Peer Review File
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Reviewer A

The manuscript investigated the use of line probe assay for detection and mutational analysis of selected drug resistance genes (rpoB, katG & inhA) and claimed that GenoType MTBDRplus and MTBDRsl assay could be used for quick detection of drug resistance in M. tuberculosis.

Though the study is interesting for scientific community and general public but the following comments should be properly addressed.

Comments:
Comment 1. Is the study idea novel, as we can see number of articles published from Pakistan on the same area? What new information will be added to the literature from this study?
Reply1: Respected Editor, we have added the particular portion in the revised manuscript. Page 12 line 330-336.
Changes in the text: The study provides a substantial sample size and comprehensive analysis of the mutational patterns in Mtb strains from Pakistan. It can contribute valuable data to the global understanding of MDR-TB epidemiology and genotypic characteristics. The study's findings regarding the prevalence of MDR isolates, specific mutations in the rpoB, katG, and inhA genes, and the effectiveness of GenoType MTBDRplus and MTBDRsl assays can potentially provide important insights into the local situation and contribute to the global knowledge and fast molecular diagnosis based on MDR-TB.

Comment 2. Generally, GenXpert can't differentiate various Mycobacterial species, so we use the term M. tuberculosis complex instead of M. tuberculosis.
Reply 2: We agree with the reviewer and thanks for the reminder. The term is changed to M. tuberculosis complex.
Changes in the text: On page 6 line 135-37, GeneXpert MTB/RIF which is used to rapidly identify the RIF resistance (RIFR) in Mtb complex (MTBC).

Comment 3. The author has used the term M. tuberculosis strains but the term strain is used following gene sequencing. Is sequencing performed in the present study?
Reply 3: The term strains is deleted from text, page 2 line 34, page 2 line 43. We did not sequence the genes.
Changes in the text: "inhA genes to identify multi-drug resistant Mtb."
"provide insights into the genetic profiles of Mtb in Pakistan".

Comment 4. Abstract should clearly mention the rationale and objectives of the study.

The results presented should be given in more details and quantitative manner.
Reply 4: The abstract has been modified according to the suggestion of the reviewer on page 2, line 24-44.
Changes in the text:
Background: Tuberculosis (TB) remains a significant global health emergency caused by Mycobacterium tuberculosis (Mtb). While the epidemiology, transmission, genotypes, mutational patterns, and clinical consequences of TB have been extensively studied worldwide, there is a lack of information regarding the epidemiology and mutation patterns of Mtb in Pakistan, specifically concerning the prevalence of multi-drug resistant TB (MDR-TB).
Methods: This study aimed to investigate the incidence of Mtb and associated mutational patterns using the Line Probe Assay (LPA). Previous studies have reported a high frequency of mutations in the rpoB, inhA, and katG genes, which are associated with resistance to rifampicin (RIF) and isoniazid (INH). Therefore, the current study utilized LPA to detect mutations in the rpoB, katG, and inhA genes to identify multi-drug resistant Mtb.
Results: LPA analysis of a large pool of Mtb isolates, including samples from 241 sputum-positive patients, revealed that 34.85% of isolates were identified as MDR-TB, consistent with reports from various regions worldwide. The most prevalent mutations observed were rpoB S531L and inhA promoter C15T, which were associated with resistance to RIF and INH, respectively.
Conclusions: This study highlights the effectiveness of GenoType MTBDRplus and MTBDRsl assays as valuable tools for TB management. These assays enable rapid detection of resistance to RIF, INH, and fluoroquinolones (FQs) in Mtb clinical isolates, surpassing the limitations of solid and liquid media-based methods. The findings contribute to our understanding of MDR-TB epidemiology and provide insights into the genetic profiles of Mtb in Pakistan, which are essential for effective TB control strategies.

Comment 5. What was the inclusion and exclusion criteria for selection of TB patients or samples?
Reply 5: Inclusion criteria: Confirmed TB diagnosed cases, newly diagnosed or previously treated TB cases, geographical location, time period and drug resistant.
Exclusion criteria: Extra pulmonary TB, co-infections or comorbidities, insufficient data, and duplicate cases.
Changes in the text: Page 5, line 110-115 of revised manuscript.

