IDENTIFICATION OF *rpoB, gyrA* AND *embB* GENE MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM RETREATMENT TUBERCULOSIS PATIENTS IN NEPAL

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ABSTRACT

Introduction:

Tuberculosis remains one of the major public health problems in Nepal and increasing trend of multi drug resistant and extensively drug resistant tuberculosis (MDR /XDR TB) is a big challenge. Rapid diagnosis and appropriate treatment of MDR/XDR TB is crucial. Identification and comparison of MDR TB using rapid molecular techniques (for *rpob, gyrA, rrs* and *embB* gene mutations) with reference to drug susceptibility test (DST) were the main objectives of this study.

Methodology:

A cross sectional study was carried out in National TB Centre (NTC). Gene Xpert, proportion method and Line Probe Assay (LPA) were used for first and second line drugs susceptibility testing (FLD-DST and SLD-DST). A total of 29 mucopurulent sputum samples were freshly collected from retreatment TB patients (Female 41.4%, Male 58.6%) with median age of 40 years attending to the four MDR TB treatment centres of eastern and central Nepal (via private courier and directly to National TB Reference Laboratory (NRL) at NTC from April 2013 to October 2017.

Results:

Among 29 sputum samples (Female 41.4%; all smear+ve, Male 58.6%; 16 smear+ve and 1 smear-ve), Gene Xpert MTB/RIF assay detected 100% *M. tuberculosis* and rifampicin resistance (*rpoB* gene resistant) of which, 100% were culture positive by conventional Lowenstein–Jensen (LJ) method. FLD-DST was performed on all culture positives of which, 96.6% showed MDR TB and 3.4% showed mono resistance to isonizid only. SLD-DST on 29 MDRTB strains by LPA showed 100%, 58.6%, 44.8% wild type for *rrs*, *gyrA* and *emb B* genes respectively. Mutation for *gyrA* and *emb B* genes was 41.4% and 51.2% respectively, *rrs* genes none. Twelve (Female 6, Male 6) MDR TB strains were identified as pre-XDR-TB. Chi square (^{2}) for trend was used to analyze Gene Xpert, smear, FLD-DST and LPA results.

Conclusion:

From this study, 29(100%) MDRTB were detected from retreatment TB cases by Gene Xpert and FLD-DST. Almost 41.4% MDR TB strains were detected as pre-XDR TB by LPA, which were found to be higher in 15-60 years group of females and males. Samples from retreatment TB patients need to be tested by rapid molecular techniques with reference to culture and DST.

Key words:

Mycobacterium tuberculosis, Gene Xpert, Line Probe Assay, Multi and Extensively Drug Resistance.

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INTRODUCTION

Tuberculosis (TB) is a top infectious killer disease worldwide. Over 95% of TB deaths occur in low- and middle-income countries, and it is among the top 5 causes of death for women aged 15 to 44. Globally in 2014, an estimated 480, 000 (an estimated 3.3% of new TB cases and 20% of previously treated cases) people developed multidrug-resistant TB (MDR-TB) and 190, 000 people died of MDR-TB. An estimated 43 million lives were saved through TB diagnosis and treatment between 2000 and 2014. *M. tuberculosis* strains identified as MDR TB (resistant to isoniazid and rifampicin with or without other first line anti-TB drugs plus any fluoroquinolone and at least one of three injectable second-line drugs is defined XDR TB. MDR TB resistant to either of fluoroquinolones or injectable aminoglycosides is categorized as pre-XDR-TB. An estimated 9.7% of people with MDR-TB have XDR-TB.[1] The SAARC region, with 34% of the global burden of TB, a total 81,142 estimated cases of MDR-TB among notified cases were notified in 2013, of which 59% were previously treated cases.[2]

Tuberculosis (TB) remains one of the major public health problems in Nepal. In 2014, total of 37,025 cases of TB were registered. Most cases were reported among the middle aged group with the highest among 15-24 years of age (20%). TB-HIV co-infection rate in Nepal is 2.4% (HIV among TB) and 11.6% (TB among HIV) based on the sentinel survey, 2011/12. Nationwide, the proportion of multidrug-resistant TB (MDR-TB) was 2.2% among new cases and 15.4% among retreatment cases based on survey carried out in 2011/12. In 2014, total of 349 MDR TB and 25 XDR TB were enrolled for treatment. WHO estimated 4.6 (2.1-7.5) thousand people died from TB in 2014 (NTP Annual report Nepal 2070/71 or 2014).[3] One of the concerned aspects of drug resistance in Nepal is the high level of resistance to fluoroquinolones (26.4%), which leads to heavy burden of pre-XDR and XDR-TB among MDR-TB patients (8% of the cases were found to be XDR among MDR cases in the same survey). To combat the excess mortality related to XDR-TB, it is recommended to perform DST for second line-drugs to all MDR-TB cases at the start of treatment.[4]

Isoniazid (INH) with rifampicin (RIF) forms the cornerstone of short course chemotherapy for tuberculosis and resistance to either drug hampers the complete cure of patients. *M. tuberculosis* strains resistant to at least these two major frontline drugs (INH and RIF) develop multi-drug resistant tuberculosis (MDR-TB).[5]

More than approximately 95% RIF resistant *M. tuberculosis* strains have mutations in an 81 bp hot spot region (codon 507-533) of *rpoB* gene that encodes RNA polymerase beta subunit.[6,7,8,9]

Globally, more than half of all TB cases are not detected the result of health care system weakness and the inadequacy of available technology. If a diagnosis is absent, patients are not treated, transmission may continue, patients suffer needlessly and may eventually die.[10]

Cepheid (Cepheid, Sunnyvale, CA) has recently introduced the GeneXpert MTB/ RIF assay for research use only.[11] The GeneXpert assay is a real-time PCR test that will simultaneously identify *M. tuberculosis* and detect rifampin resistance directly from clinical specimens.[12,13,14] Rifampin resistance can serve as a marker for multidrug-resistant tuberculosis (MDR-TB) and has been reported in 95% of the multidrug-resistant *M. tuberculosis* isolates. The GeneXpert assay detects an 81-bp "core" region of the *rpoB* gene.

