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A study on the mycobacterial burden and phenotypic drug resistance pattern with reference to the GeneXpert Cycle Threshold values in pulmonary tuberculosis

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ABSTRACT

Introduction and aim. Tuberculosis (TB) remains a significant global health challenge. Early and accurate diagnosis is crucial to prevent further transmission. The present study aimed to correlate cycle threshold values with smear microscopy and culture positivity, and determine cut-off cycle threshold values for levels of smear grade and culture positivity.

Material and methods. Forty presumptive cases of pulmonary TB were included and subjected to Ziehl-Neelsen stain, culture on Lowenstein Jensen media, CBNAAT and drug susceptibility testing for first line anti-tubercular drugs.

Results. Our study predicts 3+, 2+, and 1+ sputum smear grade at a cut-off of Ct value ≤ 16.74 , ≤ 19.68 , and ≤ 22.32 respectively. A strong positive correlation was found between time to culture positivity and Ct value. A cut-off of Ct value ≤ 22.32 predicts culture positivity with a sensitivity of 92%, and a specificity of 67%. None of the isolates showed rifampicin resistance by 1% proportion method.

Conclusion. Understanding the appropriate utilization of CBNAAT Ct values and their correlation with smear microscopy grade, culture, and drug susceptibility testing can assist clinicians in early identification and prompt initiation of appropriate treatment. This knowledge can contribute to the prevention of drug resistance, reduced transmission, and a decreased disease burden associated with TB.

Keywords. CBNAAT, cycle threshold values, rifampicin resistance, smear microscopy, tuberculosis

Introduction

Tuberculosis (TB) continues to affect millions of people worldwide despite intensified standard TB control measures. Many patients with active TB, especially in TB and HIV endemic areas remain undiagnosed and continue to spread the disease in the community.¹ Mycobacterial load, measured as smear positivity grades (scanty, 1+, 2+, and 3+) in accordance with Revised National TB Control Programme (RNTCP) is used to determine infectiousness and severity of the disease.² Another measure of mycobacterial burden is the time to culture positivity, a culture-based method routinely used to assess the bacillary burden in TB.³ However, conventional solid culture has a slow turnaround time of up to 10–12 weeks, while smear microscopy has low sensitivity and quality control issues.⁴ Liquid culture techniques were developed for the early detection of mycobacterial growth, but the turnaround time is still quite long for a diagnostic test to control transmission.⁵ The cartridge-based nucleic acid amplification test (CBNAAT), an automated molecular test for the detection of *Mycobacterium tuberculosis* (MTB) has been recommended by WHO as a first line TB diagnostic test as an alternative to smear microscopy.⁶ The GeneXpert is based on real-time PCR technique that simultaneously detects DNA of *Mycobacterium tuberculosis* and rifampicin resistance.⁷ The diagnostic techniques for the detection of resistance in *M. tuberculosis* isolates include phenotypic methods like 1% proportion method, absolute concentration method and resistance ratio methods, which are based on assessment of growth of *M. tuberculosis* in culture media containing a critical concentration of specific anti-TB drugs. Genotypic or molecular methods for detection of drug resistance are the Xpert MTB/RIF assay and two commercial line probe assays, the MTB-DR plus assay and the Nipro NTM plus MDR-TB detection kit 2 which detects resistance to rifampicin alone or in combination with isoniazid. Molecular methods are highly efficacious and provide faster results.⁸

Emerging technologies aim to maintain the trend of highly accurate TB tests that have been made possible by advancements in molecular TB diagnostics over the past few years. Despite notable scientific advancements, TB is still not properly diagnosed and monitored. Concerns with improper diagnosis of latent cases and the alarming emergence of drug resistance continue.

Aim

Hence, the present study aimed to correlate cycle threshold values with RNTCP smear microscopy grades and culture positivity, and also to determine cut-off cycle threshold values to predict the different levels of smear grade and culture positivity in pulmonary TB. We further wanted to study the phenotypic drug resistance pattern to first line antitubercular drugs among various levels of cycle threshold values in pulmonary TB.