Comment 6. The demographic and clinical characteristics of patients including new, retreated, Beijing family and non-Beijing should be properly described in methodology.
Reply 6: Methodology section, page 4-5, line 103-109.
Changes in the text: The study participants were selected based on specific demographic and clinical characteristics. Demographic information such as age, gender, and geographical location was collected for each patient. Clinical characteristics, including the TB treatment history, were also recorded. In addition, patients were

categorized into subgroups based on their treatment status, distinguishing between new and retreated cases. Furthermore, strains were classified as belonging to the Beijing family or non-Beijing strains based on genotyping methods, such as polymerase chain reactions.

Comment 7. We can see statistical analysis for table (3, 4) but there is no description of statistical analysis in the in the methodology section.
Reply 7: The data interpretation and statistical summary were conducted using the Statistical Package for Social Science (SPSS) version 16 and GraphPad Prism 5. All the variable outcomes were presented as percentages (%), and odd ratio (OR). Fisher's exact test and Chi-Square test were employed to assess the associations between categorical variables. The results were reported as either mean values or the number of patients. Statistical significance was defined as a P value less than 0.05.
Changes in the text: Methodology section page 7, line 176-181.

Comment 8. Statistical analysis should be performed for data presented in table 1 and 2.
Reply 8: The dataset consists of only a single value, statistical analysis may not be meaningful or appropriate. So we did not perform statistical analysis.

Comment 9. Replicates in each experiment should be mentioned.
Reply 9: Included in this investigation, and a three replicates were performed for each case.
Changes in the text: Page 5, line 124.

Comment 10. The presentation and labeling (X-axis/Y-axis) of figures should be improved.
Reply10: Following the instructions we have improved figures.

Comment 11. Headings of tables should be scientifically revised. The abbreviations used in tables should be defined in table footnotes.
Reply 11: The heading are revised and footnotes are included in the revised version of the manuscript. Page 14, line 363.
Changes in text: Footnote: Reporting Checklist: The authors have completed the STROBE, reporting checklist.

Comment 12. The limitation of the study should be given.
Reply 12: Page 13, line 338-339 the limitation is added.
Changes in the text: However, the current study had a limited number of data, and further studies are warranted in other areas of Pakistan, to access the accurate number and burden of TB.

Comment 13. In discussion part, the results should be compared with previous studies from Pakistan.

Reply 13: The results are compared both with previous studies from Pakistan and other countries.

Reviewer B

The authors investigated Mtb incidences in Pakistan, examined associated mutations via Line Probe Assay and reported a very high percentage (34.85%) of strains to be Multi Drug Resistant (MDR). Quite expectedly, they found most of the mutations to be present in rpoB, inhA and katG genes, associated with rifampicin and isoniazid resistance. Given such epidemiological studies are carried out less in Asian countries like Pakistan, the study presents important findings for the TB field. However, the authors must address the following concerns for the study to be considered acceptable for publication.

Comment 1. Line38: The authors should add poor compliance to the list. How does illiteracy contribute to MDR strain development?
Reply1: These MDR-TB develop due to ignorance, illiteracy (less awareness about TB), poor compliance, improper treatment or poor management of drug-susceptible TB (DS-TB).
Changes in the text: Revised manuscript, page 3 line 52.

Comment 2. Line 45-47: The sentence is factually incorrect. Only active TB patients can spread infection.
Reply 2: Despite significant medical advancements and social interventions, TB management remains challenging due to the potential for hidden transmission. Infected individuals can unknowingly spread the disease to others, as the progression from latent TB infection to active TB disease can occur gradually over time.
Changes in the text: Revised manuscript, page 3, line 58-62.

Comment 3. The study has a lot of grammar issues throughout the manuscript such as in Line 49, 59.
Reply3: We revised manuscript carefully. Thanks for the comments.
Changes in the text: Page 3, line 63-67, page 3-4, line 68-81.

Comment 4. What is the percent MDR case in new vs retreated TB patients?
Reply 4: Reply: we have addressed the comment according to the reviewer comment. Page 7 line 187-89, Table 1
Changes in text: Based on the patients' treatment history, new cases constitute more than half (126, 52%) of the cases, whereas the remaining (115, 48%) constitute the number of retreated cases.

Comment 5. Line 155: "rpoB" is written as "ropB"
Reply5: Corrected in revised manuscript.
Changes in the text: It was determined that rpoB is almost the unique gene for RIFR.

Please see page 8, line 193.

Comment 6. Line 151: “There were 97 incidences of mono-resistance or multiple gene resistance”. What does the author mean by “multiple gene resistance” in case of mono-resistance?
Reply 6: We have corrected the mistake, it was a typing error. Page 8 line 190.
Change in text: There were 97 incidences of mono-resistance among the 241 positive TB-patients

Comment 7. Line 201: The authors state “female patients constitute 84% of the study population”. However, its 54 as per table 1. Which one is correct?
Reply 7: The statement is corrected in revised manuscript, page 9, line 242.
Changes in the text: It was observed in this study that female patients constitute 54% of the study population in contrast to their male counterparts.

Comment 8. Line 202: The authors state “females are at higher risk of contracting TB in this study”. Since this observation is not in line with the literature, did authors compare % females in the study who were TB negative? What was the % of females that enrolled in the study?
Reply 8: We compared the positive cases of females with positive males. It was found that positive female’s percentage 54% (cases 129) was higher than males.
Changes in text: Please see the Table 1

Comment 9. Line 211: Can authors elaborate on “particularly when using conventional first- and second-line regimens.” What other kinds of treatment are available apart from conventional regimens?
Reply 9: Page 3-4, line 75-81.
Change in text: Also other kinds of treatment include regimens containing newer drugs, adjunctive therapies (immunomodulatory drugs, host-directed therapies, and nutritional supplementation to support the patient's immune system), and other medicine such as traditional Chinese medicine and even surgery are alternative treatment options and may vary depending on the specific healthcare setting, drug accessibility, and local guidelines. The choice of treatment approach is typically made by healthcare professionals based on individual patient factors, drug resistance patterns, and the expertise available.

Comment 10. Figure 1: The authors should present Figure 1 drug resistance distribution as a Split bar graph between new and retreated TB cases
Reply 10: The inclusion of a split bar graph might not align with the primary objectives or research questions of the study. And also we prioritized other relevant visuals or data that they deemed more important for conveying their findings within the space limitations.

Comment 11. Table 1: Does positive case age-wise distribution vary between sexes?

Reply 11: Yes, the positive case age-wise distribution vary between sexes, but we prioritized other relevant visuals or data that they deemed more important for conveying their findings within the space limitations.

Comment 12. Authors should briefly discuss about whether resistance to antibiotics vary between sexes?
Reply 12: Page 9 line 220-24 of revised manuscript.
Changes in the text: Rate of FQR was seen to be associated with gender [(95% CI: to 0.9616), OR: 0.53; P < 0.040] and age [(95% CI: to 2.874), OR: 2.143; P < 0.012], with higher incidents of FQR in females and individuals below 30 years of age (Table 5). This differences was due to healthcare-seeking behavior, treatment adherence, or exposure to risk factors. Which could potentially contributed to varying rates of drug resistance between sexes.

Comment 13. It would be useful to Include patient wise dataset in the supplementary to make it publicly available for vigorous analysis by other groups
Reply 13: Thank you for the suggestion, but due to data privacy and confidentiality we cannot add the patient wise data set.

Reviewer C

A good attempt to address the distribution of drug resistance pattern of M. Tuberculosis in the region. The study is important epidemiologically.

The manuscript text is clear and easy to read, well-structured with all necessary sections.

I would request further clarification on the following points.

Comment 1. The methodology used for the study needs to be more detailed like GenXpert MTB/RIF assay and Line probe assay are mentioned in methods, however the results sections does not mention percentage of Rifampicin resistance due to both these methods separately. More data is required whether GenXpert and LPA (MTBDR plus) and AFB culrtures were done on all samples or only smear positive samples?
Reply 1: We did only on the smear positive samples and we include only those samples which were resistant on both these methods, thereby we did not mentioned the percentages separately.
Changes in the text: Page 5 line 114-15, page 6 line 137-38, page 6 line 156-58 in revised manuscript.
“Also samples which was not resistant on both GenXpert and LPA methods were excluded. “
“For the early detection and appropriate treatment the smear positive samples were subjected to the WHO recommended assay. GeneXpert MTB/RIF which is used to rapidly identify the RIFR in Mtb complex (MTBC)”.
“The Hain protocol was used for hybridization according to manufacturer's instruction.

The smear positive samples were incubated in a "Twin-Cubator," a semiautomatic washing and shaking apparatus that can accommodate 12 samples at once".

Comment 2. It will be worthwhile to mention in methods section how the cross resistance to Rifampcin and INH with other anti tubercular drugs is determined?
Reply 2: Thanks a lot to the reviewer. We made a mistake for cross-resistance in our previous manuscript. We think we used a wrong concept of "cross-resistance" instead of "co-resistance". "Cross-resistance" means that if there are two drugs, if an isolate is resistant to one drug and it is highly possible that it is also resistant to the other drug. Usually, they have some similar mechanism of action or resistance mechanism, for example, they shared the same target(s). So here we correct the term as "co-resistance" which is more accurate as a Mtb isolate may happen to be resistant to both drugs ("coresistant") but not necessarily "cross-resistant" to each other. Now the concept is clear. So we did not emphasize this in the methods section.
Changes in the text: We have replaced "cross-resistance" to "co-resistance" at several places as needed. We added "Following the GeneXpert assay, the clinical samples resistance to other antibiotics were assessed using the Bactec MGIT 960 system at the MDR-TB Center, Saidu Teaching Hospital Swat, Pakistan, in accordance with the manufacturer's instructions and approved guidelines (4). In line 143-145, page 6.

Comment 3. Consider review of Table 3 and Table 4, the INH and Rifampcin-resistant cases are higher than susceptible cases? Is it a typing error?
Reply 3: The cases are accessed within the particular drug resistance, for instance in table 3, the total RIFR cases were 224 and susceptible were 17. So among the 224 RIFR cases, the 125 were resistant to INH as well. In both tables the susceptible cases are less than resistant ones.

Comment 4. More data on resistance to anti tubercular drugs and clinical correlation is possible in the present study, incidence of MDR and mono resistance especially among the newly detected cases and clinical correlation.
Reply 4: The suggestion to provide more data on resistance to anti-tubercular drugs and clinical correlation, specifically regarding the incidence of multidrug-resistant (MDR) and mono-resistance among newly detected cases, is a valuable point to consider. But we prioritized other relevant visuals or data that they deemed more important for conveying their findings within the space limitations.

Reviewer D

Summary: Clinical isolates from 241 sputum-positive patients from Pakistan were assayed for rifampicin resistance using the GeneXpert assay, rifampicin, isoniazid, and fluoroquinalone resistance using the MGIT growth tube system. First-line drug and fluoroquinalone resistance mutations were identified using the GeneType MTBDRplus assay and MTBDRsl assays, respectively. The overall frequency of drug-resistance mutations in this patient cohort were summarized. The association of rifampicin,

isoniazid, and fluoroquinalone resistance with gender, age, treatment history, drug-resistance status, and other anti-tuberculosis drugs were summarized.

Overall, this manuscript contributes data about drug-resistant tuberculosis from an underreported country with a high burden of DR-TB. The authors also demonstrate the utility of line probe assays for diagnosis to provide more effective treatment of drug-resistant TB infections.

Major revisions

Comment 1. Methodology: Must include methods used for data analysis, especially the statistical tests used and the software used to perform them.
Reply 1: Page 7 line 176-81 in revised manuscript.
Changes in the text: The data interpretation and statistical summary were conducted using the Statistical Package for Social Science (SPSS) version 16 and GraphPad Prism 5. All the variable outcomes were presented as percentages (%), and odd ratio (OR). Fisher's exact test and Chi-Square test were employed to assess the associations between categorical variables. The results were reported as either mean values or the number of patients. Statistical significance was defined as a P value less than 0.05.

Comment 2. Results: Were any cases found to be DR by MGIT culture testing and sensitive by MTBDRplus or MTBDRls? It is important to mention mismatch cases as the LPAs assay specific mutations which might not be responsible for drug resistance in the samples being tested.
Reply 2: It’s an important point, but we didn’t not encounter such case in the present study.

