

Gene Xpert based detection of drug resistant tuberculosis among retreatment patients visiting National Tuberculosis Centre, Nepal

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Abstract

Introduction: Tuberculosis is still one of the major public health problems in Nepal and multi drug resistant and extensively drug resistant tuberculosis (MDR/XDRTB) additionally has become serious issue. Prompt diagnosis and effective treatment of MDR/XDRTB is urgently needed. The main objective of this study was to detect MDR TB using novel molecular techniques (*rpob* gene mutations) in reference with drug susceptibility test (DST).

Methods: A cross sectional study was carried out identifying MDRTB among retreatment patients using Gene Xpert, culture and DST on first line drugs (FLD-DST). A total of 159 sputum samples were collected from retreatment TB patients (Female 40.3%, Male 59.7%) with median age of 30 years visiting to the DR 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 August 2017.

Results: *M. tuberculosis* and rifampicin resistance were detected on all 159 (100%) samples by Gene Xpert of which, 73.3%, 21.4% and 6.3% were positive, negative and contaminated respectively by culture. FLD-DST was performed on 115 cultures positives of which, 94.78% showed MDRTB, 1.74% showed mono resistance to isoniazid or rifampicin, 0.87% to streptomycin and isoniazid and 3.47% were pan susceptible.

Conclusion: One hundred fifteen of 159 cases detected rifampicin resistances (RR) by Gene Xpert were culture positive and almost 95% strains were MDRTB by FLD-DST, which was found to be higher in 15-60 years group. Sputa from retreatment TB patients required to be tested by rapid diagnostics with reference to culture and DST.

Key words: Gene Xpert, Culture, Drug Susceptibility Test, Multi Drug Resistant Tuberculosis, Sputa.

Introduction

Tuberculosis is a global threat because nearly two billion people (one third of the world's population) harboring latent infection. In 2014, 9.6 million people fell ill with TB and 1.5 million died from the disease. An estimated 9.7% of people with MDRTB have XDRTB.¹ The SAARC region, with 34% of the global burden of TB, where a total of 81,142 estimated cases of MDRTB among notified cases of which, 41% were

new pulmonary cases and 59% were previously treated cases.²

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%). Nationwide, the proportion of new cases with MDRTB 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 MDRTB and 25 XDR TB were enrolled for treatment. WHO estimated 4.6 (2.1-7.5) thousand people died from TB in 2014.³

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 MDRTB.⁴

More than 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.⁵ This region is therefore an ideal target for molecular tests for RIF resistance.^{6,7,8}

If a diagnosis is absent, patients are not treated, transmission may continue, patients suffer needlessly and may eventually die. Sputum smear microscopy, as a diagnostic tool of tuberculosis, is varying between 30% and 70% depending on a number of factors relating to how the test is implemented.⁹

Cepheid (Cepheid, Sunnyvale, CA) has recently introduced the GeneXpert MTB/ RIF assay, which is a real-time PCR test that will simultaneously identify *M. tuberculosis* and detect rifampin resistance directly from clinical specimens.^{10,11} Rifampin resistance can serve as a marker for MDRTB and has been reported in 95% of the MDR TB isolates.¹² The GeneXpert assay detects an 81-bp "core" region of the *rpoB* gene.¹³

The Gene Xpert MTB/RIF assay, conventional culture and FLD-DST are the choice of DR/MDR 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. So, the present study has been giving priority to identify MDRTB among retreatment cases using Gene Xpert MDR/RIF assay with reference to conventional culture and FLD-DST.

Methodology

The study was a cross sectional study design to identify RR/MDRTB among retreatment TB cases using Gene Xpert MTB/RIF assay and to compare the prospective data obtained with reference to conventional culture and proportional FLD-DST.

Study site: This study was carried out from April 2013 to November 2017 in National Tuberculosis Reference Laboratory at National Tuberculosis Centre (NRL/NTC), Thimi, Bhaktapur, Nepal.

Sample size: One hundred and fifty nine (159) retreatment tuberculosis patients 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 regimen were enrolled in this study.

Patient's consent: The samples were collected and examined at NRL/NTC regularly from the same selected sites before starting this study.

Inclusion/exclusion criteria: Retreatment TB cases 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. 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 excluded from the study.

