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Original Article

Microbiology Section

Characterisation of Mutations in *rpoB* Gene and Assessment of Risk Factors in Patients with Rifampicin-resistant Tuberculosis: A Cross-sectional Study from Eastern India

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ABSTRACT

Introduction: Tuberculosis (TB) continues to be a significant public health challenge in India, with alarming prevalence rates and unique socio-demographic risk factors. Resistance to Rifampicin (RIF), is caused by mutations in the *rpoB* gene of the causative agent. It is usually situated in a region at the 507-533 amino acid residues (81 bp) within the *rpoB* gene, known as the Rifampicin Resistance Determining Region (RRDR).

Aim: To assess the prevalence of Rifampicin Resistance (RR) and identify mutations in the RRDR of the *rpoB* gene.

Materials and Methods: This cross-sectional study was conducted at ESIC Medical College, Patna, in collaboration with the NABL-accredited laboratory associated with Tertiary care Hospitals, Patna, Bihar, India from July 2024 to January 2025. A total of 502 clinical samples from suspected cases of TB were analysed by GeneXpert MTB/RIF testing. Deoxyribonucleic Acid (DNA) extraction was done only for the MTB complex with RR followed by automated DNA sequencing. Data obtained from these 68 RR-MTB clinical samples were enrolled in this study. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software with the association of multivariate logistic regression with p-value <0.05.

Results: In this study, 46.57% (68/146) of all MTB-positive patients were RR. The highest alteration was at the Ser/Leu substitution at codon 531 present in 45.6% (31/68) isolates, followed by His/Tyr substitution at codon 526 in 25% (17/68), mutation at codon 516 in 16.17% (11/68) isolates and mutations at codon 511 demonstrated in 13.23% (9/68) isolates.

Conclusion: Mutations within the RRDR of the *rpoB* gene, particularly at codons 531 and 526, were identified as the predominant drivers of RR among pulmonary TB patients in Eastern India. The study highlights the importance of integrating rapid molecular diagnostics with socio-demographic risk assessment to enable early detection, guide individualised treatment, and strengthen regional TB control strategies.

Keywords: GeneXpert MTB/RIF Assay, Multidrug-resistant tuberculosis, *Mycobacterium tuberculosis*, *rpoB* gene mutation

INTRODUCTION

TB continues to be a significant public health challenge in India, with alarming prevalence rates and unique risk factors. The Global Tuberculosis Report of World Health Organisation (WHO) 2021 highlighted India as one of the countries accounting for two-thirds of the global burden, with high prevalence of multidrug resistance and underreporting of cases [1]. The prevalence of pulmonary TB is alarmingly high at 295.9 cases per 1 lac population, with even higher rates among Indian men, according to the Global Tuberculosis Report 2013 of WHO. Tuberculosis infection is caused by a bacterium *Mycobacterium tuberculosis* (MTB) [2]. According to the Centers for Disease Control and Prevention (CDC), MDR-TB is caused by TB bacteria that are resistant to at least isoniazid (INH) and rifampin (RIF)- the two most effective first-line TB drugs. While pyrazinamide (PZA) and ethambutol (EMB) are also first-line drugs, resistance to all four is more characteristic of Extensively Drug-Resistant TB (XDR-TB) [3]. The RR has a precise epidemiological variety and is a valuable marker for MDR-TB strains since 90% or above of those strains resistant to RIF are also resistant to INH, which are said to be MDR-TB. RIF is the key first-line anti-TB drug that works by inhibiting the synthesis of Ribonucleic Acid (RNA) directed by the DNA of *Mycobacterium tuberculosis* proteins by binding to the β subunit of the bacterial DNA-dependent RNA polymerase enzyme protein (*rpoB*) [4,5].