The suggested target sites of first line anti-TB drugs and the sites for most frequent mutations occur; for streptomycin, isoniazid, rifampicin, ethambutol, pyrazinamide were described. [15, 16, 17, 18, 19, 20, 21, 22] Similarly, the target sites of second line anti-TB drugs and the most frequent mutations occur for fluoroquinolone, injectable capreomycin and kanamycin were suggested.[23, 24]

The Gene Xpert MTB/RIF assay, conventional culture and FLD-DST and LPA (Genotype MTBDR*sl)* are the choice of DR/MDR TB and XDR TB diagnostics tools. Culture and FLD-DST method takes usually longer time but always being considered as the gold standard that gives the viable organisms and can be used for various research purposes.

The occurrence of MDR TB among retreatment (Cat1 and Cat 2 treatment failures) cases alerts the NTP managers for prompt diagnosis of TB/DR/MDR/XDR TB using reliable and rapid diagnostics tools based on molecular biological techniques. The main objective of this study was to identify and compare the findings of DR/MDR TB using Gene Xpert MTB/RIF assay (*rpoB* gene mutations) with reference to culture and drug susceptibility test (DST) and XDR TB (gyrA, rrs and embB gene mutations) by line probe assay (LPA).

METHODOLOGY

The study was descriptive type cross sectional study to identify rifampicin resistant or multidrug resistant tuberculosis (RR/MDR TB) among retreatment TB cases using Gene Xpert MTB/RIF assay and to compare the prospective data obtained with reference to conventional culture and FLD-DST as

well as to identify XDR TB among those MDR TB cases by LPA (Genotype MTBDR*sl* for SLD-DST).

Study site

This study was carried out from April 2013 to October 2017 in NRL at NTC, Thimi, Bhaktapur, Nepal.

Sample size

Twenty nine (29) retreatment tuberculosis patients (Female 41.4%; all smear+ve, Male 58.6%; 1 smear-ve, 16 smear+ve) with median age of 40 years were involved in this study before they were registered for starting second line anti-tuberculosis treatment.

Study population

Retreatment pulmonary TB cases (relapse, treatment after failure, and treatment after loss to follow-up) previously treated with Cat I and Cat II treatment regimen as per National TB Programme (NTP) guidelines based on WHO recommendations were enrolled in this study.

Inclusion/exclusion criteria

Retreatment TB cases (Cat I and Cat II failure) visiting for further diagnosis and diagnosed as sputum smear positive or negative before being registered for and started MDR treatment were included in this study. But the cases already registered and recently undergoing second line anti tuberculosis treatment, blood stained sputum, sputum with food particles, with saliva in greater amount, leaking, dried or if not freshly collected and patients suspected of extra-pulmonary tuberculosis were excluded from this study. The samples showing contamination during culture were not further included in the study.

Sample collection

Twenty nine early morning sputum samples (stuffy and mucopurulent, 3-5ml each) were collected from retreatment TB patients in leak proof, wide mouthed, transparent and sterile 50 ml disposable plastic centrifuge tube (Falcon BD, USA); then were appropriately labeled and stored at refrigerated temperature (2-8°C) until dispatched or processed. Out of total 29 samples 16 were received; 11(F3/M8) from Nepal Anti TB Association (NATA) Biranagar Morang, 5(F4/M1) from BP Koirala Institute of Health Sciences (BPKIHS) Dharan Sunsari of the eastern development region. Similarly, the remaining 13 samples were received; 3(F2/M1) from National TB Centre (NTC) Thimi Bhaktapur, 3(F1/M2) from United Mission to Nepal Hospital (UMN) Lalgarh Janakpur and 7(F2/M5) from National Medical College (NMC) Birgunj Parsa of central development region. The samples from the centres other than NTC were transported through private courier and the duration of sample transportation was not more than 48 hours. Some patients being treated at National TB Centre (one of the MDR TB treatment centres in central development region) submitted fresh samples directly to NRL/NTC.

Sample processing

Sputum samples were processed inside a Biological Safety Cabinet class II (BSC-II AIRTECH, Japan) directly by adding twice the volume of 4.0% NaOH digestion method (modified Petroff's method), vortex mixed and left for 15 minutes at room temperature with occasional shaking. Then phosphate buffer (pH 6.8) were added up to level of 45 ml graduation mark, vortex mixed and centrifuged at 3000x g for 15 minutes in a refrigerated centrifuge at 4°C (KUBOTA, Japan).

Culture on Lowenstein Jensen (LJ) medium

The supernatants were discarded and pellets were used for culture; 0.2ml of pellet was inoculated on duplicate LJ media, incubated at 37°C for 4-8 weeks in an incubator (MEMMERT, Germany). The tubes were examined on 7th day for rapid growers and were checked for growth at 2, 3, 4, 5, 6, 7, 8 weeks until negative. If there was any contamination in the culture tube, was recorded.

