Original Article

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Study of Isoniazid and Rifampicin Resistance among New Sputum Smear Positive Pulmonary Tuberculosis Patients by Line Probe Assay in Bikaner

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Abstract

Introduction: An alarming increase in the global incidence of drug resistant *Mycobacterium tuberculosis* (TB) infection has created a critical need for methods that can rapidly identify drug resistant cases, why isoniazid and rifampicin (RIF) so important, isoniazid is the most powerful Mycobactericidal drug available that ensures early sputum conversion and helps in decreasing the TB transmission.

Materials and Methods: A total of 100 patients with new sputum smear positive for acid-fast bacilli were enrolled for study and patient excluded with the following criteria, took TB treatment for >1 month, sputum negative pulmonary TB, age >12 years, all extra pulmonary TB cases, patients who could not participate actively. We used molecular based mechanism to detect of the drug resistant mycobacterium, Line probe assay. Statistical package for the social sciences version 10 was used to analyze the results with $P < 0.05$ taken as significant.

Result: Among the 100 new sputum positive patients, one of multi-drug resistant (MDR) TB, 12 are isonicotinic acid hydrazide (INH) mono resistant and none of the RIF mono resistant found in Bikaner, India. Our results also match with others previous studies, the prevalence of initial MDR was 1.1% in Bangalore (1980), 0.8% in Pondicherry (1985-1991), 0.9% in Jaipur (1989-1991), 1% in Pune (1992-1993), and many more study support our study and World Health Organization data of drug resistant.

Conclusion: Prevalence of MDR-TB in new sputum positive patient 1%, INH monoresistance is 12%, no monoresistance found for RIF. The status of initial MDR-TB is low in Bikaner district, which reflects the success of directly observed treatment, short-course in effective treatment of drug-susceptible TB and preventing the emergence of drug resistance. Since MDR-TB is rare among new TB cases, all new cases of pulmonary TB can be treated with empirical Category I regimen.

Key words: Antibacterial drug resistance, Multi drug-resistant, Tuberculosis

INTRODUCTION

Tuberculosis (TB) is curable and preventable disease, which is caused by *Mycobacterium* TB (MTB). It most commonly affects the lungs but can potentially involve any system or organ of the body. It is estimated that one untreated infectious TB patient is likely to infect 10-15[1,2] people because when they cough or sneeze, they expel large amount of droplets containing large number of bacteria, sputum smear positive (SSP) pulmonary TB patients are the most significant source of droplet nuclei, which carry infectious bacilli.[3] The situation is made worse by the emergence of drug resistant TB, particularly the multi-drug resistant (MDR) and extensively drug-resistant (XDR)[4,5] TB. A case of MDR-TB is about 20-40 times more expensive to manage than a case of drug-sensitive

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TB.[6] Although drug resistance was observed in MTB isolates even in the early days of chemotherapy, the emergence of strains resistant to the two most potent anti-TB drugs-Isoniazid (H) and rifampicin (RIF) i.e. MDR-TB. The level of initial drug resistance is considered to be an epidemiological indicator to assess the success of the National TB Programmed.[7]

An alarming increase in the global incidence of drug-resistant MTB infection has created a critical need for methods that can rapidly identify drug-resistant cases.[8,9]

The choice of technology to be used for diagnosis of MDR-TB has been determined as per recommendations of the National Laboratory Committee.[10,11] Thus, for the drug sensitivity testing at certified laboratory wherever available molecular Department of Science and Technology (DST) (e.g. line probe assay [LPA]) is preferred diagnostic method because of the rapid and highly-accurate RIF results.[12]

The emergence and spread of drug resistant TB are threatening to destabilize global TB control. The prevalence of drug resistant TB is increasing throughout the world both among new TB cases as well as among previously-treated ones (Figure 1). Depicts a pie chart showing proportion of estimated incident cases of MDR TB in 2012 World Wide.