Sample collection

Early morning sputum samples (stuffy and mucopurulent, 3-5ml each) were collected from 159 retreatment TB patients out of which, 76 (F23/M53) from Nepal Anti TB Association (NATA) Biratnagar Morang and 19 (F8/M11) from BP Koirala Institute of Health Sciences (BPKIHS), Dharan, Sunsari of the eastern Nepal, 24 (F11/M13) from United Mission to Nepal Hospital (UMN), Lalgarh, Janakpur, 18 (F7/M11) from National Medical College (NMC), Birgunj, Parsa, and 22 (F15/M7) from National TB Centre (NTC), Thimi, Bhaktapur of central Nepal) in leak proof, wide mouthed, transparent and sterile 50 ml disposable plastic centrifuge tube (Falcon BD, USA), appropriately labeled and stored at 2-8°C until dispatched or processed. The samples were transported through private courier and the duration of sample transportation was not > 48 hours to reach to NRL, but patients being treated at NTC had submitted fresh samples directly to NRL. The age, sex and smear result wise data are shown in table 1.

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Table 1: Age /sex wise distribution of retreatment TB cases enrolled in this study

Age (years)	Sex		Total N (%)
	Female N (%)	Male N (%)	
<15	3 (1.9)	0 (0.0)	3 (1.9)
15-29	34 (21.4)	41 (25.8)	75 (47.2)
30-45	14 (8.8)	21 (13.2)	35 (22.0)
46-60	10 (6.3)	22 (13.8)	32 (20.1)
>60	3 (1.9)	11 (6.9)	14 (8.8)
Total	64 (40.3)	95 (59.7)	159 (100)
Age range (Min-Max)	11-72		
Mean age (years) ± SD	35.86 ±15.95		
Median age (years)	30		

Sample processing and culture on Lowenstein Jensen (LJ) medium

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) was 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).

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 checked for growth at 2, 3, 4, 5, 6, 7, 8 weeks until negative. If any contamination seen in the culture tube, that was recorded.

Gene Xpert MTB/RIF assay

Gene Xpert MTB/RIF tests were performed as per the instructions provided by the manufacturers (Cepheid Sunnyvale, CA, USA). Sputum pellets were decontaminated and treated with sample reagent (SR; a mixture of NaOH and iso-propanol) as to make 3:1 ratio to the pellets and left for 15 minutes at room temperature; 2 ml of the treated samples were transferred to the Gene Xpert cartridges (Cepheid, France), and 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).

Microscopy observation

A smear of the processed sediment was prepared (size of 2 x 3 cm), air dried, heat fixed, stained by Ziehl-Neelsen method and read under binocular light microscope at the total magnification of 1000X (Olympus, Japan) and reported according WHO/IUATLD grading scale.

Preparation of bacillary suspension and inoculation for DST (1% proportion method)

One loopful (4mg approximately) of mycobacterial colonies from 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, transferred to a McCartney bottle; turbidity was compared and adjusted with McFarland standard no.1 Nephelometer (1 mg/ml or 10⁶-10⁸ CFU/ml of bacteria). Made 100 fold dilutions from McFarland standard no.1 bacillary suspension; 1 loopful (nichrome wire loop 24 SWG and 3mm diameter delivering 0.01ml) was transferred to 1ml of SDW in Bijou bottles and vortexed to make 10⁻² dilution or 10,000 CFU/ml, from which 10⁻⁴ dilution (100 CFU/ml) was prepared.

One loopful of each dilution (10^{-2} and 10^{-4}) was inoculated on two plain LJ slopes (controls) and one set each of slopes with 4 drugs (streptomycin (S); 4.0 µg/ml, isoniazid (I); 0.2 µg/ml, rifampicin (R); 40.0 µg/ml, ethambutol (E); 2.0 µg/ml or SIRE; manufactured by SIGMA-Aldrich, USA), incubated at 37°C, read on 4th and 6th week for resistant (growth on drug medium $\geq$ 1% colonies on control) and final susceptible (no or <1% colonies on control) patterns respectively. Internal quality control was routinely performed (for each batch of new drug media) using the pan-susceptible reference strain *M. tuberculosis* H37Rv (ATCC-27294).

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 *p*-nitrobenzioc acid (PNB) containing media

A loopful of neat bacterial suspension (McFarland standard No. 1) was inoculated into one plain and other slope of LJ with PNB at a concentration of 500 µg/ml and incubated at 37°C for each set and read on 28th day. *M. tuberculosis* H₃₇Rv as negative (PNB susceptible) and *M. kansasii* as positive control (PNB resistant) were used.

Results and interpretation No growth on PNB medium: *M. tuberculosis*; growth on PNB medium: *M. kansasii*

2. Niacin production test

Niacin test was performed according to the manufacturer's instruction. With a sterile transfer pipette, approximately 0.6 ml of the positive culture extract was transferred to the bottom of 20 mm × 125 mm screw cap test tube along with *M. tuberculosis* H₃₇Rv as positive and *M. kansasii* as negative control. The niacin test strips (BBL Taxo TB strips, Becton and Dickinson, USA), were dropped with arrow downward into the tubes. The colors of the extracts were then compared after 15 minutes.

