistant pattern                 | Number | %   |
|-----------------------------------|--------|-----|
| Rifampicin and Isoniazid sensitive| 2291   | 81% |
| Rifampicin and Isoniazid resistant| 347    | 12% |
| Rifampicin mono-resistant         | 117    | 4%  |
| Isoniazid mono-resistant          | 86     | 3%  |

It has been observed that most common mutation in gene associated with rifampicin resistant were *rpoB* MUT3 and in gene associated with isoniazid resistant were *katG* MUT1. Among 117 rifampicin mono-resistant isolates, 2.36% had *rpoB* MUT1, 4.36% had *rpoB* MUT2A and 14.54% had *rpoB* MUT3. Whereas in 86 isoniazid mono-resistant isolates, 9.63% had *katG* MUT1, 3.63% had *inhA* MUT1 and 2.36% had *katG* MUT1 plus *inhA* MUT1. In 347 MDR isolates, 3.81% had *rpoB* MUT1 plus *katG* MUT1, 5.27% had *rpoB* MUT1 plus *inhA* MUT1, 39.81% had *rpoB* MUT3 plus *katG* MUT1, 4.90% had *rpoB* MUT3 plus *katG* MUT1 plus *inhA* MUT1, 2% had *rpoB* MUT3 plus *inhA* MUT1, 4.90% had *rpoB* MUT3 plus *inhA* MUT2 and 2.36% had *rpoB* MUT3 plus *katG* MUT1 plus *inhA* MUT3A (Table 4).

Table 4: Mutations in genes associated with rifampicin and isoniazid resistance.

| *rpoB* (gene mutation) | *katG* (gene mutation) | *inhA* (gene mutation) | Frequency (N=550) | %   |
|------------------------|------------------------|------------------------|-------------------|-----|
| MUT1                   | -                      | -                      | 13                | 2.36|
| MUT1                   | MUT1                   | -                      | 21                | 3.81|
| MUT1                   | -                      | MUT1                  | 29                | 5.27|
| MUT2A                  | -                      | -                      | 24                | 4.36|
| MUT3                   | -                      | -                      | 80                | 14.54|
| MUT3                   | MUT1                   | -                      | 219               | 39.81|
| MUT3                   | MUT1                   | MUT1                  | 27                | 4.90|
| -                      | MUT1                   | -                      | 53                | 9.63|
| -                      | -                      | MUT1                 | 20                | 3.63|
| -                      | MUT1                   | MUT1                  | 13                | 2.36|
| MUT3                   | -                      | MUT2                 | 27                | 4.90|
| MUT3                   | MUT1                   | MUT3A                | 13                | 2.36|

DISCUSSION

In present study we found that among the LPA positive samples, 73% were sputum smear microscopy positive and rest were sputum smear microscopy negative. Whereas result from different study indicated that among the smear negative Pulmonary tuberculosis sensitivity of LPA was 78%, in comparison to present study they found good positivity of LPA in smear negative PTB cases. A comparative study between LPA and culture from Vellore, showed positivity of LPA and culture were 60% and 54% respectively, whereas in our study we found positivity of culture was 91% in LPA positive clinical samples. A study by Prabha D et al, found that among the diagnosed cases of Tuberculosis using LPA, 19% strains were MDR, 11% and 8% strains were rifampicin mono-resistant and isoniazid mono-resistant respectively. In comparison to present study they found more number of MDR, rifampicin mono-resistant and isoniazid mono-resistant cases. A study on prevalence of MDR TB from other part of Bihar reported 15%. Study conducted in Gujarat reported 17% of MDR-TB among the diagnosed cases of tuberculosis. When some international studies were compare from present study we found that a study from Karachi, Pakistan reported prevalence of MDR-TB was 18%, whereas present study reported 12%. In a report from Southeast of Iran, indicated 16% of MDR-TB cases in Pulmonary tuberculosis patients. Prevalence of mutation of genes associated with rifampicin and isoniazid resistance vary...
widely in different geographic locations. Present study have indicated that most common genes associated with rifampicin resistant was MUT3 and MUT1, which were seen in 69% and 11% of cases among drug resistant Pulmonary tuberculosis respectively. Whereas in isoniazid resistant isolates MUT1 was the only patter of resistant in katG gene, and MUT1 and MUT2 was in inhA gene, which were isolated from 18% and 2% of isolates respectively. A study from New Delhi indicated most common mutation type was MUT3 followed by MUT1 in rifampicin resistance, which were reported from 47% and 9% of cases respectively. Among the isoniazid resistant isolates MUT1 was the only type of pattern in katG gene, which were seen in 71% of cases and MUT1, MUT3A and MUT3B were seen in inhA gene, which were isolated from 12, 2 and 1 number of cases respectively. An international study from South Africa reported MUT3 was the most common mutation pattern followed by MUT1, which were seen in 47% and 28% of rifampicin resistant isolates respectively, whereas MUT1 was seen in katG gene and MUT1 and MUT3B were seen in inhA gene, which were seen in 33%, 14% and 8% respectively. Hence, among the available conventional and molecular methods LPA will be the better option, since drug resistant cases is increasing exponentially. LPA has potential to reduce the turnaround time, resulting better outcome in treatment of drug resistant Pulmonary tuberculosis. However, conventional culture method must be followed for diagnosis along with LPA test.

CONCLUSION

Rapid diagnosis of multidrug resistant and extremely drug resistant tuberculosis is a serious issue worldwide. Using LPA result can be made available within same day of performance, which will guide the clinician about drug resistant patter helping in treatment. Diagnostic utility of LPA is also good in smear negative tuberculosis. Our study recommend LPA can be an alternative diagnostic tool for diagnosis of suspected cases of drug resistant and smear negative Pulmonary tuberculosis, since sensitivity and specificity of the test is high.

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