Although previous treatment for TB is the strongest risk factor for the development of drug resistant TB,[13-15] naïve patients are also at risk due to either spontaneous mutations or transmission of resistant strains.[16,17] The risk of transmission of resistant strains from close contacts is increasing day-by-day because of the growing burden of drug resistant TB patients.[18] Therefore in the context to Bikaner division this study sought to determine the status of isoniazid and RIF resistance by LPA in Bikaner district. MTB undergoes spontaneous, slow but constant mutation resulting in resistant mutant organism.[19,20] This natural phenomenon varies for different anti-TB drug and

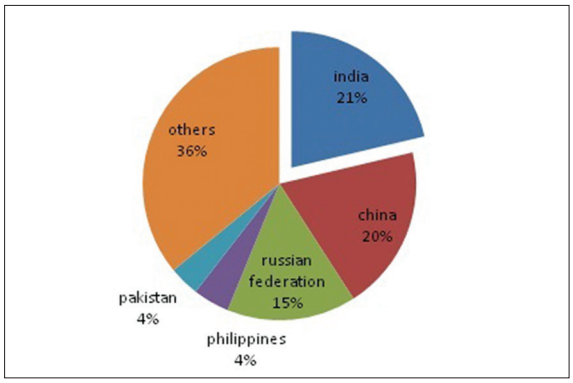

Figure 1: Proportion of estimated incident cases of MDR TB in 2012

is genetically determinant. Spontaneous occurrence of drug-resistant mutants in wild strains of mycobacterium for RIF is 1 strain in 10^8 bacilli, for isoniazid, streptomycin, ethambutol, kanamycin and p-amainosalicylic acid is 1 strain in 10^6 bacilli and for ethionamide, cycloserine, capreomycin and thiacetazone is 1 strain in 10^3 bacilli. Spontaneous occurrence of resistant mutants for RIF and isoniazid both simultaneously is 1 strain in 10^{14} bacilli.[21]

Thus in a 2.5 cm cavity, which harbors 10^8-10^9 bacilli only one naturally RIF resistant and 100-1000 naturally isoniazid resistant strains may be found. This illustrates a very fundamental principle that MDR-TB is a man-made problem.[22] A high bacterial load and several cycles of inappropriate treatment are therefore needed for significant numbers of drug resistance bacilli to emerge (acquired drug resistance). These strains can also be transmitted to individuals who have never before had TB and they can present with drug resistance TB (primary drug resistance), resistance can be for mono drug, poly drug, MDR (resistant to isonicotinic acid hydrazide [INH] and regional integrated pest management) with and without other drug, XDR (MDR plus resistant to floroquinolons, kanamycin, amikacin, capreomycin. Poly drug resistant other than INH and RIF at a time, Total drug resistant, resistant to all first line and second line drug.[23]

Why INH and RIF so important**,** isoniazid is the most powerful Mycobactericidal drug available that ensures early sputum conversion and helps in decreasing the TB transmission. RIF because of its mycobactericidal and sterilizing activities is crucial for preventing relapses. Thus, Isoniazid and RIF are the keystones in TB management. Molecular mechanism of drug resistant of isoniazid resistant, mutation in katG is the main mechanism of INH resistance.[24] KatG S315T mutation is the most common mutation in INH-resistant strains, especially in high level resistant strains (minimum inhibitory concentration [MIC]>5 μg/ml) low-level resistant strains (MIC<1 μg/ml) often still possess catalase activity.[25,26] Accounting for 50−95% of INH-resistant clinical isolates.[27] Mutations in inhA or its promoter region are usually associated with low-level resistance (MICs = 0.2-1 μg/ml) and are less frequent than katG mutations. INH-resistant MTB harboring inhA mutations could have additional mutations in katG, conferring higher levels of INH resistance[28,29] mutations in inhA not only cause INH resistance, they also confer cross-resistance to the structurally related drug, ethionamide. RIF resistant, RIF is a potent inhibitor of DNA dependent RNA polymerase. RNA polymerase consists a core enzyme having 4 polypeptide chains $\alpha_2\beta\beta'$ and an additional subunit σ that allows promoter recognition for initiation of transcription. These proteins and subunits ($\alpha\beta\beta'\sigma$) are coded by different genes known as rpoA, rpoB, rpoC and rpoD respectively.

