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Original Research Paper

Quantitative Assessment of Gene Xpert Assay with sputum microscopy

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ABSTRACT:

Background: The assessment of mycobacterial burden plays a very important role in evaluating the infectiousness and severity of disease. Smear microscopy is used to assess the load of mycobacteria in the sputum sample by clinicians. Gene Xpert has been recommended by WHO as a first-line tuberculosis (TB) diagnostic test as an alternative to smear microscopy. Gene Xpert offers a quantitative estimation of mycobacterial load in the form of cycle threshold values (Ct).
Objective: To evaluate mycobacterial quantitation by Gene Xpert and compare with grades of staining methods**.**
Methods: This prospective comparative study was done in the Department of Microbiology and Department of Pulmonary Medicine, Faculty of Medicine and Health Sciences, SGT University, Budhera, Gurugram (Haryana) from November 2018 to May 2020. Early morning sputum samples were processed for smear microscopy (by Ziehl Neelsen Staining and Auramine Staining) and Gene Xpert as per the NTEP guidelines. **Results:** A strong correlation was found between decreasing Ct values of Gene Xpert and increasing estimates of bacterial load by smear microscopy. The majority of low and very low Ct values quantified by Gene Xpert were negative by smear microscopy.

Keywords: ZN Staining, AO Staining, Gene Xpert, Ct values.

INTRODUCTION:

Rapid diagnosis of tuberculosis is paramount to limit its worldwide spread. Early diagnosis leads to early and efficient initiation of anti-tubercular treatment, adequate isolation and contact tracing.[1] Microscopic examination of sputum smears is widely used method for detection of acid-fast bacilli (AFB). It helps in quantifying mycobacterial load at the time of diagnosis. Mycobacterial load quantification is helpful in evaluating the severity of the disease and the risk of transmission in pulmonary tuberculosis patients. [2,3] Despite its low sensitivity and specificity, smear microscopy is the initial microbiological examination carried out when a patient's clinical presentation is consistent with pulmonary tuberculosis.[1] Culture is regarded as the gold standard, but it is not widely available due to the long turnaround time, which causes patient treatment delays. It is also prone to contamination and needs specialized infrastructure. Although PCR-based methods have substantially improved and accelerated tuberculosis diagnosis, they require a particular molecular diagnostic laboratory setup. Due to these limitations, WHO has approved Gene Xpert/Xpert MTB/RIF/CBNAAT as the initial test for tuberculosis diagnosis. [4] Gene Xpert is a simple point-of-care test (POCT) that can simultaneously detect, *Mycobacterium tuberculosis* (MTB) and the main mutations associated with resistance to rifampicin in 2 hours. It measures mycobacterial burden quantitatively using cycle threshold values (Ct), which correlate inversely with the concentration of TB. High bacilli load is implied by low Ct values, and vice versa.[5] The World Health Organization has suggested the Gene Xpert as the initial test for tuberculosis microbiological

detection in all suspected tuberculosis patients, including new cases and retreatment cases, as well as suspected multidrug-resistant (MDR) tuberculosis. It is very useful in HIV-infected patients as well as in children due to its high sensitivity and specificity combined with an incredibly fast turnaround time, where microbiological diagnosis is still challenging. [6,7]

Earlier, smear microscopy was the only diagnostic method to find out the infectiousness of patients. A positive smear result increases the likelihood of tuberculosis transmission and confirms the suspicion of the disease. [8-12]. It has been found that the risk of transmission in smear negative patients is considerably low as compared to smear-positive patients. [13,14]

However, various studies have demonstrated that smear-negative people are still capable of transmitting TB, with a minimum relative transmission rate estimated to be 0.22. [15-18] Studies have shown the relationship between the bacillary load as determined by conventional methods, such as colony counts on solid agar growth media or measurements of Time-to-Detection (TTD) in liquid culture with the Ct values of Gene Xpert. [19-21] To study its diagnostic value for predicting the smear status in patients with pulmonary tuberculosis is yet to be explored. [1,22-26]

The aim of the current study was to ascertain the possible benefit that the Gene Xpert Ct values may add when used as a rule-in test for smear positivity status. The correlation between Gene Xpert Ct and the smear grade has been included in the study. With the aim of determining the mycobacterial load by smear status and Gene Xpert Ct values we conducted a study in patients suffering from pulmonary tuberculosis.