The GeneXpert MTB/RIF testing is a rapid molecular diagnostic test that is performed with an automated cartridge-based GeneXpert machine (Cepheid, USA). The test function based on nucleic-acid amplification assay that detects MTB bacilli and RR pattern from the patient's sputum and other body fluids [6], and the conventional method of ZN staining followed by culture and drug susceptibility tests for detection of RR-MTB strains are the two methods commonly practiced. The socio-demographic factors associated with TB are family income, employment status, and nutritional status. Behavioural factors that are related are knowledge, attitude, smoking, and history of contact with TB patients [7].

Mutations in the *rpoB* gene are reported as 95 to 97% of RR-MTB strains throughout the world, which is usually situated in a region at the 507-533 amino acid residues (81bp) within the *rpoB* gene known as RRDR [8]. Despite the growing burden of drug-resistant TB in India, detailed local epidemiological data on RR mutations are scarce. This study aimed to assess the prevalence of RR and to identify mutations in the RRDR of the *rpoB* gene among *Mycobacterium tuberculosis* isolates. The primary objective was to characterise the specific mutation in (RRDR) of the *rpoB* gene using DNA sequencing. The secondary objective was to evaluate the socio-demographic and risk factors.

MATERIALS AND METHODS

This cross-sectional study was conducted in the ESIC Medical College, Patna, Bihar, India in collaboration with a NABL-accredited laboratory (Lifecell International), from July 2024 to January 2025, after ethical approval from the Institutional Ethical Committee (IEC)

(No-CREC/2024/12), and informed consent for all subsequent study procedures was taken from the participants. The role of Lifecell International was to perform Next Generation Sequencing.

Inclusion criteria: Patients of all ages and genders presenting with clinical signs and symptoms suggestive of pulmonary TB were included in the study.

Exclusion criteria: Participants unwilling to provide informed consent or if they had already commenced Anti-Tubercular Treatment (ATT) prior to the screening visit were excluded from the study.

Sample size: A total of 502 non-identical patients' samples were included. This was a time-bound study; all subjects available in the study were taken into consideration.

Study Procedure

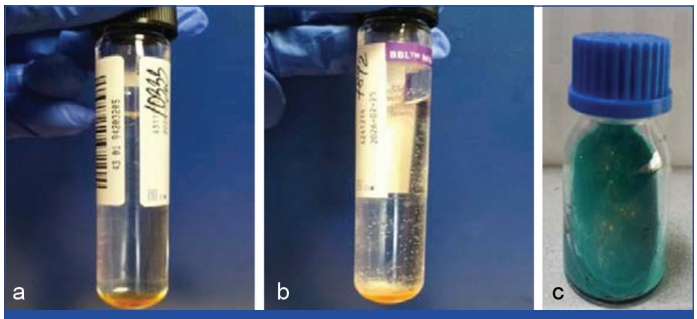
Following appropriate instructions to rinse with water to clear your mouth [Table/Fig-1a], followed by deep breathing and coughing [Table/Fig-1b], the samples from the suspected patient were self-collected in an open-air setting within a universal sterile container.

[Table/Fig-1a,b]: Preparation and self-collection of deep cough sputum from suspected Tuberculosis (TB) patients in open-air settings.

The samples were processed using the standard N-Acetyl-L-cysteine-NaOH decontamination method [9], followed by analysis with the GeneXpert MTB/RIF assay, which concurrently detects MTB complex and RR. Out of 502 patient samples, individuals were first classified based on the presence of *Mycobacterium tuberculosis*: either detected or not detected. Those with *Mycobacterium tuberculosis* detection were then further assessed for RR and categorised as RIF detected, RIF indeterminate, or RIF not detected. In alignment with the study's objective of characterising mutations associated with RIF resistance, patients from the RIF-detected and RIF-indeterminate strata were prioritised for inclusion. Although these strata did not represent the largest proportions within the overall population, their diagnostic relevance justified deliberate oversampling to achieve sufficient statistical power and depth of analysis. For this study, stratified sampling was applied to select patients from the RIF-detected and RIF-indeterminate subgroups within the MTB-positive population.