RIF resistance (>96%) has been associated with mutations in the rpoB gene. Genetic probes which detect drug resistance to RIF with >95% accuracy are very suggestive of MDR-TB; <10% of RIF resistance is monoresistant so the RIF resistance is a surrogate marker for MDR-TB in >90% of cases. In an Indian study Siddhiq *et al.*[30,31] found that most isolates had mutations at cod on 531. Mutations at 516 and 521 are associated with low level resistance (MIC <40 μg/ml) and mutations at 510, 526, 527, 528, 531 are associated with high level resistance (MIC >64 μg/ml). LPA It is a manual and automated system, which is validated for SSP samples. Two commercial assay are available INNO-Lipa test a geno type MTB complex assay use for rpoB gene mutation for RIF. InhA and KatG mutation for isoniazid, these also can be used for disease differentiation form MOTT. LPA s are highly sensitive (≥97%) and specific (≥99%) for the detection of RIF resistance, alone or in combination with isoniazid (sensitivity ≥90%; specificity ≥99%), on isolates of MTB and on smear-positive sputum specimens.[32,33] Overall accuracy for detection of MDR-TB was equally high at 99% and retained when RIF resistance alone was used as a marker for MDR.[34]

LPA s are not a complete replacement for conventional culture and DST, as mycobacterial culture is still required for smear-negative specimens while conventional DST is still necessary to confirm XDR-TB.[35,36] Limitation of LPA: Not applicable for sputum negative and extra pulmonary TB cases. Requires highly sophisticated infrastructure. Skilled and highly trained persons are required. The global incidence of primary and secondary resistance was 3.6% and 20.2%, respectively. Drug XDR TB had been reported by 92 countries globally by the end of 2012.

Revised National TB Control Programme (RNTCP) has recently undertaken three communities based state level drug resistance surveillance studies in Gujarat, Maharashtra and Andhra Pradesh and estimated the prevalence of MDR-TB to be about 3% in new cases and 12-17% in re-treatment cases.

MATERIALS AND METHODS

One hundred patients with new SSP for acid-fast bacilli (AFB) were included in this prospective study.

The patients who had taken TB treatment for >1 month, sputum negative pulmonary TB, age >12 years, all extra pulmonary TB cases, patients who could not participate actively were excluded from the study. Thorough examination of the respiratory system and sputum for AFB smears × 2 samples as per RNTCP guidelines the samples were transported immediately to intermediate reference laboratory (IRL), Ajmer.[37] Other investigation including hematology (complete blood count, rythrocyte sedimentation rate), X-ray chest posterioranterior View, blood Sugar and HIV were done. LPA technology involves the following steps.[38] DNA is extracted from MTB isolates or directly from clinical specimens. Polymerase chain reaction (PCR) amplification of the resistance-determining region of the gene under question is performed using biotinylated primers. Following amplification, labeled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled hybrids are detected by colorimetric development, enabling detection of the presence of MTB complex, as well as the presence of wild-type and mutation probes for resistance. LPA testing should be performed in three separate rooms and World Health Organization (WHO) recommendation manner. DNA extraction should be performed in the biosafety level-3 (BSL-3) laboratory, master mix preparation in a second room, and PCR and hybridization were performed in a third laboratory. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by the lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations.[39] The post-hybridization reaction leads to the development of colored bands on the strip at the site of probe binding and is observed by eye.

LPA are highly sensitive (≥97%) and specific (≥99%) for the detection of RIF resistance, alone or in combination with isoniazid (sensitivity ≥90%; specificity ≥99%), on isolates of MTB and on smear-positive sputum specimens. Overall accuracy for detection of MDR was equally high at 99% and retained when RIF resistance alone was used as a marker for MDR. LPA are not a complete replacement for conventional culture and DST, as mycobacterial culture is still required for smear-negative specimens while conventional DST is still necessary to confirm XDR-TB. Sputum samples, which were found positive by ziehl-neelsen staining at the IRL lab, Ajmer, were subjected to LPA testing to detect isoniazid and RIF resistance.

The geno type MTB plus LPA was performed according to the manufacturer's (Hain Lifescience, Nehren, Germany) instructions. Three steps for LPA test included DNA extraction, multiplex PCR amplification and reverse hybridization. These steps were carried out in three separate rooms with restricted access and unidirectional workflow. Mycobacterium DNA was extracted in BSL-3 laboratory according to manufacturers' instructions. Briefly, 500 μl of decontaminated sputum sample was centrifuged at 10,000 × g for 15 min, the supernatant was discarded, 100 μl lysis buffer (A-LYS) was added. Then it was resuspended and incubated for 5 min at 95°C, 100 μl neutralization buffer (A-NB) was added and centrifuged for 5 min at