METHODOLOGY:

Study Design and Study Area:

The Study was approved by the Institutional Ethical Committee (IEC), and written informed consent was taken by all the participants to use their sputum for TB Research and Diagnostics. In the research study, sputum samples were included from both inpatients and outpatients.

Inclusion Criteria:

1. All the clinically suspected cases of pulmonary tuberculosis based on the presence of clinical characteristics, suggestive radiological findings, and/or a history of exposure to an infected case of TB were enrolled in the study. Patients came with symptoms such as fever and cough with or without expectoration for 2 weeks, unexplained significant weight loss, loss of appetite, fatigue, hemoptysis, and suggestive chest radiography.
2. Immunocompromised and patients with or without previous history of TB

Exclusion Criteria:

1. Patients ≤ 14years of age
2. Patients already on Anti Tubercular Drugs

Sample Collection:

Two samples of sputum from suspected tuberculosis cases were collected in the early morning hours of the day using leak-proof, disposable, and labelled containers. One sample was collected in a sterile, heat-proof, wide-mouth container for staining, and the other was collected in a sterile 50 ml falcon tube for Gene Xpert at the hospital's Designated Microscopic Center(DMC), following standard protocol. We retrospectively compared the results of the Gene Xpert with two staining methods (Ziehl Neelsen Staining and Auramine Staining) on sputum samples collected in patients with suspected pulmonary tuberculosis. Two sputum smears were prepared from the wide mouth container on glass slides: one for the Ziehl Neelsen (ZN) stain and the other for the Auramine Stain (AO). A proforma/questionnaire was used to collect patient data at the time of sample collection. Sample processing was carried out inside a Type II A 2 Biosafety Cabinet.

Measurement of Mycobacterial Burden:

Sputum samples collected in the early morning were prepared according to the NTEP (formerly RNTCP) protocols for Gene Xpert and smear microscopy. Smears were graded as scanty, 1+, 2+ and 3+ smear grades as per the International Union Against Tuberculosis and Lung Disease (IUATLD) scale. [27] Gene Xpert used the mean of the five probes (A, B, C, D, and E) to quantify bacilli, and the latter was reported as the mean Ct value. [25]

RESULTS:

Comparison of smear grading in ZN Staining with Gene Xpert

Smear Grading (ZN Staining)	Gene Xpert Positive results on the basis of cycle threshold					Total
	Ct (>28)	Ct (22-28)	Ct (16-22)	Ct (<16)	ND	
Neg	30	32	43	19	0	124
Scanty	2	3	3	4	1	13
1+	1	4	14	29	7	55
2+	0	4	29	23	2	58
3+	2	0	10	23	0	35
Total	35	43	99	98	10	285

Correlation of Gene Xpert Ct Values with ZN Staining:

Out of the 285 sputum samples, 161 samples were found to be positive for AFB by ZN staining while *Mycobacterium tuberculosis* was detected by Gene Xpert in 275 samples. Of these 124 samples negative by ZN staining but positive by Gene Xpert the bacillary load was found to vary from very high to very low. Nineteen ZN negative samples had high bacillary load Ct value (<16) while 43 samples had medium bacillary load Ct value (16-22) in rest of the 62 samples bacillary load was low in 32 samples Ct value (22-28) and very low in 30 samples Ct value (>28). The mean Ct values of Gene Xpert and the smear grades of ZN staining had a strong inverse correlation. (-0.353). The results were found to be statistically significant ($p < 0.05$)