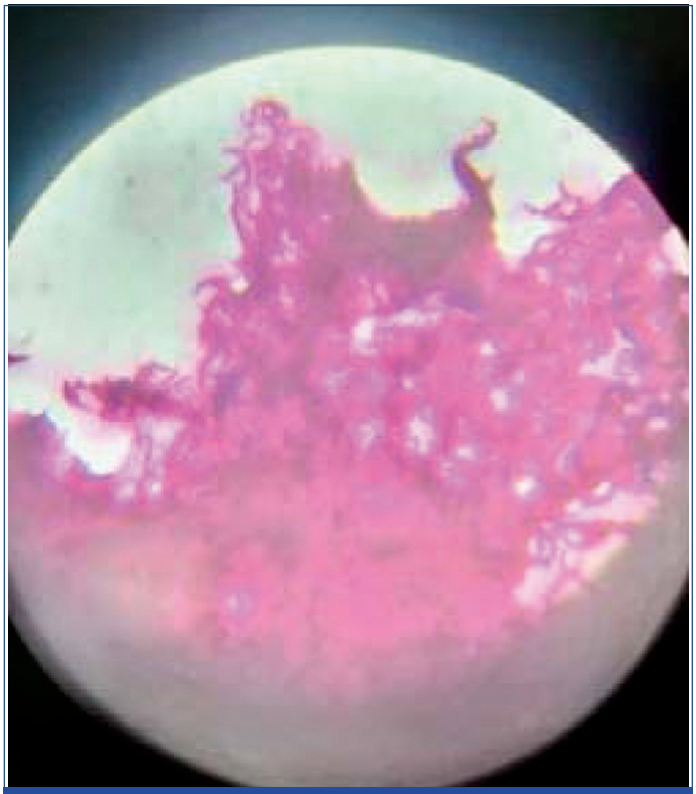
All patients diagnosed with RR-TB completed questionnaire forms [Annexure-1], provided written consent, and their medical information was recorded. The study variables included information on the history of TB, defaulted TB treatment, age and gender of the patient, co-morbidities, consumption of alcohol and tobacco, and other socio-economic information was correctly recorded. The educational level was self-reported by participants and categorised as follows: No Education (illiterate), Primary (up to 5th grade), Secondary (6th to 10th grade), and Higher Education (college or university) [10].

Isolation of bacterial strain and DNA extraction: The samples that were tested positive for *Mycobacterium tuberculosis* with RR were further tested by growing in a Middlebrook 7H9 liquid medium with fluorescent indicator in the BACTEC MGIT 960 system. It was monitored throughout the incubation period for fluorescence signals indicative of mycobacterial growth [11], further, *Mycobacterium* colonies were subcultured onto Löwenstein-Jensen (LJ) medium, which showed positive growth after incubation of three weeks at 37°C [Table/Fig-2a-c].



[Table/Fig-2]: Shows (a) Sterile Middlebrook 7H9 liquid medium in MGIT tube; b) Growth of *Mycobacterium tuberculosis* (MTB) in middlebrook 7H9 liquid medium; c) Growth of colonies of *Mycobacterium tuberculosis* over Löwenstein-Jensen (LJ) medium.

Isolated strains were confirmed by the Ziehl-Neelsen staining technique for acid-fast bacilli [Table/Fig-3].



[Table/Fig-3]: Cord formation of acid-fast bacilli visualised by Ziehl-Neelsen staining from colonies of *Mycobacterium tuberculosis* (1000x).