Comment 3. Results: The results of the statistical analysis of relationships among drug resistance statuses must be described more explicitly than “statistically significant”. The particular relationship being tested needs to be used to describe the test results (association, correlation, etc.).
Reply 3: We did our best to describe the significant data, such as page 9 line 220-24 in revised manuscript.
Changes in the text: Rate of FQR was seen to be associated with gender [(95% CI: to 0.9616), OR: 0.53; $P < 0.040$] and age [(95% CI: to 2.874), OR: 2.143; $P < 0.012$], with higher incidents of FQR in females and individuals below 30 years of age (Table 5). This differences was due to healthcare-seeking behavior, treatment adherence, or exposure to risk factors. Which could potentially contributed to varying rates of drug resistance between sexes.

Comment 4. Discussion: In Table 3 there are more RIF genotype isolates than RIF-resistant cases (227 vs. 224) but fewer INH genotype isolates than INH-resistant cases (137 vs. 138) and fewer FQ genotype isolates than FQ-resistant cases (61 vs. 63). These numbers imply multiple DR mutation in genes for RIF resistance but not INH or FQ

resistance. The deficits in the INH and FQ cases need to be discussed.
Reply 4: The RIFR case was 224, INH-resistant was 138 and FQ-resistant was 63. The deficit in the INH and FQ cases indicating the mono-drug resistance, MDR and XDR cases. Another possibility is that there are many genes related to INH resistance, but only limited gene fragments were tested.

Comment 5. Discussion: The discussion of RIF resistance is confusing. Mutations in the RRDR of the rpoB gene are know to reduce pathogen fitness. Drawing a conclusion about using RIF resistance alone as a marker for MDR cases based on a small number of samples with a limited geographic distribution is over-interpreting of results.
Reply 5: Page 10 line 265-69 in revised manuscript.
Changes in the text: RIFR may serve as an alternative marker for MDR-TB as majority of RIFR Mtb isolates are also resistant to INH (17) as the INH spontaneous rate is very high. However, in regions with low prevalence of RIFR, fast testing for RIFR alone cannot reliably predict MDR-TB (18). These tests might be useful in an MDR-TB management plan in regions with high RIFR and MDR-TB prevalence.

Comment 6. Discussion: The discussion of cross-resistance for multiple theraputics needs to include a discussion of the presence of multiple drug-resistance mutations driving this phenomenon. The accumulation of drug resistance mutations occurs in a cumulative manner, so the presence of cross-resistant strains implies that these strains have been subject to multiple therapies over the lifetime of the strain lineage.
Reply 6: Thanks for the reviewer and we have added them on page 9-12 line 231-324 in revised manuscript.
Changes in the text: Mutations within regulatory and coding regions of drug resistance-determining genes have been implicated in resistance to other antibiotics (21, 31-34). Incidence of resistance to other anti-tubercular drugs is quite evident in this study (Tables 3, 4 and 5), which implies the prevalence of MDR-TB in the studied population. For instance, a significant level of co-resistance was observed between RIF and INH as well as between RIF and MOX ($P < 0.05$), which are important first- and second-line anti-tubercular drugs. Co-resistance to RIF and INH potentially renders the first-line drugs PZA, and EMB ineffective, and resistance to MOX may reduce the effectiveness of the second-line regimens. This will increase the burden of MDR-TB cases and eventually that of Pre-XDR-TB. Significant level of co-resistance ($P < 0.001$) was also seen between INH and five other drugs (EMB, PZA, STR, LFX and MOX) (Table 4). Co-resistance between INH and other drugs may possibly be due to the high spontaneous mutation rate of INH or due to inhA as such mutations have been previously implicated in co-resistance to INH and PTO as they have the same target, InhA (35,36). The accumulation of drug resistance mutations occurs in a cumulative manner, so the presence of co-resistant strains implies that these strains have been subject to multiple therapies over the lifetime of the strain lineage.

Comment 7. Discussion: The discussion of treatment failure needs to refer to the drug-resistance mutation status of the strains in these cases. Failure could be the result of the limitations of LPA to assay mutations for resistance the latest group of anti-tuberculosis drugs (linezolid, delamanid, etc.) or novel mutations for resistance to FLDs and SLDs..
Reply 7: Page 15 line 404-410 of revised manuscript.
Changes in the text: However, others succumbed to TB, had failed treatment, were lost to follow up or are still under treatment perhaps due to extensive drug resistance. Treatment failed in 2% of the patients and 22% are still under treatment. The treatment failure could be the result of the limitations of LPA to assay mutations for resistance the latest group of anti-tuberculosis drugs (linezolid, delamanid, etc.) or novel mutations for resistance to FLDs and SLDs. This paints a rather stark picture of the extent of resistance in these patients and warrants controlled use of antibiotics among TB patients.

Minor revisions

Comment 1. Line 16: “two important anti-TB drugs i.e. rifampicin (RIF) and isoniazid (INH).” remove “i.e.”
Reply 1: Corrected on page 2, line 32-33 of revised manuscript.
Changes in the text: Which are associated with resistance to rifampicin (RIF) and isoniazid (INH).

Comment 2. Line 35: “DR-TB becomes an advance to MDR-TB ...”. This sentence is difficult to understand and must be re-written.
Reply 2: Page 3 line 49 of revised manuscript.
Changes in the text: DR-TB leads to MDR-TB.

Comment 3. Line 43: MTBC needs to be defined when first used.
Reply 3: Page 3 and line 57 of revised manuscript.
Changes in the text: Treated cases of Mtb complex (MTBC) infections.

Comment 4. Line 74-75: list the SLDs detected by MTBDRsl.
Reply 4: Page 4, line 97-98 of revised manuscript.
Changes in the text: While MTBDRsl is used to detect mutation in SLDs (levofloxacin (LFX) and moxifloxacin (MOX).

Comment 5. Line 83: This sentence would read better as “Over a one year time span we collected early-morning sputum samples in sterile leak-proof containers from 241 patients who were smear positive with a grade of +1 or higher.”
Reply 5: Page 5 line 117-19 of revised manuscript.
Changes in the text: Over a one year time span we collected early-morning sputum samples in sterile leak-proof containers from 241 patients who were smear positive with a grade of +1 or higher. The sample was kept at freezer a temperature of 2-8oC for a maximum of four days.

Comment 6. Line 84: “The samples were kept ...”
Reply 6: Page 5 line 118-19 of revised manuscript.
Changes in the text: The samples were kept at freezer a temperature of 2-8oC for a maximum of four days.

Comment 7. Line 95-96: “… and sodium citrate (2.9%). Followed by ...” should be “… and sodium citrate (2.9%), followed by ...”.
Reply 7: Page 5 line 128 of revised manuscript.
Changes in the text: and sodium citrate (2.9%), followed.

Comment 8. Line 119: “...of distal water…” should be “… of distilled water ...”.
Reply8: Page 6 line 152 of revised manuscript.
Changes in the text: of distilled water and incubated in a water bath at 95°C for 20 minutes.

Comment 9. Line 132: “… the kit protocol of stringent and rinse ...”. “Stringent” is not the correct word.
Reply 9: Page 7 line 165 of revised manuscript.
Changes in text: “For the washing process, the kit protocol of stringent wash was followed.”

Comment 10. Line 151: Does “multiple gene resistance” actually mean “non-MDR multiple drug resistance”?
Reply 10: Please refer to question 6 of Reviewer B, page 8 line 190 of revised manuscript.
Changes in the text: There were 97 incidences of mono-resistance among the 241 positive TB- patients.

Comment 11. Line 155 “ropB” should be “rpoB”.
Reply 11: Page 8 line 193 of revised manuscript.
Changes in the text: It was determined that rpoB is almost the unique gene for RIFR.

Comment 12. Line 161: “and D94Y (6) in gyrA.” This should be D94N according to Table 2.
Reply 12: Page 8 line 199 of revised manuscript.
Changes in the text: and D94N (6) in gyrA.

Comment 13. Line 164: “OR” needs to be defined when it first occurs. The best place would be in the Methods, where the calculation of OR needs to be described.
Reply 13: Added in the method section.
Changes in text: Line 179, and page 7 of revised manuscript.

Comment 14. Line 166: The use of “RIFS” for RIF-sensitive and “RIFR” for RIF-resistant needs to be defined. This is also true for “INHS”, “INHR”, “FQS”, and”FQR”.

Reply 14: Page 8 line 204-5, page 8 line 213, page 8-9 line 219-20 of revised manuscript.
Changes in the text: rifampicin-susceptible (RIFS) or rifampicin-resistant (RIFR), isoniazid-susceptible (INHS) and isoniazid-resistant (INHR)
FQ- susceptible (FQS) or FQ-resistant (FQR).

Comment 15. Line 168-170: "While among other factors, ...". This sentence needs more detail. The relationship among the "RIFS, RIFR, and MDR cases needs to be described better.
Reply 15: Page 8 line 206-7 of revised manuscript.
Changes in the text: In the other factors, the statistical significant data were observed among the RIFS, RIFR, and Pre-XDR cases [(95% CI: 0.8034-0.9841), OR: 0.25; P 0.02].

Comment 16. Lines181-183: Association of drug resistant or sensitive status is mentioned for FQ, but not for RIF and INH. These associations need to be described in the appropriate paragraphs.
Reply 16: Page 9 line 220-24 of revised manuscript.
Changes in the text: Rate of FQR was seen to be associated with gender [(95% CI: to 0.9616), OR: 0.53; P < 0.040] and age [(95% CI: to 2.874), OR: 2.143; P < 0.012], with higher incidents of FQR in females and individuals below 30 years of age (Table 5). This difference was due to healthcare-seeking behavior, treatment adherence, or exposure to risk factors. Which could potentially contributed to varying rates of drug resistance between sexes.

Comment 17. Line 201: Gender imbalance in the cases studied could be the result of many different factors other than innate susceptibility. If reference 11 provides an explanation for this it should be explicitly stated.
Reply 17: Page 9 line 242-46 of revised manuscript.
Changes in the text: It was observed in this study that female patients constitute 54% of the study population in contrast to their male counterparts, suggesting that females are at higher risk of contracting TB in this study (11) This differences was due to healthcare-seeking behavior, treatment adherence, or exposure to risk factors, which could potentially contributed to varying rates of drug resistance between sexes.

Comment 18. Line 215: "… multiple mutations on the same gene." The number of samples with multiple drug resistance mutations in the same gene were not presented in the results. They need to be if they are going to be included in the Discussion.
Reply 18: The comment has been addressed in revised manuscript. The information is corrected now.

Comment 19. Line 216: "… which spells the likelihood ...". Not clear what this is supposed to mean.
Reply 19: Page 10 line 259-60 of revised manuscript.
Changes in the text: There is evidence of resistance to RIF, INH and FQs among the

studied patients which influences the possibility for treatment failure using first-line and perhaps second-line regimens.

Comment 20. Line 230: If the mutation in the inhA promoter is the most common in these sample the frequency needs to be provided. A frequency is given for mutations in the latG gene.
Reply 20: Page 10 line 271-73 of revised manuscript.
Changes in the text: This study, a mutation at the -15 nucleic acid position of the promoter region of inhA (C15T) (74 cases) is seen to be most commonly associated with INH resistance.

Comment 21. Line 261: “PZA, EMB” should be “PZA and EMB”.
Reply 21: We have changed the manuscript according to the comment. Page 11, line 304.
Change in text: PZA and EMB

Comment 22. Figure 2: The entry for the total is not necessary and should be removed.
Reply22: Respected reviewer, we have removed the entry of total from figure 2.

Comment 23. Table 3: The upper portion of this table appears to be a summary of all samples, not the samples that are RIF resistant. This should be presented as a separate table. Rearrange the “No of cases” entry so both Beijing and Non-Beijing are separate rows under the Positive column. Having them under the Positive and Negative columns is confusing.
Reply 23: Thank the reviewer. The table portions are separated by highlighted mark lines in the revised manuscript, as the journal have specific number for inclusion of tables in article. This is why we merged the summary of all sample in the beginning of table 3.

Comment 24. Multiple locations: The numbers used for the rpoB mutations (S531L, etc.) are the coordinates in the E. coli protein, not the M. tuberculosis protein. The correct coordinates can be found in the WHO catalog of M. tuberculosis drug resistance mutations which is available from https://www.who.int/publications/i/item/9789240028173.
Reply 24: The amino acids in the RpoB protein were numbered according to the Mtb RpoB.

Reviewer E

The manuscript is well written with proper data. I would like to ask a few questions to the author

Comment 1. In Figure 2, you have given the treatment outcomes of TB-positive cases. There you have shown 136 cases were cured out of 241 cases. Among those 136 cases,

how many cases are of drug-resistant cases? (Include that data)
Reply 1: All the cases were drug-resistant TB. The 241 samples in this study were all drug-resistant.

Comment 2. Whether this work was carried out under the safety measures/guidelines issued by your government?
Reply 2: Yes, the study was carried out under WHO guidelines and Pakistan government guidelines.

Reviewer F

Comment 1. Line Probe Assay (LPA) technology is an effective clinical detection tool, but the convenience of operation and cost are not friendly.
Reply 1: We agree that the LPA is only relatively cost effective and not convenient.

Comment 2. The LPA technology is relatively good for detecting clinical samples, but to clarify the Distribution of common and rare drug resistance patterns in Mycobacterium tuberculosis is not enough. With the continuous application and popularization of new technology, such as mWGS, tNGS, et al. These technologies are more suitable for this study.
Reply 2: For further studies we will consider the mWGS, tNGS technologies as they are relatively more expensive and more complicated in Pakistan.

Reviewer G

The manuscript investigated Mtb incidences and associated mutational patterns by using Line Probe Assay (LPA) and reported the multi-drug resistant Mtb strains by mapping the rpoB, katG & inhA genes mutations. Though many similar studies were reported from different parts of the world, the information provided here could also be useful in managing tuberculosis.

Major concern:

Comment 1. The result section is a simple description. The authors need to include statements that explain the meaning of their data. So include the meaning of each table or graph at the end of the paragraph in a few sentences.
Reply 1: We have added the description in the results section for the tables and figures.

Minor concern:
Comment 1. Correct spelling errors. For example, in the result section, in Table 3 add “t” to EMB resistan
Reply 1: We have corrected the spellings errors and checked typo errors carefully according to your precious comments.

Reviewer H

Comment 1. Good attempt in conduct of the study and nice write up.
Reply 1: Thank you for the encouraging words.

Comment 2. However needs some modification in the write up and content
Reply 2: We did all the possible modifications in the revised manuscript.

Comment 3. In the abstract, methods should say how the study was conducted, The sentence -For instance the studies reported high14 frequency of mutations- could be avoided and rather be in Results section
Reply 3: We have modified the all the sections following the comment and comments from other reviewers, line 30-38 page 2.

Comment 4. In main manuscript, Introduction is detailed, which is ok, but ultimately the rationale for the study is to be clearly spelt (Is it to compare the utility of LPA in your setup to solid and automated liquid culture systems and to establish the prevalence of primary drug resistance within the study population?.)
Reply 4: This study highlights the effectiveness of GenoType MTBDRplus and MTBDRsl assays as valuable tools for TB management. These assays enable rapid detection of resistance to RIF, INH, and fluoroquinolones in Mtb clinical isolates, surpassing the limitations of solid and liquid media-based methods.

Comment 5. Methodology starts with Sample assembly and storage, it would be good if clearly and briefly the method how study was conducted is spelt and then the lab procedures described
Reply 5: Respected reviewer, we modified the section in the revised manuscript, line 103-115, page 4-5. Thank you.

Comment 6. Results section may be briefly described with main findings
Reply 6: Following the comment, we have described the main findings in the result section, line 182-230, and page 7-9.

Comment 7. Discussion should speak the implications of the finding of the study, need to be focused on the topic
Reply 7: Following the comment, we modified the discussion portion by focusing on this topic.
Changes in text: Line 231-324, and page 9-13.

Comment 8. Conclusions should restrict to what was concluded from the study findings. Though the statement in the conclusion may be true, it the conclusion from the study findings?
Reply 8: The conclusions are mainly based on the current study findings. Changes in text: Line 325-339, page 12-13.

Comment 9. Figure 1 is confusing. Is it DR or DS?
Reply 8: It is drug resistant (DR) and we marked this time in in figure 1.
Changes in text: Please see figure 1

Comment 10. Presentation in Fig 2 could also be modified
Reply 10: The figure is modified in the modified manuscript.

Comment 11. Too many tables. No of tables could be reduced and selected and important information could be presented.
Reply 11: Thanks for the comment to make the data more precise but respected reviewer we added all the tables carefully and selected based on their importance and valuable data.