Comparison of smear grading in AO Staining with Gene Xpert

Smear Grading (AO Staining)	Gene Xpert positive results on the basis of Cycle threshold Total					Total
	Ct (>28)	Ct (22-28)	Ct (16-22)	Ct (< 16)	ND	
Neg	14	16	19	12	0	61
Scanty	11	12	22	3	0	48
1+	7	10	15	25	8	65
2+	1	5	19	28	1	54
3+	2	0	25	29	1	57
Total	35	43	100	97	10	285

Correlation of Gene Xpert Ct Values with AO Staining:

Out of 285 samples positive for pulmonary tuberculosis, 275 samples showed the presence of *mycobacterium tuberculosis* by Gene Xpert while 224 samples were positive for AFB by AO staining. Of the 51 samples negative by AO staining but positive for *Mycobacterium tuberculosis* by Gene Xpert, the bacillary load varied from very high to very low. Twelve samples had very high bacillary load Ct value (< 16) while 19 had medium bacillary load Ct value (16-22), in rest of the 30 negative samples the bacillary load was low in 16 samples Ct value (22-28) and very low in 14 samples Ct (>28) respectively. It was found that the mean Ct values of Gene Xpert and the smear grades of AO staining had a weak correlation between them (0.008) that was not statistically significant ($p > 0.05$)

DISCUSSION:

Sputum smear microscopy is still the primary microbiological investigation used to diagnose tuberculosis and to determine patient infectiousness

associated with a high transmission risk. [8-10,12] On the other hand it is time and labour intensive, requires specialized personnel, and has a limited sensitivity. Moreover, AFB staining is unable to distinguish between MTB and non-tuberculosis mycobacteria, which contributes to the low specificity of smear microscopy. The World Health Organization has recommended Gene Xpert as confirmatory test for TB diagnosis in order to overcome these limitations. [6]

Gene Xpert / Cartridge based nucleic acid amplification test (CBNAAT) is a real time polymerase chain reaction-based method used for the detection of TB. It is an automated, cartridge-based test specifically for MTB complex. It fully automates the amplification and detection processes, using real-time PCR, and results are available within 100 minutes.[29] It has a highly specific primer and five unique molecular probes to target the *rpoB* gene associated with rifampicin resistance.[30] In the assay lyophilized *Bacillus globigii* spores act as an internal sample processing and PCR control. *Mycobacterium tuberculosis* is detected by five overlapping molecular probes (A-E) that are complementary to the entire 81 bp *rpoB* core region. [29,30]. *M. tuberculosis* is detected when at least two of the five probes generate positive signals with a cycle threshold (Ct) of 38 cycles that differ by no more than a predetermined number of cycles. *B. globigii* internal control is found positive when the single *B. globigii*-specific probe produces a Ct of ≤38 cycles. [29,30]. The standard user interface indicates the presence or absence of *M. tuberculosis* and rifampicin resistance, and the Ct range (high, <16; medium, 16–22; low, 22–28; extremely low, >28) defines a semiquantitative assessment of the concentration of bacilli. Assays are considered as invalid when they test negative for both the *B. globigii* internal control and *M. tuberculosis*. [29,30] There were no cross reactions with any other bacterial species examined, including a comprehensive band of mycobacteria, there by excluding non-tubercular mycobacteria.

The infectious dosage of tuberculosis comprises of less than ten bacilli. In sputum, the limit of detection for Gene Xpert is found to be 131 (95% CI: 106-176) CFU/ml. [23] whereas the sensitivity of microscopy ranges from 5000 to 10,000 AFB/ml, so it suggests that smear microscopy has more chances of missing many potentially infectious patients than Gene Xpert. [1]

Thus, the current study was carried out to evaluate the efficacy of Gene Xpert Ct values as a rule-in test for smear positivity status. Additionally, we investigated the relationship between the smear grade and Gene Xpert Ct values.

In this study, we compared the performance of the Gene Xpert to smear microscopy for determining the potential added value of the molecular Point of Care Test (POCT) as a first-line test for tuberculosis diagnosis and to assess patient transmission potential.