full speed in a centrifuge. A volume of 5 µl of the DNA supernatant was used for PCR while the remainder was stored at 20°C. Master mixture for amplification consisted of 10 µl amplification mix-A (provided with kit), 35 µl of amplification mix -B (provided with kit) and 5 µl of DNA supernatant in a final volume of 50 µl. The amplification protocol consisted of 15 min of denaturation at 95°C, followed by 20 cycles comprising denaturation at 95°C for 30 s and 65°C for 2 min. This was followed by 30 cycles comprising 95°C for 25 s, 50°C for 40 s and 70°C for 40 sec and a final extraction at 70°C for 8 min. Hybridization was performed with the automatic machine (GT blot-48). After hybridization and washing, strips were removed, fixed on paper and results were interpreted.[39] Each strip of LPA had 27 reaction zones (bands), including six controls (conjugate, amplification, MTB complex (TUB), rpoB, katG and inhA controls), eight rpoB wild-type (wt1-wt8) and four mutant probes (rpoBMUT1, rpoBMUT2A, rpoBMUT2B, and rpoBMUT3), one katG wild-type and two mutant probes (katGMUT1 and katGMUT2), and two inhA wild type and four mutant probes (inhAMUT1,inhAMUT2, inhAMUT3A, inhAMUT3B). Either missing of wild-type band or the presence of mutant band was taken as an indication of a resistant strain. Incomplete amplification of RIF and/or INH genes was considered as an invalid result.

Statistical Analysis

According to the reports of IRL lab Ajmer, statistical analysis was done. Appropriate test was applied as and when required using statistical package for the Social Sciences Software version 10.0 and isoniazide and RIF and both resistances were calculated. Ethical approval for the study was obtained by the Institutional Review Board.

OBSERVATION AND RESULTS

Observations of 100 patients in different parameters e g., data according geographical distribution, out of that most of the patient form rural (57.1%) of Bikaner and there were 43% from urban back ground. Majority of the cases were farmers (28%) followed by house wives (27%). Laborer and students constituted to the tune of 13%, and others were 12%. Seven percent of cases were Government employees. The addiction to smoking in 25% cases and 9% were for alcohol, and 5% were for tobacco. Only one case was consuming opium, 68% cases had no addiction symptomatically Majority of cases were having cough (94%), fever in 89% cases. 49% cases reported expectoration, 39% loss of appetite, 46% had weight loss (Table 1). Breathlessness and chest pain was reported by 24 and 34% cases, respectively. Comorbiditize were: Diabetes 7%, HIV 3%, Hypertension 2%, ischemic heart disease 2%. Resistance to isoniazid 12%, RIF 0 and MDR in only one case. According to age, it is evident that out of 12 cases having resistance with Isoniazid, 4 cases were present in age group 51-60 years followed by 3 cases each in age group 12-20 and 21-30 years (Table 2). One case was in 41-50 age group and one was in 61-70 age group. Only 1 MDR case found in age group 61-70 year of age. The data show that out of 12 isoniazid resistant cases, 6 were males and 6 were females, and 1 MDR case was male. Of 12 cases having resistance to isoniazid, 3 were farmers, 4 were house wives, 2 were students, 1 was Government employee, 1 laborer and others. The 1 case of MDR was farmer. None of the patients had RIF monoresistance. Perusal of data reveals that out of 12 cases resistant to isoniazid, 7 were having no addiction. However, 5 cases were addicted to smoking and alcohol both, out of which 1 case was addict to opium also in addition to smoking and alcohol. The only case of MDR was habitual of smoking and alcoholism.

Table 1: Distribution of the study patients according to their symptoms

Symptoms	Number *n*=100	Percentage
Cough	94	94.00
Expectoration	49	49.00
Loss of appetite	39	39.00
Weakness	59	59.00
Weight loss	46	46.00
Fever	89	89.00
Hemoptysis	17	17.00
Breathlessness	24	24.00
Chest pain	34	34.00

Out of total resistant cases, only one (diabetes) co morbidity was found. Majority of cases having isoniazid resistance were recorded in socio-economic status (SES) Class III-V ($P < 0.001$). Data reveals that the 7 isoniazid resistant cases had a history of contact with known case of TB and 1 multi drug resistant case had a history of contact with known case of MDR-TB.