Material and methods

All presumptive cases of pulmonary tuberculosis diagnosed by Ziehl-Neelsen (ZN) staining for acid fast bacilli and/or CBNAAT. All the general and COVID-19 precautionary measures were taken before sample collection. Two sputum samples (one spot and one early morning) were collected for each patient. The study was conducted in the Direct Observation Treatment Short-course (DOTS) Centre of University College of Medical Sciences and Guru Teg Bahadur Hospital, New Delhi from November 2019 to October 2021. One sample was subjected to CBNAAT, while the other to microscopy and culture. Smears were made from the growth and identified on ZN staining, and further confirmed by MPT64 antigen detection assay. Drug susceptibility testing was performed by 1% Proportion method to Isoniazid, Rifampicin and Ethambutol as per RNTCP guidelines. We utilized the *H37RV* strain as a control to assess the drug susceptibility testing method for first-line drugs.

Ethical approval

Informed written consent was taken from the study subjects prior to conducting the study. Participation was fully voluntary based including the right to withdraw from the study at any time without presenting an explanation for their withdrawal. Data confidentiality was maintained through anonymity by avoiding any personal identifiers. Clearance for the study was taken from IEC-HR Institutional Ethics Committee-Human Research of the University College of Medical Sciences (IEC-HR/ 2019/41/68).

Data management and statistical analysis

MS Excel spreadsheet program was used to record the data. SPSS v23 (IBM Corp., Armonk, NY, USA) was used for data analysis. Descriptive statistics were elaborated in the form of means/standard deviations and medians/IQRs for continuous variables. Frequencies and percentages were used for categorical variables. Data were presented in a graphical manner wherever appropriate for data visualization using histograms/box-and-whisker plots/column charts for continuous data and bar/pie charts for categorical data. Coefficient of correlation (Spearman correlation coefficient) for Ct value with smear grade, time to culture positivity, serum adenosine levels and clinical scores were calculated. The optimum value of sensitivity and specificity of Ct value were calculated at an optimum cut-off after obtaining the ROC curve. Statistical significance was kept at p value <0.05.

Results

Our study had population range between 18 to 53 years, with male to female ratio 1.6:1. Out of the 40 samples tested, 2 were smear-negative, 12 (30%) had 1+ sputum smear grade, 15 (37.5%) had 2+, while 11 (27.5%) had 3+ sputum smear grade as per RNTCP sputum smear grading. Our study reported 37 (92.5%) of the total samples to be culture positive. Out of the total 37 culture positive isolates, 8.10% were culture

positive at the end of 3 weeks, 18.90% were positive at 4 weeks, followed by 13.50% at 5 weeks, 24.30% at 6 weeks, 16.20% at 7 weeks and 18.90% at 8 weeks. The mean (SD) of time to culture positivity was 5.78 (1.6) weeks. The time to culture positivity ranged from 3-8 weeks. Eleven samples (27.5%) of the total had a high CBNAAT category, while 22 (55%) and 7 (17.5%) had medium and low CBNAAT categories respectively. None (0%) showed rifampicin resistance.

Correlation of Ct value (mean) and sputum smear microscopy (RNTCP sputum smear grade)

Table 1 depicts the comparison of the 4 subgroups of sputum smear microscopy grades in terms of the Ct value (mean). There was a significant difference between the 4 groups of sputum smear grade in terms of Ct value (mean) ($\chi^2=30.799$, $p<0.001$), and the strength of association (Kendall's Tau) was 0.72 (large effect size).

Table 1. Comparison of the 4 Sputum smear microscopy grades in terms of Ct value (mean) (n=40)

Ct value (Mean)	Sputum smear microscopy grade				Kruskal Wallis Test	
	Negative	1+	2+	3+	χ^2	p
Mean (SD)	20.67 (2.45)	22.25 (1.63)	19.39 (1.05)	14.53 (1.84)		
Median (IQR)	20.67 (19.8– 21.53)	21.86 (21.09– 23.89)	19.26 (18.98– 19.49)	14.9 (14.71– 15.09)	30.799	<0.001
Range	18.94–22.4	19.5–24.56	17.12–21.58	11.08–16.74		

The area under the ROC curve (Fig. 1) predicts 3+ sputum smear grade at a cutoff of Ct value (Mean) \leq 16.74, with a sensitivity of 100%, and a specificity of 100%, thus demonstrating an excellent diagnostic performance. It was statistically significant ($p<0.001$). The odds ratio (95% CI) for smear grade 3+ when Ct value (mean) \leq 16.74 was 413 (15.58–10944.98), while the relative risk (95% CI) was 30 (6–169.25).