Gene Xpert MTB/RIF Assay

From the remaining of the pellets, Gene Xpert MTB/RIF tests were performed by following the procedures provided by the manufacturers (Cepheid Sunnyvale, CA, USA, and Gene Xpert IV Cephied, France). Sputum pellets were decontaminated and treated with sample reagent (SR); a mixture of NaOH and iso-propanol. The SR was added at a 3:1 ratio to the sputum pellets and left for 15 minutes at room temperature; 2 ml of the treated samples were transferred to the Gene Xpert cartridges (Cephied, France), which were then loaded into the programmed Gene Xpert modules. Gene Xpert device was kept on and results were observed after the whole process completed (within about 2 hours).

Smear microscopy

A smear of the processed pellets was prepared (size of 2*3 cm), air dried at room temperature (RT), heat fixed, stained by Ziehl-Neelsen method and read under binocular light microscope at the total magnification of 1000X (Olympus, Japan) and reported according to NTP Nepal grading scale that is adopted exactly same scale from WHO/IUATLD grading scale.

Preparation of Bacillary Suspension and inoculation for DST

One looful (4mg approximately) of mycobacterial colonies grown on LJ media was harvested and emulsified with 1ml of sterile distilled water (SDW) in a sterile bijou bottle, vortex mixed and allowed to stand for 15 minutes, supernatant was transferred into a McCartney bottle. Turbidity of supernatant was compared with McFarland Standard No.1 Nephelometer (standardized at 1 mg/ml equivalent to 10^6-10^8 CFU/ml). Made 100 fold dilutions from McFarland Standard No.1 suspension; 1 loopful (nichrome wire loop 24 SWG and 3mm diameter delivering 0.01ml) of bacillary suspension was transferred to 1ml of SDW in bijou bottles and vortexed (10^{-2}: 10,000 CFU/ml), similarly 10^{-4}(l00 CFU/ml) was prepared. One loopful of each dilution (10^{-2} and 10^{-4}) was inoculated on two slopes of plain LJ medium (controls) and one set each of slopes with 4 drugs (streptomycin, isoniazid, rifampicin, ethamputol), incubated at 37°C, read on 4th and 6th week for resistant (growth on drug medium 1% colonies on control) and final susceptible (no or <1% colonies on control) patterns respectively.

Biochemical identification tests

From the positive growth, identification tests were performed by biochemical methods i.e. growth on PNB containing LJ medium and niacin production tests.

1. *Growth on PNB containing media*

A loopful of neat bacterial suspension (McFarland standard No. 1) was inoculated into one slope of LJ medium and one slope of p-nitrobenzioc acid (PNB) at a concentration of 500 g/ml and incubated at 37°C for each set. Read on 28th day.

M. tuberculosis does not grow but all other mycobacteria are resistant to PNB.

M. tuberculosis $H_{37}Rv$ as negative control (PNB susceptible) and *M. kansasii* as positive control (PNB resistant) were used.

Results and interpretations

No growth on PNB medium: *M. tuberculosis*

Growth on PNB medium: *M. kansasii*

2. *Niacin production test*

BBL Taxo TB Niacin test strips (Becton and Dickinson, USA), absorbent paper strips and TB niacin positive test control paper discs were used according to the manufacturer's instruction. With a sterile transfer pipette, approximately 0.6 ml of the positive culture broth extract was transferred to the bottom of 20 mm × 125 mm screw cap test tube. Negative control was also prepared. The strips were dropped with arrow downward into the tubes. Positive controls, negative controls and test culture tubes were recapped immediately. The colors of the extracts were then compared after 15 minutes.

M. tuberculosis $H_{37}Rv$ as positive control and *M. kansaii* as negative control were used. Niacin accumulation was indicated by vivid appearance of a yellow color in the extract.

Results and interpretation

M. tuberculosis $H_{37}Rv$: yellow colour (niacin positive)

M. kansaii: colourless (niacin negative)

All the positive cultures have shown PNB negative and niacin positive.

Drug Susceptibility Test on First Line Drugs (proportion method)

Drug susceptibility test (DST) on first line anti tuberculosis drugs (FLD); streptomycin(4.0 µg/ ml), isoniazid(0.2 µg/ ml), rifampicin(40.0 µg/ml), and ethambutol(2.0 µg/ml) (SIRE; manufactured by SIGMA-Aldrich, USA) was performed on all the culture positive samples in duplicated drug tubes as well as two LJ slopes without drug (control) using 1% proportion method. Internal quality control was routinely performed (for each batch of new drug media) using the reference strain *M. tuberculosis* H37Rv (ATCC-27294), which was susceptible to all the 4 drugs. All the inoculated tubes were then incubated at 37°C, resistance pattern of the SIRE was checked at 4th week and 6th week.

Drug Susceptibility Test on Second Line Drugs by Line Probe Assay

The whole process of the LPA was followed as per the guideline provided by the manufacturer-MTBDR*sl* 96 version 1.0 (HAIN Life Science, GmbH Germany) following the steps of DNA extraction, PNM mix, amplification by PCR, hybridization and detection as mentioned below:

1. DNA extraction

Homogenized bacterial suspension was prepared by harvesting 1-2 colonies of organisms from LJ tube with sterile inoculating wire loop inside a BSC-IIA (Micro Flow, Bioquell, UK), re-suspended in 300 µl of molecular grade water in a cryovial (1.5-2ml), mixed by vortexing (SONAR, India), heat inactivated at 95°C for 20 minutes, incubated in an ultrasonic water bath (LABTECH, India) for 15 minutes, centrifuged for 5 minutes at 13000*g (Microfuge, KINTARO) and supernatant containing DNA was transferred to another cryotube and stored at 4°C to -20°C until processed in a refrigerator (SANYO, Japan).

2. Primer nucleotides mix (PNM)

With micropipettes 35µl of PNM, 5µl of 10x buffer (15mM MgCl2), 2 µl MgCl2 (25mM), 3 µl H_2O, 0.2 µl Taq polymerase (Hot star Thermis aqaticus) were added into a cryotube and mixed well carefully. Prepared a master mix for the determined number of samples, mixed and aliquoted (45 µl) in 1.5ml PCR tubes. PNM process was completed inside a LPA Safety Hood (LAB COMPANION). The molecular grade water to bring the master mix to volume was used as conjugate control (CC) and the LPA strip (functions as both the internal "PCR positive control" and the "inhibition positive control) was used as amplification control (AC).

3. Amplification by PCR (Thermal Cycler, USA)

To the aliquoted 45 µl master amplification mix, 5 µl DNA was added (inside a BSC-IIA), gently vortexed to mix, placed into the thermal cycler (Genotype Hot 30 specific progamme). The DNA amplification was performed for 30 cycles following an initial denaturation; 10 cycles of initial denaturation followed by denaturation at 95°C for 30 seconds, chain elongation at 58°C for 2 minutes followed by additional 20 cycles of denaturation at 95°C for 25 seconds, primer annealing at 53°C for 40 seconds and elongation at 70°C for 40 seconds and final extension at 70°C for 8 minutes.

4. Hybridization and Detection

The hybridization buffer (HYB) and stringent wash solution (STR) were prewarmed at 45°C to dissolve the undissolved precipitates, rinse solution (RIN) and DW were prewarmed at RT, freshly diluted Con-C and Sub-C 1:100 in the respective dilution buffer and protected from light. Twincubator set with P1 programme was used for Hybridization and Detection.

4.1 Hybridization probe

Denaturing buffer (DEN) 20 µl was added with 20 µl amplicon, mixed well and incubated for 5 minutes at RT on the shaking platform, 1ml HYB was added using micropipette and filter tips, mixed by tilting the tray back and forth carefully (purple DEN and green HYB mixed well), tray was then placed on the TwinCubator (HAIN Life Science, GmbH Germany). DNA strip was placed in each well (with a forceps) of TwinCubator tray and covered by the liquid. When the temperature reached to 45°C, tray cover was closed and incubated for 30 minutes at 45°C.

4.2 Detection probe

HYB was aspirated completely and 1 ml STR was added and incubated for 15 seconds at 45°C, STR removed completely, 1ml RIN added, incubated for 1 minute at RT, RIN removed completely, 1ml diluted conjugate (10 µl Con-C /conjugate C and 990 µl Con-D/conjugate D) were added and incubated for 30 minutes at RT and conjugate was removed completely. Added 1ml RIN, incubated for 1minute at RT, removed RIN completely and rinsed with H_2O for 1 minute, 1ml diluted substrate added (10 µl Sub-C and 990µl Sub-D), incubated 2-10 minutes at RT and removed substrate completely. Stopped reaction by rinsing twice with H_2O for 1 minute then removed DNA strip from tray and dried it on absorbent paper. Detection process was completed using a TwinCubator (HAIN Life Science, GmbH Germany).

Individual strip after colour development was adhered to the corresponding column of the HAIN Life Science, GmbH Germany provided format and resistance pattern was identified. The original strips showing positive bands were kept for NRL record after being scanned for the present study purpose (Figure 1).

Statistical data analysis

The statistical analysis of the study data were analyzed using SPSS version 16.0 software. The Chi square test was used to compare age and sex wise distribution of negative and positive sputum smear results, smear and culture results, MDR TB identified by Gene Xpert MTB/RIF assay and conventional culture and FLD-DST results, identification of XDR/ pre XDR TB cases by LPA (MTBDR*sl*). The *P*-value <0.05 was considered statistically significant.

RESULTS

The sputum smear microscopy results for female (12/41.4%) were all +ve, whereas for male (17/58.6%); 16 +ve and 1-ve. The age wise distribution of smear results was; 3.4% was smear-ve in 15-29 years and 51.7%, 20.7%, 13.8% and 10.3% in 15-29, 30-45, 46-60 and above 60 years of age groups respectively were smear positives (Table 1).

Table 1: Age (years)/sex wise distribution of retreatment TB cases from different treatment centres

Treatment centre	15-29		30-45		46-60		>60		Total		Remarks
	F	M	F	M	F	M	F	M	F	M	
E+E1	3	2		2		2		2	3	8	*NATA Morang*
E2	2		2			1			4	1	*BPKIHS*
C	2	1							2	1	*NTC*
C1	1	2							1	2	*UMN Lalgarh*
C2	1	2	1	1		1		1	2	5	*NMC Parsa*

A total of 29 freshly collected good quality sputum samples from 5 DR/MDR treatment centres; E+E1: Nepal Anti TB Association (NATA) Morang, 11 (F3/M8) samples and E2: BP Koirala Institute of Health Sciences (BPKIHS) Sunsari Eastern Nepal; 5 (F4/M1) samples. C: National TB Centre (NTC) Kathmandu; 3 (F2/M1) samples, C1: United Mission to Nepal Hospital (UMN) Lalgarh Dhanusha; 3 (F1/M2) samples and C2: National Medical College (NMC) Birgunj Parsa Central Nepal; 7 (F2/M5) samples. There was no significant difference of age and sex wise smear results (p>0.05). All 29 sputum specimens showed rifmapicin resistance (RR/MDR) by Gene Xpert MTB/RIF assay in which 55.2%, 20.7%, 13.8% and 10.3% in 15-29, 30-45, 46-60 and above 60 years age group and 41.4%, 59.6% females and males respectively (Table 2).