Quantitative Gene Xpert results have been compared in various studies with conventional methods of measuring bacterial load, such as time-to-detection (TTD) in liquid culture, counts of CFU on solid agar growth media and direct or concentrated sputum smears. [1,23-26]

A study in Cape Town (South Africa) by Theron et al found that Gene Xpert Ct values had limited clinical relevance as a rule-in test for smear positivity, however, a Ct cut-off value of 31.8 offered a relatively excellent rule-out value for smear positivity. [22]

Robert Blackmore et al did a multisite assessment of the bacterial burden by Xpert MTB/RIF and compared it with conventional quantitative methods in sputum samples. They estimated the bacterial burden of Xpert MTB/RIF by measuring the threshold-cycle. A Ct cut off of around 27.7 accurately indicated smear-positive status. According to their findings, Xpert MTB/RIF quantification provides a novel, standardized technique for assessing bacterial burden in the sputum of patients suffering from tuberculosis. [23]

Isabel Fradejas et al in Madrid, Spain calculated the association between Ct values, smear status and TTD on liquid culture. In 65 patients Ct value and TTD yielded a positive correlation, while Ct and smear grade yielded an inverse correlation. It was determined that 21.1 cycles was the ideal Ct value for ruling out patients with positive smear results. The study confirmed that the value of Gene Xpert Ct levels acts as a predictor of positive smear status for quantifying mycobacterial load especially at Ct values below 21. [24]

A recent study in Uganda by Irene Najjingo et al revealed a decreasing trend in CT values with increasing smear grades. They compared GeneXpert cycle threshold results to smear microscopy and culture as a measure of mycobacterial load. A weak correlation of 0.37 was found between Xpert Ct levels and MGIT time to positive, whereas 0.34 was found between Xpert Ct values and LJ culture. While there was a relatively strong correlation of 0.55 between Xpert Ct value and smear grade. A cutoff Ct value of 23.62 turned out to be the best predictor of smear positivity. [25]

O. Opota et al. assessed the feasibility of substituting the microscopy results with Xpert MTB/RIF in order to assess patient's transmission risk and promptly advise for airborne isolation. They proved that Xpert MTB/RIF is more effective than smear-based techniques for tuberculosis diagnosis and determining patient infectiousness. [1]

In a study conducted by Bineeta Kashyap et al, both respiratory and non-respiratory samples of pediatric TB cases were processed to evaluate the relationship

between CBNAAT grading based on the cycle threshold value and conventional microbiological methods of diagnosis, AFB staining, and Solid Culture. They came to the conclusion that CBNAAT grading is significantly associated with smear and culture positivity. Increased accessibility and wider availability of CBNAAT may aid in the diagnosis of certain cases that remains undiagnosed by conventional microbiological methods. [26]

All the previous studies are in support of the findings of present study which shows inverse correlation between the grades of smear microscopy and Ct values of Gene Xpert.

According to Gene Xpert, a large number of sputum samples classified as smear-negative showed a rather high mycobacterial burden in the present study. This implies that a low Ct value can identify samples with a significant number of MTB bacilli, even if the sample is smear negative. As smear-negative patients are a significant potential source of TB transmission [15], Gene Xpert quantitation could be utilized to detect the infectious smear-negative patients and aid in the spread of disease.

The only study in which smear samples were stained with the auramine thiazine red technique and examined under a fluorescence microscope was carried out by Isabel Fradejas et al [24].

To the best of my knowledge, no such study has been conducted to compare two staining techniques (ZN and AO) with Gene Xpert quantification.

CONCLUSION:

There is a strong relationship between decreasing Gene Xpert Ct values and increasing estimations of bacterial load reported by smear microscopy-based techniques. Most of the extremely low and low Ct values reported by Gene Xpert were found to be negative by smear microscopy, posing a danger of TB to the community. This suggests that Gene Xpert have the quantitative capability and can be used in settings where microscopy-based techniques are used.

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TRANSPARENCY DECLARATION:

There are no conflicts of interest for the authors in this research work.

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