PCR and Sequencing: Extracted DNA was analysed using the Qubit dsDNA HS Assay kit on a Qubit Fluorimeter for DNA concentration [12]. The number of PCR cycles was set at 40 to amplify the DNA of the targeting *rpoB* gene to a detectable level. It involved an initial denaturation for 10 minutes at 95°C, followed by 60 seconds at 95°C, 30 seconds at 61°C, and 30 seconds at 72°C, followed by 10 minutes at 72°C. The total volume of PCR was 30 µL, which contained 5 µL of DNA sample, 5 µL of primer and 20 µL of Taq DNA Polymerase mastermix. Nextera XT DNA Library Preparation Kit was used as recommended by the manufacturer [13]. An automated DNA sequencer was used to sequence the amplified products to locate the exact location of the mutation. The Illumina Nova Seq 6000 System with in-built DRAGEN interpretation software provides accurate, rapid secondary analysis. DNA fragments, sequenced by the NGS method, were analysed in-silico in order to identify mutations over the RRDR *rpoB* [14]. Frequent mutation sites and probes identified were reported. The sequence data obtained from the sequencer were then processed and analysed bioinformatically. This includes quality control, read mapping to a reference genome, variant calling to identify Single-Nucleotide Polymorphisms (SNPs)

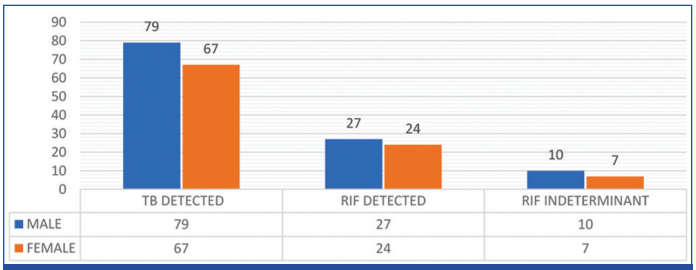
and annotation of detected variants. SNPs from individual samples were subjected to multiple sequence alignment using multiple sequence alignment software.

STATISTICAL ANALYSIS

Data were entered and analysed using SPSS version 20.0. The multivariate logistic regression analysis was used for association between socio-demographic and clinical risk factors and the occurrence of RR-TB. The p-value was calculated by using Chi-square test and p-value ≤ 0.05 was considered statistically significant.

RESULTS

Out of 502 samples, 146 (29.08%) were MTB positive, which included RIF-not detected as well as RIF-detected and RIF indeterminate. The prevalence of RR-TB was found to be 68 (46.57%) among MTB-positive patients. Males showed a slightly higher prevalence, with 37 (54.41%) cases compared to females [Table/Fig-4].



[Table/Fig-4]: Gender-wise distribution among TB detected patient and their Rifampicin Resistance (RR) status.

A total of 9 (13.23 %) patients were above the age of 70 years, and 24 (35.29 %) were from the 0-18 years age group. The socio-demographic profile, including educational level of the study population, is presented in [Table/Fig-5].

Variable	Category	n (%)
Gender	Female	31 (45.59)
	Male	37 (54.41)
Educational level	Illiterate	6 (08.82)
	Primary	17 (25.00)
	Secondary	24 (35.29)
	Higher education	21 (30.88)
Marital status	Married	27 (39.71)
	Single	41 (60.29)
Age (years)	≤18	24 (35.29)
	19- 69	35 (51.48)
	≥70	9 (13.23)

[Table/Fig-5]: Demographic characteristics of RR-TB patients (n = 68): Gender, education level, and marital status.