Results and interpretation: *M. tuberculosis* H₃₇Rv: yellow colour (niacin positive); *M. kansasii*: colourless (niacin negative). All the cultures have shown PNB negative and niacin positive.

Statistical data analysis

The statistical analysis of the study data were analyzed using SPSS version 16.0 software. The χ^2 test was used to compare age and sex wise distribution of smear and culture, Gene Xpert and culture and FLD-DST results. The *P*-value <0.05 was considered statistically significant. The sensitivity, specificity, PPV, and NPV of the GeneXpert and FLD-DST were calculated using MedCalc software and 95% confidence intervals were estimated.

Results

Out of 159 (64 F; 40.3%, 95 M; 59.7%, Male:Female ratio 1.48:1), 42 (13F/29M; 26.4%) and 117 (51F/66M; 73.6%) were sputum smear negative (ss -ve) and sputum smear positive (ss +ve) respectively. The grading was high positives for 111 cases (1+, 2+, 3+) and 6 were scanty positives (2-6 AFB) as shown in figure 1. There was no significant difference in age and sex wise smear results (*p* >0.05). Inconclusive results for 10 (15.9%) cases (4 ss-ve; F1/M3, and 6 ss+ve; F1/M5) were found to be contaminated, so excluded from the study.

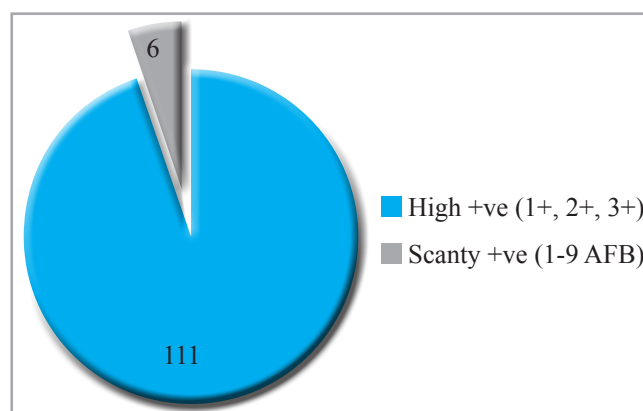


Figure 1 Grading distributions of smear positive results

Table 2 shows age and sex wise distribution of smear and culture results, in which 115 (72.3%) of 159 cases (F48/M67; ss -ve 9/ss +ve 106) were culture positive (F45/M70); s+/c+ (F44/M62), s+/c- (F2/M3), s-/c+ (F1/M8) and s-/c- (F12/M17). There was no significant difference in gender wise culture positivity (*p* = 0.213). The culture positives were high among smear positive cases and majority fell within 15-60 years age group and among males. There was an association between smear and culture positive results (*p* < 0.001).

Table 2: Age/sex wise Comparison of smear and culture results

Results	Age & sex distribution												Grand Total
	<15 years		15-29 years		30-45 years		46-60 years		>60 years		Total culture positive		
	F	M	F	M	F	M	F	M	F	M	F	M	
S+ C+	3	0	26	29	8	16	5	12	2	5	44	62	106
S+ C-	0	0	1	2	1	0	0	0	0	1	2	3	5
S- C+	0	0	1	5	0	2	0	1	0	0	1	8	9
S- C-	0	0	4	5	4	3	4	4	0	5	12	17	29
Contamination	0	0	0	2	0	2	1	2	1	2	2	8	10

F = female M = male S + = smear +ve S - = smear -ve, C+ = culture +ve

Detection of *M. tuberculosis* with RR/MDR was found in all 159 samples (40.3% F, 59.7% M) by Gene Xpert MTB/RIF assay (figure 2) and was occurred in all age categories (<15, 15-29, 30-45, 46-60 and >60 years), were 1.9%, 47.2%, 22.0%, 20.1% 8.8% respectively but the majority in 15-60 years and even above (14 cases). There was a significant difference in distribution of MDR TB among age groups ($p = 0.050$), but no significant difference of MDR TB between females and males ($p = 0.225$).