The area under the ROC curve (Fig. 2) predicts 2+ sputum smear grade at a cutoff of Ct value (mean) \leq 19.68, with a sensitivity of 92%, and a specificity of 86%, thus demonstrating excellent diagnostic performance. It was statistically significant ($p = <0.001$). The odds ratio (95% CI) for sputum smear grade 2+ when Ct value (mean) \leq 19.68 was 46 (6.74–313.92), while the relative risk (95% CI) was 4.6 (2.01–13.12).

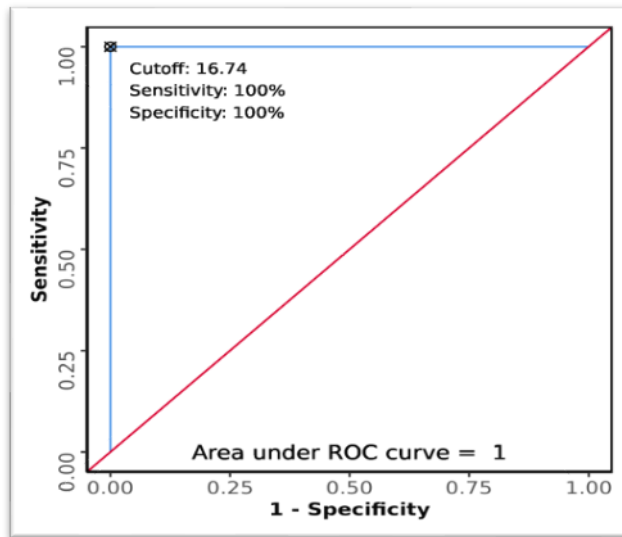


Fig. 1. ROC curve analysis showing diagnostic performance of Ct value (Mean) in predicting 3+ sputum smear grade (n=40)

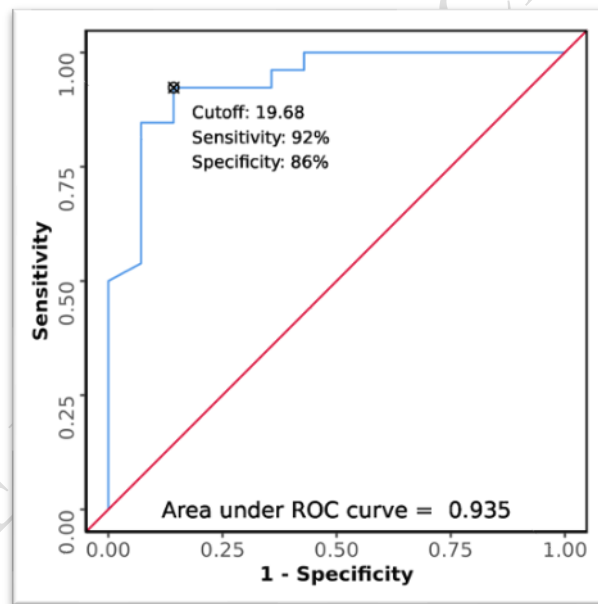


Fig. 2. ROC curve analysis showing diagnostic performance of Ct value (Mean) in predicting 2+ sputum smear grade (n=40)

Figure 3 depicts the ROC curve analysis showing the diagnostic performance of Ct value (Mean) in predicting 1+ sputum smear grade. At a cut-off of Ct value (mean) ≤ 22.32 , it predicts sputum smear grade 1+, with a sensitivity of 90%, and a specificity of 50%.

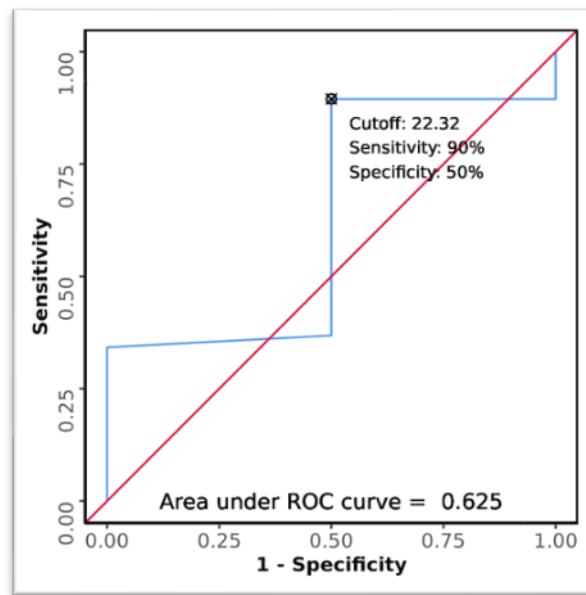


Fig. 3. ROC curve analysis showing diagnostic performance of Ct value (Mean) in predicting 1+ sputum smear grade (n=40)

Correlation of Ct value (mean) and culture positivity

Table 2 depicts the correlation of Ct value (mean) and culture positivity. The mean (SD) and median (IQR) of Ct value (mean) in the culture positive group were 18.73 (3.31) and 19.26 (16.62–21.1) respectively.

Table 2. Comparison of the 2 Subgroups of Culture in terms of Ct value (mean) (n=40)

CT Value (Mean)	Culture		Wilcoxon-Mann-Whitney U Test	
	Positive	Negative	W	p
Mean (SD)	18.73 (3.31)	21.97 (2.83)		
Median (IQR)	19.26 (16.62–21.1)	22.4 (20.67–23.48)	26.500	0.143
Range	11.08–24.34	18.94–24.56		

Correlation of Ct value (mean) and time to culture positivity

Scatterplot in the figure (Fig. 4) shows a strong positive correlation between time to culture positivity (weeks) and Ct value (Mean), and this correlation was statistically significant ($\rho=0.88$, $p<0.001$). For every 1 unit increase in time to culture positivity (weeks), the Ct Value (mean) increases by 1.75 units.

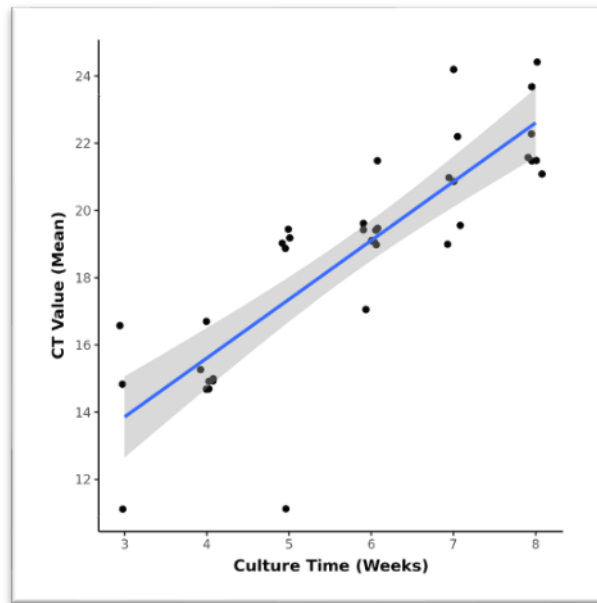


Fig. 4. Correlation between time to culture positivity (weeks) and Ct value (mean) (n=37)

The area under the ROC curve (Fig. 5) for Ct value (mean) in predicting culture positive vs culture negative was 0.761 (95% CI: 0.365–1), thus demonstrating fair diagnostic performance. At a cutoff of Ct value (mean) ≤ 22.32 , it predicts culture positivity with a sensitivity of 92%, and a specificity of 67%.

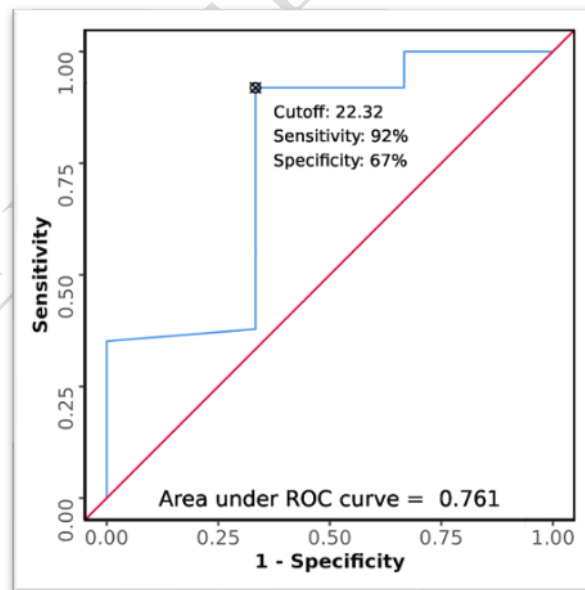


Fig. 5. ROC curve analysis showing diagnostic performance of Ct value (mean) in predicting culture positivity (n=37)

Phenotypic drug susceptibility testing (DST) pattern to 1st line anti-tubercular drugs

Table 3 shows the phenotypic drug susceptibility pattern to 1st line anti-tubercular drugs. Out of the 37 culture positive isolates, none showed resistance to rifampicin and ethambutol by 1% proportion method. However, 1 (2.7%) isolate showed isoniazid mono-drug resistance. No significant association was found between isoniazid mono-resistance and the various other patients' parameters like smear microscopy grade, culture positivity and Ct value (mean).

Table 3. Phenotypic DST pattern to 1st line anti-tubercular drugs (n=37)

Rifampicin resistance by CBNAAT	Number of isolates resistant to 1st line anti-tubercular drug in DST by 1% proportion method		
	Isoniazid	Rifampicin	Ethambutol
0	1	0	0

Discussion

Lack of adequate diagnostic facilities for TB is one of the key barriers to TB control in the majority of high-burden countries. The steadily rising number of cases of drug-resistant TB emphasizes the critical need for precise and rapid diagnostic techniques for their detection.⁹ The laboratory detection turnaround time should be as short as possible with a large capacity in order to reach the large number of patients efficiently.

Our study has demonstrated an excellent diagnostic performance of Ct value in predicting the various grades of smear positivity, with higher sensitivity as well as specificity. We also found a significant difference between sputum smear grade and Ct value (mean) ($\chi^2=30.799$, $p<0.001$), with the strength of association (Kendall's Tau) of 0.72 (large effect size). A study conducted in Uganda among pulmonary TB patients found Xpert Ct values comparable with smear microscopy, similar to our findings. Their study reported a cut-off of 23.62, which had the highest sensitivity and specificity in predicting +1 smear grade, comparable to our study findings.¹⁰

In a study conducted by Kassa et al. on pulmonary drug-resistant tuberculosis patients, 16.35% (95% CI: 13.40, 19.79) were smear-negative, which is a slightly higher proportion compared to our study (5%).¹¹ Furthermore, the distribution of smear grades also varied. While our study showed a higher proportion of 1+ smear grade (30.0%) compared to Kassa et al. (18.27%), their study reported a higher percentage of 3+ smear grade (34.42%) compared to our study (27.5%). It is evident that there are differences in the proportions of smear-negative cases and the distribution of smear grades which could be attributed to the variations in study populations, sample sizes, and different geographical locations.¹¹

Our study found a strong positive correlation (Spearman correlation coefficient=0.9) between time to culture positivity and Ct value (Mean), and this correlation was statistically significant ($\rho=0.88$, $p<0.001$). For every 1 unit increase in time to culture positivity (in weeks), the Ct value (mean) increases by 1.75 units. Najjingo et al. found a correlation of 0.37 between the Xpert Ct values and time to culture positivity in their study on pulmonary TB patients.¹⁰ Their study has also shown a linear relationship between time to culture positivity and Ct values; the Ct values were found to increase by 2.57 for every unit increase in days to culture positivity, which is comparable to our study findings. However, their study compared the time to culture positivity of liquid culture media with Ct values, in contrast to solid culture media used in our study.¹⁰