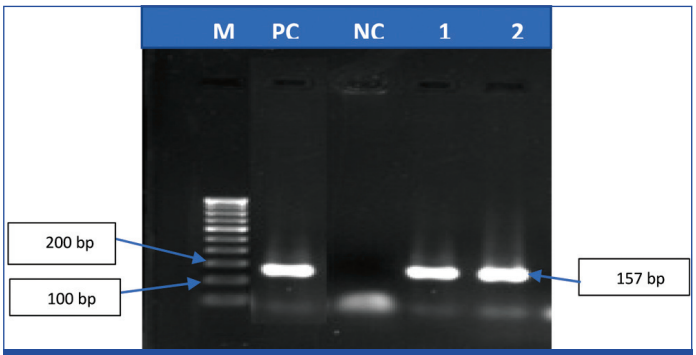
On univariate analysis, smoking (7/146; 4.79%; χ^2=4.65; p-value=0.031), treatment default (8.21%; χ^2=5.34; p-value=0.021), previous contact with MDR-TB (8.90%; χ^2=4.41; p=0.036), poor knowledge of MDR-TB (6.16%; χ^2=4.14; p-value=0.042), and prior TB treatment (5.47%; χ^2=4.26; p-value=0.039) were all significantly associated with RR. After adjusting for age, sex, and education in multivariate logistic regression, treatment default and previous contact with MDR-TB remained independent predictors, while co-morbidity (p-value=0.052), alcohol use (p-value=0.076), and drug side-effects (p-value=0.071) did not reach statistical significance [Table/Fig-6].

The presence of the *rpoB* gene was further validated through gel electrophoresis [Table/Fig-7],

These PCR products were subsequently subjected to gene sequencing, and the resulting data were analysed. The Frequent sites of mutation in the RRDR region (507-533 amino acids) of the *rpoB* gene were codon-531 (TCG/TTG Serine/Leucine) noted in

Variables	n (%)	Chi-square (χ^2) value	p-value	95 % CI	
				Lower	Upper
Co-morbidity	4 (2.73)	3.77	0.052	1.03	4.43
Alcohol consumption	3 (2.05)	3.15	0.076	0.34	3.76
Smoking	7 (4.79)	4.65	0.031	2.76	6.82
Defaulted TB treatment	12 (8.21)	5.34	0.021	5.01	11.41
Previous contact with MDR-TB	13 (8.90)	4.41	0.036	5.83	11.97
Knowledge about MDR-TB	9 (6.16)	4.14	0.042	3.95	8.37
Previously treated for TB	8 (5.47)	4.26	0.039	3.27	7.67
Experienced TB drug side-effects.	6 (4.10)	3.28	0.071	2.27	5.93

[Table/Fig-6]: Shows risk factors associated with confirmed *Mycobacterium tuberculosis* (MTB) cases n=146.
p-value was calculated using the Chi-square test



[Table/Fig-7]: Shows gel electrophoresis of 157 bp *rpoB* gene PCR product on 3% agarose gel: lanes 1-2, clinical isolates; lane NC, negative control; lane PC, positive control (*M. tuberculosis* H37Rv); lane M, molecular size marker.

31 (45.6%) followed by codon-526 (CAC/TAC Histidine/Tyrosine) observed in 17 (25%) [Table/Fig-8].

Codon	Change in nucleotide sequence	Change in amino acid sequence	Type of Mutation	n (%)
511	CTG/CCG	Leucine/Proline	SNP	9 (13.23)
516	GAC/GTC	Aspartic acid/Valine	SNP	11 (16.17)
526	CAC/TAC	Histidine/Tyrosine	SNP	17 (25.00)
531	TCG/TTG	Serine/Leucine	SNP	31 (45.60)

[Table/Fig-8]: Frequency and type of mutations in the RRDR region (codons 507–533) of the *rpoB* gene in rifampicin-resistant *M. tuberculosis* (MTB) isolates.

DISCUSSION

Questionnaire responses from RR-TB patients demonstrate they were generally unaware of the causes and symptoms of TB, as well as its prevention and treatment. Recurrent TB patients contribute to a significant proportion of the TB burden in India. A nationwide survey was conducted during 2019-2021 across India among adults to estimate the prevalence of TB, which shows 27.1% cases of recurrent TB [15]. The majority of TB positive patients having RR were males 54.41% of whom 17 were either unemployed or uneducated/had a primary level of education. The development of RR can be attributed to a lack of awareness about its treatment and exposure to other patients with MDR-TB. The socio-demographic factors, such as young age, illiteracy, unemployment, poverty, overcrowding, and improper sputum disposal practices, were associated with RR TB [16]. RR in MDR-TB cases, as observed in many studies, is due to RRDR of the *rpoB* gene mutation [17].