Table 2: Age (years)/sex wise distribution of MDR TB cases diagnosed by Gene Xpert from different treatment centres

	15-29		30-45		46-60		>60		Total		Grand total	Remarks
	F	M	F	M	F	M	F	M	F	M		
	3	2		2		2		2	3	8	11	NATA Morang
	2		2			1			4	1	5	BPKIHS
	2	1							2	1	3	NTC
	1	2							1	2	3	UMN Lalgarh
	1	2	1	1		1		1	2	5	7	NMC Parsa
Total	9	7	3	3		4		3	12	17	29	

The age wise distributions of MDR TB by Gene Xpert MTB/RIF assay for 15-29 years, 30-35 years, 46-60 years and above 60 years of age group were 55.2%, 20.7%, 13.8% and 10.3% respectively. Similarly, sex wise MDR TB detection by Gene Xpert was 41.4% and 59.6% for females and males respectively. Similarly, all 29 specimens showed positive growth for all age and sex groups on LJ culture media (Table 3). There was no significant difference of age and sex wise smear and culture results (p>0.05).

Table 3: Age (years)/sex wise Comparison of smear and culture results

Results	Age and sex distribution										Grand Total culture positive
	15-29		30-45		46-60		>60		Total culture positive		
	F	M	F	M	F	M	F	M	F	M	
S+ C+	9	7	3	3	0	4	0	3	12	16	28
S+ C-	0	0	0	0	0	0	0	0	0	0	0
S- C+	0	1	0	0	0	0	0	0	0	1	1
S- C-	0	0	0	0	0	0	0	0	0	0	0
Contamination	0	0	0	0	0	0	0	0	0	0	0

A. ^{2} for trend of age wise smear results=0.842, df=3, P value=0.840 (>0.05), so there was no significant difference of age wise smear results.

B. ^{2} for trend of sex wise smear results=0.731, df=1, P value=0.393 (>0.05), so there was no significant difference of sex wise smear results.

The culture positives were biochemically (PNB-ve, niacin test+ve) identified as *M. tuberculosis* which were then processed for FLD-DST following proportion method. Twenty eight (96.6%; 11F/17M; ss-ve 1M, 11 F and 16M ss+ve) of 29 FLD-DST) showed both INH and RIF resistance, 1 case (3.4%/M) detected as RRTB by Gene Xpert MTB/RIF showed mono resistance to isoniazid only (Table 4).There was no significant difference of age and sex wise MDR TB cases identified by conventional FLD-DST for smear results (p>0.05).

Table 4: Age (years)/sex wise distribution of FLD-DST patterns

DST patterns of FLDs by conventional proportion method											
	15-29		30-45		46-60		>60		Total		Grand total
	F	M	F	M	F	M	F	M	F	M	
Total Tested	9	7	3	3	0	4	0	3	12	17	29
Fully Susceptible	0	0	0	0	0	0	0	0	0	0	0
Any Resistance	1	0	0	0	0	0	0	0	1	1	
Mono Resistance	1	0	0	0	0	0	0	1	1	1	1
S	0	0	0	0	0	0	0	0	0	0	0
I	1	0	0	0	0	0	0	0	1	0	1
R	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0
Total I+ R Resistance (MDR)	8	7	3	3	0	4	0	3	11	17	28
IR	2	2	0	2	0	0	0	0	2	4	6
IRE	1	0	0	0	0	0	0	1	1	1	2
SIR	1	3	2	0	0	2	0	1	3	6	9
SIRE	4	2	1	1	0	2	0	1	5	6	11

S: streptomycin I: isoniazid R: rifampicin E: ethambutol

A. ^{2} for trend of age wise MDR TB detected by culture and DST results=0.842, df=3, P value=0.840 >0.05, so there was no significant difference of age wise MDR TB detection by culture and DST.

B. ^{2} for trend of sex wise MDR TB detected by culture and DST results=1.467, df=1, P value=0.226 >0.05, so there was no significant difference of sex wise MDR TB detection by culture and DST.

All the MDR TB cases detected by Gene Xpert MTB/RIF assay and/or FLD-DST were performed for DST on second line anti-TB drugs (SLD-DST) by LPA. During the process of LPA, negative controls were developed at CC and AC bands in the strips. MDR TB strains showed TUB bands formation in the strips. Similarly, 17(58.6%, F5/M12) for *gyrA* WT1, WT2, WT3 probes located in regions from codons 85 to 97 (binding sites for fluoquinonlones / ofloxacin or levofloxacin), 29(100%, F12/M17) for *rrs* WT1, *rrs* WT2 probes located in regions for nucleotides 1401,1402 and 1484 (binding sites for injectable aminoglycoside / capreomycin), and 13(44.8%, F4/M9) for *emb B* WT1 (binding site for ethambutol) gene probes located in regions from codons 306 were found to be susceptible sites for the corresponding drugs. Whereas, the DNA matched with the mutant probes; 12(41.4%, F6/M6) were mutants for *gyrA* gene (*gyrA* MUT1, MUT2, MUT3A to 3D) conferring most frequently mutation occurring codons (A90V, S91P, D94A, D94N/Y, D94G, and D94H), 16(51.2%, F8/M8) for *emb B* (*embB* MUT1A, MUT1B) probes conferring mutations M306V and M306I and none were for AG/CP or *rrs* genes (*rrs* MUT1, MUT2) probes conferring mutations for A1401G and G1484T. Similarly, *gyrA* gene mutations together with *embB* gene that were regarded as fluoroquinolones and ethambutol resistance (*embB* MUT1B) were found in 9 cases (31.0%, F5/M4). Any case showing single *gyrA* gene mutation or *gyrA* gene mutation together with or without other gene mutations was interpreted as pre-XDR TB (Figure 1).

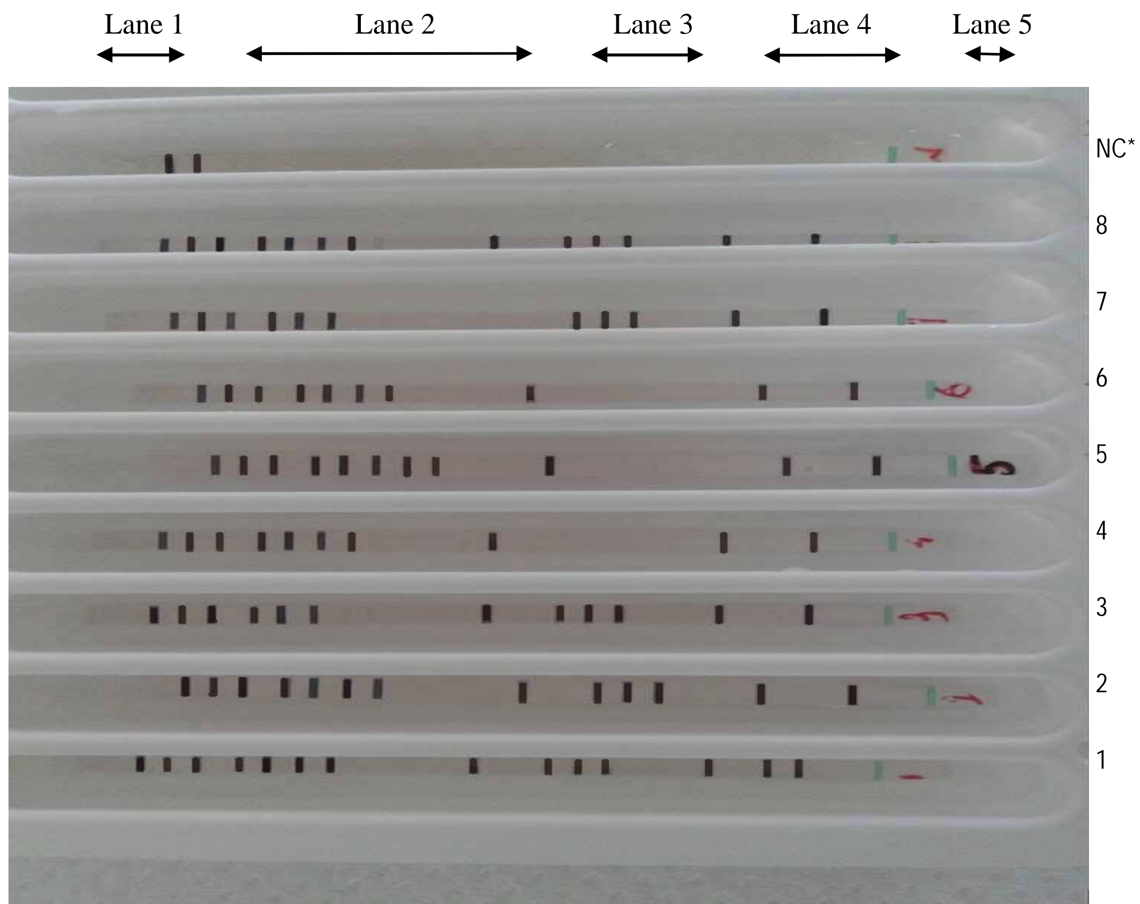

Figure 1: Hybridization process and development of different bands on MTBDR*sl* strips
Lane 1: CC/AC/TUB *(CC: conjugate control, AC: amplification control, TUB: tuberculosis)*
Lane 2: *gyrA* (WT 1-WT 3/MUT 1- MUT 2, MUT 3A- MUT 3D) (*WT: wild type, MUT: mutation)*
Lane 3: *rrs* (WT1/MUT 1-MUT 2)
Lane 4: *embB* (WT 1/MUT 1A- MUT 1B)
Lane 5: M *(Marker)*

A total of 12(43.4%; F6/M6) among 29 MDR confirmed *M. tuberculosis* strains were found to be pre-extensively drug resistant (pre-XDR-TB), but no XDR TB. The age/sex wise distribution of the SLD-DST pattern showed maximum number of pre-XDR TB among 15-45 years of age groups and similar for both females and males (50%; 4F and 2M in 15-29, 33.3%; F2 and 2M in 30-45, 8.3%; M1 in 46-60 and 8.3%; M1 above 60 years). The mutations detected for *emb B* (ethambutol) genes were also similar for both females and males (F8/M8). Twelve cases thus identified as pre-XDR TB were from the MDR TB treatment centres providing treatment to the MDR TB patients were; 4 (F1/M3) out of 11 (F3/M8) from Nepal Anti TB Association (NATA) Biranagar Morang, 1 (F1) of 5 (F4/M1) from BP Koirala Institute of Health Sciences (BPKIHS) Dharan Sunsari of the eastern development region. Similarly, 1(F1) of 3 (F2/M1) from National TB Centre (NTC) Thimi Bhaktapur, 2 (F1/M1) of 3 (F1/M2) from United Mission to Nepal Hospital (UMN) Lalgarh Janakpur and 4 (F2/M2) of 7 (F2/M5) from National Medical College (NMC) Birgunj Parsa of central development region, were found to be pre-XDR TB (Table 5). There was no significant difference between XDR TB detection by LPA on MDR TB identified by Gene Xpert and FLD-DST (p>0.05).

Table 5: Age (years)/sex wise distribution of SLD-DST patterns by Line Probe Assay on MDR TB detected by Gene Xpert and FLD-DST

Description	15-29		30-45		46-60		>60		Total		Remarks
	F	M	F	M	F	M	F	M	F	M	
TUB	9	7	3	3		4		3	12	17	29
gyrA WT	4	5	1	1		3		3	5	12	17
gyrA MUT	4	2	2	2		1		1	6	6	12
rrs WT	9	7	3	3		4		3	12	17	29
rrs MUT											0
emb BWT	4	4		1		2		2	4	9	13
emb BMUT	5	3	3	3		1		1	8	8	16
Treatment centre	Patients tested for SLDST										Remarks
*E+E1	3	2		2		2		2	3	8	NATA Morang
E2	2			2		1			4	1	BPKIHS
**C	2	1							2	1	NTC
C1	1	2							1	2	UMN Lalgarh
C2	1	2		1		1		1	2	5	NMC Parsa
Treatment centre	Patients diagnosed as Pre-XDR TB										Remarks
E+E1	1			1		1		1	1	3	NATA Morang
E2			1						1		BPKIHS
C	1								1		NTC
C1	1	1							1	1	UMN Lalgarh
C2	1	1	1	1					2	2	NMC Parsa

** E+E1, E2: NATA Morang and BPKIHS Eastern Nepal. ** C, C1, C2: NTC, UMN and NMC of Central Nepal.*

From NATA Morang 4 (F1/M3), BPKIHS 1 (F1), NTC 1 (F1), UMN Lalgarh 2 (F1/M1) and NMC Parsa 4 (F2/M2) were found to be pre-XDR TB. ^{2} for trend of MDR TB by Gene Xpert, culture and DST and XDR TB detection by LPA=0.73, df=1, P value=0.393 (>0.05), so there was no significant difference between XDR TB detection by LPA on MDR TB by Gene Xpert and culture and DST.

The age wise distributions of pre-XDR TB by LPA on MDR TB by Gene Xpert MTB/RIF assay and FLD DST for 15-29 years, 30-35 years, 46-60 years and above 60 years of age group were 50.0%, 33.3%, 8.3% and 8.3% respectively. Similarly, sex wise MDR TB detection by Gene Xpert was 50.0% and 50.0% for females and males respectively.

DISCUSSION

Microscopy is still familiar as a main diagnostic technique of diagnosing tuberculosis in resource-limited countries including Nepal. Due to shortcomings of conventional technique, novel molecular techniques are needed that combine the rapidity of microscopy and the sensitivity of culture. They can identify the mycobacterial species, and would help the clinician during the initial treatment of the patient. Though molecular techniques are not used routinely in Nepal, some investigators reported its feasibility (Sapkota et al 2007).[26]

To combat the excess mortality related to XDR-TB, it is recommended to perform DST for second line-drugs to all MDR-TB cases at the start of treatment. To comply with the above suggestion, it seems important for Nepal to strengthen its capacity to perform SLD-DST, either by culture (solid/liquid) or molecular biology e.g. Line Probe Assay (NTP Annual report Nepal 2015).[4] In order to overcome such problems, present study has evaluated a study of sputum specimens collected from retreatment TB cases by Gene Xpert MTB/RIF assay for the rapid diagnosis of DR/MDR TB. All the MDR TB cases detected by Gene Xpert MTB/RIF assay were further verified by conventional culture and DST for first line anti-tuberculosis drugs (FLD-DST). MDR TB diagnosed by both the methods were analyzed for second line drugs (SLD-DST) LPA.

In the present study, despite of smear results (1 ss-ve and 28 ss+ve), Gene Xpert MTB/RIF assay

showed 100% rifampicin resistance (RR/MDR TB), which was high among 15-60 years and in males (58.6%) than in females (41.4%). In previous study, 27 ss+ve and 23 ss-ve specimens were found to be RR/MDR TB detected by Gene Xpert MTB/RIF, whereas 8 cases were negative for MTB among ss-ve. The study report published previously for smear microscopy and Gene Xpert has revealed the similar results.[27]

In this study, concordance of sputum smear results and detection of *M. tuberculosis* along with rifampicin resistance by Gene Xpert MTB/RIF assay for all 29 samples was 100%.

The assay was successful in rapidly detecting *M. tuberculosis* as well as rifampicin susceptibility pattern. Whereas, it was reported in the similar study by Helb et al (2010)[12] from 107 sputum samples in Vietnam that the concordance for ss+ve, Gene Xpert MTB/RIF (RR TB) and culture was 100%(29/29). In the same study, it was described that 64 smear-positive sputa from retreatment tuberculosis cases in Uganda tested by Gene Xpert MTB/RIF assay detected *M. tuberculosis* among 63/64(98.4%) were also found to be culture-positive and rifampin resistance. Similar results were obtained out of total 62 pulmonary TB cases in previous study.[28] But in this study, out of 29 cases detected as MDR TB by Gene Xpert MTB/RIF assay, all the samples were found to be showing positive growth, none were contaminated. Sputum smear +ve and culture positive results were found higher in 15-60 years (even in >60 years) and in males (16) than in females (12). The distribution of ss+ve and culture results was 28(12 F/16M) and 1 male case was ss-ve out of total 29 culture positives.

Twenty eight of 29(96.6%) culture positive samples on drug susceptibility test (DST) showed both INH and RIF resistance, 1 case (3.4%) detected as RRTB by Gene Xpert MTB/RIF showed mono resistance to isoniazid only. Marlowe et al (2011)[14] has reported similar results previously. The age and sex wise distribution of MDR TB by FLD-DST was high in 15-60 years group (even in above 60 years) in males compared to females. It has been reported in one study by Rijal et al (2005)[29] that the MDR TB among previously treated patients was 19.25% (n=161) irrespective of age and sex variation.

Mboowa et al (2014)[30] stated that the resistance was conferred by four different *rpoB* gene mutations in the 81 bp rifampicin resistance detection region (RRDR) of MTB. These were detected by probes A, B, D, and E. It has also been mentioned in previous study that 96.1% *rpoB* gene mutations located in a region of 426-452 amino acid residues (81bp) of MTB *rpoB* gene (RRDR) detected by probes A-E using Gene Xpert MTB/RIF assay.[31] In this study also it can be revealed that all the MDR TB identified by Gene Xpert MTB/RIF assay has detected 100% *rpoB* gene mutations in 81bp RRDR of MTB. Majority of the MDR TB identified by both Xpert and conventional FLD-DST were males. Male dominated MDR-TB results had been described in a similar study previously.[28]

In the present study, all 29 MDR TB cases were performed for DST on second line anti-TB drugs (SLDs) by LPA. As it was mandatory for the two negative controls must be positive only at CC and AC bands in the strips that were clearly formed in the strips used in this study, so the test process was valid. Among 29 *M. tuberculosis* strains confirmed as MDR TB, all were (100%) showing TUB (*M. tuberculosis* complex). It was found that the *M. tuberculosis* probe was 100 per cent specific. All were found to be wild type for *rrs* WT1, *rrs* WT2, 17(58.6%, F5/M12) for *gyrA* WT1, WT2, WT3 and 13(44.8%, F4/M9) for *emb B* WT1 genes, whereas 12 (41.4%, F6/M6) were mutants for *gyrA*, 16(51.2%, F8/M8) for *emb B*) and none were for *rrs* genes. The mutation of *gyrA* gene was detected by the formation of positive band on the nitrocellulose membrane strip (*gyrA* MUT3A and MUT3C) alone and interpreted as fluoroquinolone/FLQ resistance in 12 cases (F6/M6). Similarly, gene mutations of *gyrA* together with *embB* gene in 9 cases (F5/M4). Any case showing single *gyrA* gene mutation or *gyrA* gene mutation together with *embB* gene was interpreted as pre-XDR TB. Seven out of 29 strains (24.1%) were found to be susceptible to all drugs (F3/M4) but none were XDR TB.

There were 12 *gyrA* mutations identified was high among 15-60 years group that were equally distributed among females and males (F6/M6) and 9 *gyrA* and *embB* gene mutations found to be high among 15-60 years group. The result showed that the distribution of pre XDR TB was higher in the male age group of 15-60 years, among which 1 case was ss-ve, which may suggest us to screen ss –ve retreatment TB cases frequently. Whereas, age wise distribution of pre-XDR TB in female was found to be higher in 15-45 years group (6 cases; all ss+ve). The sex wise pre-XDR TB was identified similar in females/males (F6/M6). None of the age or sex group showed pre-XDR TB below 15 years.

Four pre-XDR TB cases identified were from NATA Morang, 1from BPKIHS Sunsari, 1 from NTC, 2 from UMN Hospital Lalgargh and 4 from NMC Parsa. All the 12(41.4%: F6/M6) among 29 MDR confirmed *M. tuberculosis* strains were found to be *gyrA* gene mutations (pre-XDR-TB). The age/sex wise distribution of the SLD-DST pattern showed maximum number of pre-XDR TB among 15-45 years of age groups and was similar for both sexes.

All the 29 MDR TB cases were found to be *rrs* gene wild type. It has been revealed by the previous similar study.[32] It is now clear that the pre-XDR TB is prevalent and scattered in all the MDR TB treatment centres in Nepal.

CONCLUSIONS

Gene Xpert MTB/RIF assay as it is a useful method of simultaneous detecting MTB and rifampicin resistance (surrogate marker of MDR-TB) in both the sputum negative and positive samples along with the culture and DST as reference gold standard.

From this study, out of 29 retreatment TB patients enrolled, 100% were detected RR/MDR TB by Gene xpert MTB/RIF assay irrespective of sputum smear results and all were found to be culture positive. All the culture positive strains were identified as to resist isonoazid and rifampicin with or without remaining drugs resistance. All the 29 MDR TB strains were tested for SLD-DST using LPA (Genotype MTBDR***sl***) and 12 out of 29 MDR TB strains were found to be pre-XDR TB as well. The prevalence of MDR and/or pre-XDR TB was higher in the 15-60 years age group and distributed in both the females and males.

RECOMMENDATIONS

It is recommended that all the samples submitted for microscopic examination should be further processed for culture and DST, if the laboratory setting is capable to do. If not so, all the Cat1 treatment failures as well as Cat 2 treatment failure cases should be tested for RR/MDR TB using Gene Xpert MTB/RIF assay and culture and FLD-DST. Simultaneously, all the MDR-TB cases confirmed by Gene Xpert MTB/RIF assay and culture and FLD-DST should be further tested for SLD-DST by various culture/DST methods and Genotype MTBDR***sl*** to identify pre-XDR and/or XDR TB. This way, prompt diagnosis of TB/DR-MDR TB/XDR TB can be made possible and patient's treatment management as well. Further study should be frequently performed at the national, regional or provincial level on higher samples.

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