Our study showed 37 (92.5%) of the total samples to be culture positive. Out of the total 37 culture positive isolates, 8.10% were culture positive at the end of 3 weeks, 18.9% were positive at 4 weeks, followed by 13.50% at 5 weeks, 24.30% at 6 weeks, 16.20% at 7 weeks and 18.9% at 8 weeks. The mean (SD) of time to culture positivity was 5.78 (1.6) weeks. The time to culture positivity ranged from 3–8 weeks. Palange et al. in his study found that the mean of time to culture positivity on Lowenstein Jensen media for pulmonary samples was 31.32 days which ranged from 30.8–31.64 days.¹² Among 7 low CBNAAT category detected samples in our study, culture growth was not seen in 3 samples. However, one sample among the culture-negative samples showed 1+ sputum smear grade; rest two were smear-negative. Prakash et al reported similar discordance in his study in the very low category of CBNAAT.¹³ In his study, only two samples out of 34 samples in the very low category were smear positive, one of which was culture negative, whereas growth was seen in 10 samples only. The two samples which were smear positive in the very low category did not show growth on culture which is comparable to our study findings. On the contrary, ten samples which showed growth on culture had delayed time to culture positivity and were AFB smear negative.¹³ Najjingo et al. in his study concluded that majority of low and very low Ct values quantified by Xpert were negative by smear microscopy.¹⁰ This poses a higher risk of TB transmission in the community.

In our study, the following patients' parameters were significantly associated ($p<0.05$) with Ct value (mean): sputum smear microscopy findings, culture time, and CBNAAT category. The mean (SD) of Ct value (mean) in smear-negative group was 20.67 (2.45), whereas the mean (SD) of Ct value (mean) in 1+, 2+ and 3+ grades were 22.25 (1.63), 19.39 (1.05) and 14.53 (1.84) respectively. The Ct value (mean) ranged from 18.94–22.4 in the smear-negative group, whereas it ranged from 19.5–24.56, 17.12–21.58, and 11.08–16.74 in 1+, 2+ and 3+ grades respectively. Najjingo et al. reported that the median Ct values were 25.4, 23.8, 18.2, 20.1 and 16.6 for negative, scanty, 1+, 2+ and 3+ smear grades respectively.¹⁰ A recent study reported that the smear negative samples had a median Ct of 28.4 (IQR 24.2–31.44) compared to 18.7 (15.8–22.6) for smear positive samples.¹⁴ Fradejas et al. reported that the samples from smear-negative patients had a significantly higher mean Ct value than those from smear-positive patients 20.9(± 5.8) vs.

16.9 (± 4.9), which is comparable to our study findings.⁶ Another study reported a statistically significant difference in the mean Ct value between sputum smear-positive and sputum smear-negative patients (17.8 ± 4.8 and 22.3 ± 6.7 , respectively; $p=0.002$).¹⁵ This could possibly be due to variable sample size in the various studies described above. Moreover, the co-morbid conditions and various other factors associated with the study population also varied in different studies.

Conclusion

Several correlations were found between CBNAAT Ct values and various microbiological parameters. Various cutoff levels to determine different grades of smear and culture positivity were also established. Phenotypic drug resistance pattern was also analyzed. The knowledge of the proper utilization of Ct values by the treating physicians and the drug resistance epidemiology will bridge the gap between the delay in diagnosis and management of TB with further prevention of transmission of drug resistant strains.

Declarations

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Author contributions

Conceptualization, B.K. and K.S.; Methodology, K.S.; Software, K.Si.; Validation, B.K., K.S. and P.H.; Formal Analysis, K.S.; Investigation, K.S.; Resources, P.H.; Data Curation, K.Si.; Writing – Original Draft Preparation, K.S.; Writing – Review & Editing, B.K.; Visualization, K.S.; Supervision, B.K.; Project Administration, B.K.

Conflicts of interest

The authors have no conflict of interest. There is no relationship of interest with any company in the study we are responsible for. No support was received from any project or company for the research.

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Institutional Review Board of the Institutional Ethics Committee-Human Research of the University College of Medical Sciences (IEC-HR/ 2019/41/68).

References

1. Tuberculosis 2023. World Health Organization. Available from: <https://www.who.int/news-room/fact-sheets/detail/tuberculosis>. Published April 21, 2023. Accessed August 14, 2023.
2. Najjingo I, Muttamba W, Kirenga BJ, et al. Comparison of GeneXpert cycle threshold values with smear microscopy and culture as a measure of mycobacterial burden in five regional referral hospitals of Uganda- A cross-sectional study. *PLoS One*. 2019;14(5):e0216901. doi: 10.1371/journal.pone.0216901
3. Lawn SD, Nicol MP. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiol*. 2011;6(9):1067-1082. doi:10.2217/fmb.11.84
4. Steingart KR, Ng V, Henry M, et al. Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis*. 2006;6(10):664-674. doi: 10.1016/S1473-3099(06)70602-8
5. Moore DF, Guzman JA, Mikhail LT. Reduction in turnaround time for laboratory diagnosis of pulmonary tuberculosis by routine use of a nucleic acid amplification test. *Diagn Microbiol Infect Dis*. 2005;52(3):247-254. doi: 10.1016/j.diagmicrobio.2005.02.014
6. Fradejas I, Ontañón B, Muñoz-Gallego I, Ramírez-Vela MJ, López-Roa P. The value of xpert MTB/RIF-generated CT values for predicting the smear status of patients with pulmonary tuberculosis. *J Clin Tuberc Other Mycobact Dis*. 2018;13:9-12. doi: 10.1016/j.jctube.2018.04.002
7. Agrawal M, Bajaj A, Bhatia V, Dutt S. Comparative Study of GeneXpert with ZN Stain and Culture in Samples of Suspected Pulmonary Tuberculosis. *J Clin Diagn Res*. 2016;10(5):DC09-DC12. doi: 10.7860/JCDR/2016/18837.7755
8. Gilpin C, Korobitsyn A, Weyer K. Current tools available for the diagnosis of drug-resistant tuberculosis. *Ther Adv Infect Dis*. 2016;3(6):145-151. doi: 10.1177/2049936116673553
9. Laniado-Laborín R. Clinical challenges in the era of multiple and extensively drug-resistant tuberculosis. *Rev Panam Salud Publica*. 2017;41:e167. doi: 10.26633/RPSP.2017.167
10. Najjingo I, Muttamba W, Kirenga BJ, et al. Comparison of GeneXpert cycle threshold values with smear microscopy and culture as a measure of mycobacterial burden in five regional referral hospitals of Uganda- A cross-sectional study. *PLoS One*. 2019;14(5):e0216901. doi: 10.1371/journal.pone.0216901
11. Kassa GM, Merid MW, Muluneh AG, Fentie DT. Sputum smear grading and associated factors among bacteriologically confirmed pulmonary drug-resistant tuberculosis patients in Ethiopia. *BMC Infect Dis*. 2021;21(1):238. doi: 10.1186/s12879-021-05933-y

12. Palange P, Narang R, Kandi V. Evaluation of Culture Media for Isolation of Mycobacterium Species from Human Clinical Specimens. *Cureus*. 2016;8(8):e757. doi: 10.7759/cureus.757
13. Prakash AK, Datta B, Tripathy JP, Kumar N, Chatterjee P, Jaiswal A. The clinical utility of cycle of threshold value of GeneXpert MTB/RIF (CBNAAT) and its diagnostic accuracy in pulmonary and extra-pulmonary samples at a tertiary care center in India. *Indian J Tuberc*. 2018;65(4):296-302. doi: 10.1016/j.ijtb.2018.05.021
14. Beynon F, Theron G, Respeito D, et al. Correlation of Xpert MTB/RIF with measures to assess Mycobacterium tuberculosis bacillary burden in high HIV burden areas of Southern Africa. *Sci Rep*. 2018;8(1):5201. doi: 10.1038/s41598-018-23066-2
15. Pagano GC, Pereira GR, D'Ávila KG, Monaiar LR, Silva DR. Association between Xpert MTB/RIF cycle threshold values and sputum smear microscopy in patients with pulmonary tuberculosis. *J Bras Pneumol*. 2021;47(2):e20200549. doi: 10.36416/1806-3756/e20200549

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