DNA sequencing was utilised to target the RRDR of the *rpoB* gene in GeneXpert-confirmed rifampicin-resistant samples. Mutations at codons 531 (TCG → TTG), 526 (CAC → TAC), and 516 (GAC → GTC) were identified as the most frequent alterations associated

with RR. Priyanka K et al., reported a high prevalence (89.65%) of RR associated with codon 531 mutations [18]. Similarly, Barnard M et al., and Raj NY et al., observed 70.5% and 72% respectively in their studies [19,20]. In contrast, the present study findings revealed a lower proportion (45.6%) of RR due to codon 531 mutations. This discrepancy may be attributed to differences in sample size and the criteria used for sample selection. However, in some studies, the previous researchers also observed that codon-526 was the most common site of mutation associated with RR in MDR-TB cases [21,22].

The most significant benefit of the DNA sequencing method is to decrease the turnaround time between sampling and the availability of the test results for clinical decision-making. In traditional culture method and rifampicin susceptibility testing will take 6-8 weeks for a conclusive diagnosis. In contrast, the test results based on genetic alteration in the RRDR of the *rpoB* gene are available in only 3-5 days. Early identification of MDR pulmonary TB cases is crucial for a better clinical outcome. So DNA sequencing is a fast and more specific method than the conventional culture and Drug Susceptibility Testing (DST). It can determine the precise location and frequency of mutation in the RRDR of the *rpoB* gene responsible for RR in different geographical areas. The role of DNA sequencing i primarily provides accurate and rapid prediction about RR MTB and secondly provides useful data for the development of a screening protocol to detect MDR-TB in future [23]. Future research should increase the cohort size, incorporate longitudinal follow-up, and utilise whole-genome sequencing to uncover emerging resistance mechanisms and guide personalised treatment regimens.

In the current era of individualised treatment approaches gene sequencing has become the standard tool for diagnosing drug-resistant TB. Individualised treatment could significantly reduce resistance amplification and overcome the use of suboptimal treatment regimens, impacting the trajectory of the DR-TB epidemic.

Limitation(s)

This study had several limitations inherent to its cross-sectional design. Since data were collected at a single point in time, it does not allow for the assessment of changes or trends over a period. Additionally, reliance on participants' recall, particularly in health-related surveys, may introduce recall bias, potentially affecting the accuracy of reported exposures or behaviours. Selection bias is another concern, as the sample may not fully represent the broader population. The methodological complexity of gene sequencing posed a significant challenge. Furthermore, the relatively small sample size limits the generalisability of the findings.

CONCLUSION(S)

This cross-sectional study revealed that mutations in the RR-determining region of the *rpoB* gene, most notably at codons 531 and 526, are key drivers of RR among pulmonary TB patients in this region. The socio-demographic assessment demonstrated a higher RR-TB burden in males and identified illiteracy, unemployment, prior treatment defaults, and household contact with MDR-TB cases as significant risk factors. These findings highlight the necessity of pairing rapid molecular diagnostics with targeted community education, socio-economic interventions, and robust contact-tracing efforts to achieve the goal of the End TB strategy. The applicability of WGS-based individualised treatment in low-income settings is hindered by the cost and complexity of the platform and the lack of expertise required for bioinformatics analysis.

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[ANNEXURE-1]

Socio-Demographic Risk Assessment Questionnaire for Rifampicin-Resistant Tuberculosis Patients

Name- Age Sex- Date-

Sample Type- Patient ID-

Q1. Educational level: A) Illiterate B) Primary C) Intermediate D) Graduate

Q2. Employment status: A) Unemployed B) Employed

Q3. Marital status: A) Unmarried B) Married

Q4. Do you consume: A) Tobacco B) Alcohol C) Drug abuse

Q5. Had contact with a known TB patient A) Yes B) No

Q6. Previously treated for TB A) Yes B) No

Q7. Had TB treatment interruption A) Yes B) No

Q8. Had knowledge of MDR-TB A) Yes B) No

Q9. Do you have any underlying health issues/co-morbidities/take any immunosuppressant drugs?