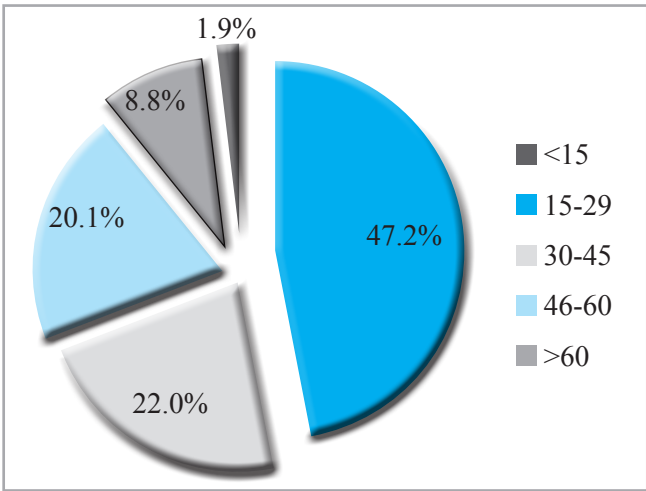


Figure 2 Age wise distribution of MDR TB case

The sensitivity (106/115; 92.2% at 95% CI; 85.1, 96.1), specificity (29/34; 85.3% at 95% CI; 68.2, 94.5), predictive value of positive test (106/111; 95.5% at 95% CI; 89.3, 98.3), predictive value of negative test (29/38; 76.3% at 95% CI; 59.4, 88.0) of Gene Xpert and smear microscopy results were evaluated with reference to culture using standard formula (Table 3).

Table 3: Comparison of smear and *R^r by Gene Xpert results with reference to culture

Smear	* Gx R ^r	Culture +ve	Culture -ve	Sensitivity 95% CI	Specificity 95% CI	PPV 95% CI	NPV 95% CI
Negative n (%)	42 (28.9)	9 (5.6) (c)	29 (18.3) (d)	a/a+c*100	d/b+d*100	a/a+b*100	d/c+d*100
Positive n (%)	117 (71.1)	106 (a) (66.7)	5 (3.1) (b)	106/115 = 92.2 % (85.1, 96.1)	29/34 = 85.3 % (68.2, 94.5)	106/111 = 95.5 % (89.3, 98.3)	29/38 = 76.3 % (59.4, 88.0)
Total n (%)	159 (100)	115 (72.3)	34 (21.4)				

*GxR^r: rifampicin resistant by Gene Xpert MTB/RIF assay

Formula: Sensitivity (Se) = $a/(a+c) \times 100$, Specificity (Sp) = $d/(b+d) \times 100$, Positive predictive value (PPV) = $a/(a+b) \times 100$, Negative predictive value (NPV) = $d/(c+d) \times 100$

Where, a = true positive, a+c = total positive (positive test): a, c = disease

d = true negative, b+d = total negative (negative test): b, d = no disease

PPV = positives among total positive predicted

NPV = negatives among total negative predicted

FLD-DST was performed on 115 culture positive isolates; of which 109 strains were identified as MDR TB. A total of 109 (94.78% 48F/61M; ss -ve 9: all M & ss +ve 100: 43F/57M) out of positives showed resistance to both I & R, 1 case each (1.74% 1F/1M) only to I and R, 1 (0.87% M) to S and I and 4 (3.7% 3F/1M) were susceptible to all SIRE. There was significant difference of culture positive results and MDRTB detection ($p = 0.001$).

Out of 109 MDR TB cases; 11.0% (F2/M10) were resistant to IR which were high among 15-45 years age group, 36.7% to IRE (F3/M4 & high in 15-45 years), 30.3% to SIR (F16/M17 were high among 15-60 years group respectively) & 50.5% (F23/M32) were resistant to SIRE that was high among 15-60 years group & even above (F2/M4). Three cases (2 resistant to SIR & 1 to SIRE) were all females below 15 years age group. The age wise distribution of MDR TB by Gene Xpert and FLD-DST was 2.7%, 49.5%, 23.9%, 19.3% and 4.6% for <15, 15-29, 30-45, 46-60 and >60 years group. There was no significant difference of age wise MDR TB cases identified by Gene Xpert and conventional C/DST ($p = 0.532$), the reference strain *M. tuberculosis* H37Rv was pan susceptible. The sex wise distribution of FLD-DST patterns was higher among males (43F/64M). There was no significant difference of sex wise distribution of MDR TB cases identified by Gene Xpert and conventional C/DST ($p = 0.775$) as shown in table 4.

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

DST patterns of FLDs by conventional proportion method													
	<15		15-29		30-45		46-60		>60		Total		Grand total
	F	M	F	M	F	M	F	M	F	M	F	M	
Total Tested	3	0	29	31	9	17	5	13	2	6	48	67	115
Fully Susceptible	0	0	2	0	1	0	0	1	0	0	3	1	4
Mono Resistance	0	0	1	0	0	0	0	0	0	1	1	1	2
S	0	0	0	0	0	0	0	0	0	0	0	0	0
I	0	0	0	0	0	0	0	0	0	1	0	1	1
R	0	0	1	0	0	0	0	0	0	0	1	0	1
E	0	0	0	0	0	0	0	0	0	0	0	0	0
Total I+ R Resistance /MDR	3	0	26	28	7	19	10	11	2	3	48	61	109
IR	0	0	1	5	0	5	1	1	1	0	3	11	14
IRE	0	0	3	0	0	2	0	1	0	1	3	4	7
SIR	2	0	8	8	4	6	4	3	0	0	18	17	35
SIRE	1	0	14	15	3	6	5	6	1	2	24	29	53

S: streptomycin I: isoniazid

R: rifampicin

E: ethambutol

Discussion

Due to shortcomings of conventional technique, novel molecular techniques are needed that combine the rapidity of microscopy and the sensitivity of culture. Though molecular techniques are not used routinely in Nepal, some investigators reported its feasibility.¹⁴ In order to overcome such problems, present study has evaluated a study of sputum specimens collected from retreatment TB cases by Gene Xpert for the rapid diagnosis of DR/MDR TB, which were further verified by FLD-DST) as gold standard.

In the present study, age wise sputum smear positivity was high among 15-60 years of age group and in males (66/159) than in females (51/159). Similar results were reported previously, in which, out of 58 patients (with age range 21–67 years) clinically suspected to have pulmonary retreatment TB, Z–N smear examination was positive for AFB in 49 (90.7%). L–J culture results revealed positive yield in 54 cases.¹⁵

In the present study, despite of smear results (42 ss-ve and 117 ss +ve), Gene Xpert showed 100% RR/MDR TB, which was high among 15-60 years and in males (58.6%) than in females (41.4%), whereas, 115 (72.3%) of 159 cases (F45/M70; 9 ss -ve and 106 ss +ve) were culture positive (F39.1%, M60.9%). From this study, sensitivity (92.2% at 95% CI; 85.1, 96.1), specificity (85.3% at 95% CI; 68.2, 94.5), PPV (95.5% at 95% CI; 89.3, 98.3) and NPV at 95% CI (76.3% at 95% CI; 85.1, 96.1), of Gene Xpert and smear microscopy results were evaluated with reference to culture. Similar results were described in previous study in which, sensitivity, specificity, PPV, & NPV was 70% at 95% CI; 60.8, 77.8, 100% at 95% CI; 98.8, 100, 100% at 95% CI; 95.2, 100, and 90.6% at 95% CI; 87.1, 93.2 respectively.¹⁶

In our study, 109 of 115 (94.8%) culture positives on FLD-DST showed both IR resistances, 1 case each (1.74%) detected as RRTB by Gene Xpert showed I mono resistance only. The assay was successful in rapidly detecting *M. tuberculosis* as well as rifampicin susceptibility pattern. It was reported in the similar studies in Vietnam and Uganda that the concordance for ss +ve, Gene Xpert (RR TB) and culture was 100% (29/29) and 63/64 (98.4%) respectively.^{12, 13, 17}

In this study, no repeated culture/FLD DST or Gene Xpert was performed. The age and sex wise distribution of MDR TB was high in 15-60 years group (even above

60 years) and in males than females by both the methods. It has been reported in one study that the MDR TB among previously treated patients was 19.25% (n=161) irrespective of age and sex variation.¹⁸

The resistance was conferred by four different rpoB gene mutations in the 81 bp rifampicin resistance detection region (RRDR) of MTB 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.^{19,20} In this study also it can be revealed that all the RR/MDR TB identified by Gene Xpert 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 MDRTB results had been described in a similar study.¹⁷ There was not a single rifampicin mono resistance by conventional FLD-DST in this study as it is very rarely occurring.

Conclusions

Gene Xpert MTB/RIF assay as it is a useful method of simultaneous detecting MTB and rifampicin resistance (surrogate marker of MDRTB) in both the sputum negative and positive samples along with the culture and DST. From this study, out of 159 retreatment TB patients enrolled, 100% were detected RR/MDR TB by Gene Xpert irrespective of smear results and 72.3% were culture positive, among which, 94.8% were MDR TB by FLD-DST. The prevalence of MDR TB was found to be high among 15-60 years age group and distributed in both the females and males by both methods. Results of this study has given a very good example that 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 (prompt diagnosis) and culture and FLD-DST (gold standard method).

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tuberculosis cases enrolled in this study and health facilities submitting good quality specimens.

Conflict of interest: None declared.

Ethical approval: This study was conducted with the approval of the Nepal Health Research Council (NHRC) Ethical Review Board (Registration No. 308/2017).

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