DISCUSSION

The present study was prospective done on consecutively enrolled 100 patients in the Department of Respiratory Medicine, Sardar Patel Medical College, Bikaner, Rajasthan. The demographic profile of our patients was similar to other series, with a majority (61.89%) of male patients in the economically productive age group.

The mean age of our cohort was 41.7 years whereas mean age for males and females was 44.14 years and 37.54 years, respectively. Majority (63%) of patients belong to economically productive age group. In a similar study at Delhi, mean age of the patients was 27.8 ± 10.2 year; 59 (27%) were females.

Table 2: Distribution of various parameters in the patients with resistance to anti-tubercular drug

Parameter	Isoniazid resistance	RIF resistance	MDR
Age group			
12-20	3	0	0
21-30	3	0	0
31-40	0	0	0
41-50	1	0	0
51-60	4	0	0
61-70	1	0	1
>70	0	0	0
Gender			
Male	6	0	0
Female	6	0	0
Occupation			
Farmer (*n*=28)	3	0	1
Government employee (*n*=07)	1	0	0
Laborer (*n*=13)	1	0	0
Housewife (*n*=27)	4	0	0
Student (*n*=13)	2	0	0
Other (*n*=12)	1	0	0
Comorbidity			
Diabetes mellitus (07)	01	0	0
HIV (03)	0	0	0
Hypertension (02)	0	0	0
IHD (02)	0	0	0
Socio economic scale (Modified kuppuswamy scale)			
I	0	0	0
II	01	0	0
III	0	0	0
IV	03	0	0
V	09	0	01
Addiction			
Nil (*n*=68)	7	0	0
Smoker (*n*=25)	5	0	1
History of contact			
Yes	7	0	1
No	5	0	0
Education			
Illiterate (*n*=61)	06	0	01
R and W (*n*=06)	0	0	0
Primary (*n*=07)	02	0	0
Middle (*n*=08)	02	0	0
Secondary (*n*=07)	02	0	0
Senior secondary (*n*=05)	01	0	0
Graduate (*n*=06)	0	0	0

MDR: Multi drug resistance, RIF: Rifampicin

According to WHO global report 2013, prevalence of primary/initial drug resistance in India was around 2.2%. The prevalence of initial MDR, isoniazid monoresistance and RIF monoresistance observed in different studies in different institutes and tertiary care centers India and result were founds, ranged from 0.5% to 3%, 10.4-15.2% and 0.5-2% respectively from years 1999 to 2005.[40]

In the previous studies the prevalence of initial MDR was 1.1% in Banglore[41] (1980), 0.8% in Pondicherry[42] (1985-1991), 0.9% in Jaipur[43] (1989-1991), 1% in Pune[44] (1992-1993), and many more study support our study. However, Anuradha *et al.*, showed 1.5% RIF monoresistance. In the present study monoresistance to, isoniazid was 12%. In 2009, Ramachandran *et al.,* also found (11%) similar results.

Risk factors for drug resistant TB: Here factors are, contact history, low SES and illiteracy were found to be associated with drug resistance.

In this study, the association between age and gender with drug resistance not significant. However, according to WHO report on MDR and XDR surveillance 2010 data from many countries showed no association between drug resistance and gender of the patient. MDR-TB surveillance data in 13 Countries of Central and Eastern Europe showed that peak of MDR-TB was seen at 35-44 years of age.[45]

Our work showed that the association known for centuries between TB and poverty also applies to MDR-TB. In this study, majority of drug resistant TB patients were of low SES and uneducated, which was similar to Turkey study.[46] In the present study, no significant association was found between addiction and initial drug resistance. The issue of HIV infection being a risk factor for drug resistant TB has been discussed for several years. Here 3% patient were positive for HIV and none of them were having drug resistance, Therefore, the HIV positive status and MDR may be events independent of each other in the present study. Bashar *et al.*, found a significant association between diabetes and MDR-TB. In present study, 7 patients were diabetic, and none of them are MDR TB, was isoniazid mononresistance. In the present study, significant association was found between contact history and drug resistance.[1]

CONCLUSIONS

The status of initial MDR-TB is low in Bikaner district, which reflects the success of directly observed treatment, short-course in effective treatment of drug-susceptible TB and preventing the emergence of drug resistance. Since MDR-TB is rare among new TB cases, all new cases of pulmonary TB can be treated with empirical Category I regimen.

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