DM2/Hypertension/Asthma/Obesity/H/O Organ Transplant/ Other:

Q10. Do you currently have any of the following symptoms? (Tick in the box given below)

1) Unexplained cough lasting more than 3 weeks?
A) Yes B) No
2) Unexplained fever lasting more than 3 weeks?
A) Yes B) No
3) Night sweats?
4) Shortness of breath?
5) Unintentional weight loss?
6) Chest pain?
7) Unexplained fatigue?
8) Blood in sputum?

Acknowledgment and consent:

I hereby acknowledge to have been informed about the research study conducted on rifampicin-resistant TB cases. I consent to give my personal information in this form to be used for this study and publication in the future. I certify that the information provided in this survey form is true and correct.

Signature:

रिफैम्पिसिन प्रतिरोधी क्षय रोग रोगियों के लिए सामाजिक-जनसांख्यिकीय जोखिम मूल्यांकन प्रश्नावली

नाम- आयु- **लिंग-** **दिनांक-**

नमूना प्रकार- **रोगी आईडी-**

प्रश्न 1. शैक्षिक स्तर: A) अशिक्षित B) प्राथमिक C) इंटरमीडिएट D) स्नातक

प्रश्न 2. रोज़गार की स्थिति: A) बेरोज़गार B) रोज़गार

प्रश्न 3. वैवाहिक स्थिति: A) अविवाहित B) विवाहित

प्रश्न 4. क्या आप सेवन करते हैं: A) तंबाकू B) शराब C) नशीली दवाओं का सेवन

प्रश्न 5. ज्ञात क्षय रोग रोगी के संपर्क में आए हैं: A) हाँ B) नहीं

प्रश्न 6. पूर्व में क्षय रोग का इलाज हुआ है: A) हाँ B) नहीं

प्रश्न 7. क्षय रोग के उपचार में रुकावट आई है: A) हाँ B) नहीं

प्रश्न 8. एमडीआर-टीबी के बारे में जानकारी थी: A) हाँ B) नहीं

प्रश्न 9. क्या आपको कोई अंतर्निहित स्वास्थ्य समस्या/सह-रुग्णता है/क्या आप कोई प्रतिरक्षा-दमनकारी दवाएँ लेते हैं?

DM2/उच्च रक्तचाप/अस्थमा/मोटापा/अंग प्रत्यारोपण/अन्य:……..

प्रश्न 10. क्या आपको वर्तमान में निम्नलिखित में से कोई भी लक्षण है?

1) 3 सप्ताह से अधिक समय तक चलने वाली अस्पष्टीकृत खांसी? A) हाँ B) नहीं

2) 3 सप्ताह से अधिक समय तक चलने वाला अस्पष्टीकृत बुखार? A) हाँ B) नहीं

3) रात में पसीना आना? 4) साँस लेने में तकलीफ? 5) अनजाने में वज़न कम होना?

6) सीने में दर्द? 7) अस्पष्टीकृत थकान? 8) थूक में खून?

स्वीकृति और सहमति:

मैं एतद्द्वारा स्वीकार करता/करती हूँ कि मुझे रिफैम्पिसिन प्रतिरोधी टीबी मामलों पर किए गए शोध अध्ययन के बारे में जानकारी दी गई है। मैं इस फॉर्म में अपनी व्यक्तिगत जानकारी इस अध्ययन और भविष्य में प्रकाशन के लिए उपयोग करने हेतु देने के लिए सहमति देता/देती हूँ। मैं प्रमाणित करता/करती हूँ कि इस सर्वेक्षण फॉर्म में दी गई जानकारी सत्य और सही है।

हस्ताक्षर: