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COMPARISON OF DIAGNOSTIC TOOLS; ZIEHL NEELSEN STAINING, FLUORESCENCE MICROSCOPY WITH GEN-EXPERT FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS IN RESOURCE LIMITED SETTINGS

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Abstract

In resource-limited nations such as Pakistan, pulmonary tuberculosis represents a predominant contributor to disease burden and fatalities. Conventional diagnostic techniques such as Ziehl-Neelsen (ZN) staining, Fluorescence Microscopy (FM), and GeneXpert each have distinct advantages and limitations. This cross-sectional study aimed to assess the diagnostic efficiency of ZN staining, FM, and GeneXpert for the detection of MTB in resource-limited, high-burden settings.

The current study was carried out at the Department of Pathology, Khalifa Gul Nawaz Medical Teaching Hospital, Bannu, Pakistan. A total of n=400 respiratory samples were collected and analyzed through Ziehl Neelsen staining, fluorescence microscopy, and GeneXpert; reference standard. Diagnostic performance metrics were determined for each diagnostic approach. The socioeconomic status of the participants was categorized according to the revised Kuppuswamy scale. Statistical analysis was performed using SPSS v 26.

The GeneXpert identified 19.3% (n=77/400) of the patients positive for Mycobacterium tuberculosis, followed by FM 15.5% (n=62/400) and ZN staining 12%(n=48/400). The GeneXpert emerged as the superior diagnostic method, achieving 95% sensitivity, 98% specificity, 96% positive predictive value, and 97% negative predictive value. The sensitivity and specificity of FM vs. ZN 88% vs.92% and 85% vs.90% respectively. The highest percentages of MTB positive cases were observed in the upper lower 31.2%(n=24/77) and lower 37.6% (n=29/77) socioeconomic groups and decreased consistently among higher socioeconomic tiers. GeneXpert remained a reliable tool for the diagnosis of MTB, with the limitation of infrastructure and associated costs.

Key Words: *GeneXpert, Microscopy, MTB/RIF, Pakistan, Tuberculosis*

INTRODUCTION

Pulmonary tuberculosis is an infectious disease with significant transmissibility; predominantly affecting the respiratory system with the potential of dissemination to other organs. Despite effective prevention and treatment strategies, tuberculosis remains a leading cause of mortality in resource limited settings (1). Globally, an estimated 10.8 million new cases were reported in the year 2023 and the predominant form was pulmonary tuberculosis (2). Early and accurate detection of Mycobacterium tuberculosis (MTB) is very essential to prevent its spread, associated morbidity, and mortality. Different traditional laboratory diagnostic tests such as acid-fast bacilli (AFB): Ziehl Neelsen (ZN) microscopy, fluorescence microscopy (FM), and nucleic acid amplification such as GeneXpert MTB/RIF, each have their advantages and limitations.

In low-resource settings, ZN staining is a gold standard for the diagnosis of MTB due to its low cost, simplicity, and minimal infrastructure requirements (3). However, wide variation in its sensitivity (20-60%), particularly poor in smear-negative or borderline samples has been observed (3). Furthermore, the prognostic value of ZN staining microscopy is limited as it cannot quantify the bacterial load nor detect

Rifampicin resistance, whereas due to its high specificity may miss the early MTB cases. Alternative to ZN staining, Fluorescence microscopy is more sensitive and time efficient and its acquisition in traditional diagnosis particularly in MTB endemic countries has improved diagnostic capabilities (3). The FM requires specialized laboratory equipment with its high cost but offers higher sensitivity and rapid scanning. However, it cannot still identify drug resistance and relies on operator expertise (4).

The introduction of GeneXpert transformed the MTB diagnosis by combining nucleic acid amplification with the detection of Rifampicin resistance. As per WHO guidelines GeneXpert is considered a gold standard in a facility lack of culture (5). It's a rapid test and took one and a half hours to detect MTB DNA and rifampicin resistance in comparison to culture latency by weeks. The reported sensitivity and specificity are around 89%-99% in smear-positive cases and 60-74% in smear-negative cases with consistently around 98% specificity (3). However, these strengths of GeneXpert come at higher costs including capital investment, instruments, cartridges, supply chain reliability, and stable electricity (3).

The MTB culture (Liquid/solid) is considered a gold standard and remains essential for the confirmation of smear-negative cases and for testing antibiotic susceptibility, yet the slow turnaround limits its clinical utilization. Given these outcomes, this study evaluated the comparative diagnostic accuracy of Ziehl Neelsen staining, fluorescence microscopy, and GeneXpert for the detection of pulmonary tuberculosis in Bannu District, Khyber Pakhtunkhwa, a high-burden, resource-constrained region of Pakistan.

MATERIALS AND METHODS

STUDY DESIGN AND SETTING

The current prospective observational study was carried out between July to November 2023 at the Department of Pathology, Khalifa Gul Nawaz Medical Teaching Hospital, Bannu, Khyber Pukhtoonkhawa (KPK), Pakistan. The Hospital has a capacity of 385 beds with 210 beds currently in function. The hospital has an emergency, intensive care unit and basic diagnostic and supportive services. The hospital provides tertiary care services to the southern region of the province, particularly to districts Bannu, Karak, North Waziristan, and Lakki Marwat. Study participants comprised individuals presenting with clinical manifestations suggestive of pulmonary tuberculosis, recruited from outpatient department (OPD).

SAMPLE COLLECTION AND PROCESSING

Altogether, four hundred (n=400) suspected patients were included in the current study. Early morning deep cough, mucopurulent sputum with the volume of 3-5ml in a clean, sterile, wide mouth, leak-proof, and screw-capped 50 ml containers were collected from each study participant. The containers were labeled with patient's demographics and the clinical specimens fulfilling the Bartlett criteria were included (6). The sputum specimens mixed with pus, blood, saliva and less than 3ml were excluded from the current study. Each collected sputum sample was divided into three parts; ZN staining, FM, and GeneXpert analysis. Duplicate smears were prepared for both ZN and Auramine phenol staining and staining was carried out as described elsewhere (7). Parallel to test smears, positive and negative control smears were prepared and stained for quality control. The ZN-stained smears were observed under a light microscope at 100X, whereas, Auramine-phenol stained specimens underwent microscopic examination utilizing a 40× dry objective lens on an LED-based fluorescence microscopy system (Zeiss Primo Star iLED). The interpretation was carried out for both types of staining as per Revised National Tuberculosis Program (RNTCP) guidelines. The stained smears were graded as negative, doubtful, 1+,2+, or 3+ by WHO recommendations. The GeneXpert MTB/RIF assay was carried out as per the protocol of the manufacturer (Cepheid Inc., Sunnyvale, CA, USA) and as described previously (7). The results were obtained after one and a half hours. Furthermore, a questionnaire regarding socioeconomic status was recorded from each participant, and the socioeconomic status of the participant was determined by revised socioeconomic status scales (8).

ETHICS STATEMENT

Ethical approval was obtained from the Institutional Ethical Board of the Institute of Para Medical Sciences, Khyber Medical University. Written informed consent was procured from all study participants, with data maintained under strict confidentiality protocols.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS version 26.0 (IBM Corporation, Armonk, NY). Categorical variables were presented as frequencies and percentages, with a chi-square test applied for statistical comparisons. Statistical significance was established at $p<0.05$. Ziehl Neelsen staining and fluorescence microscopy findings were evaluated against GeneXpert as the gold standard reference method. GeneXpert is considered the reference standard for true positive and true negative classifications. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated to assess diagnostic performance.

RESULTS

A total of four hundred (n=400) suspected pulmonary tuberculosis patients {51.3%(n=205/400) male and 48.7% (n=195/400) female patients} were scrutinized using three different diagnostic techniques: Ziehl Neelsen staining, FM, and GeneXpert (reference standard). The GeneXpert identified 19.3% (n=77/400) of the patients positive for Mycobacterium tuberculosis. 53.3% (n=41) of the GeneXpert-positive patients were male whereas 46.7% (n=36) were female as shown in table 1. The MTB detection by GeneXpert was further stratified by the bacterial load as follows; high: 4.5%(n=18/77), medium:5%(n=20/77), low: 5%(n=20/77), and very low: 3.25% (n=13/77) detection cases whereas 1.5%(n=6/77) yielded in-determined results.

Table I. Age, gender, and socioeconomic distributions of GeneXpert Positive cases (n=77) of MTB suspected patients attending Khalifa Gul Nawaz Medical Teaching Hospital, Bannu, from July to November 2023

Age	Male 53.3% (n=41)	Female 46.7% (n=36)	Total n=77	Class	Negative (n=323)	Positive (n=77)	Total (n=400)
1-20 yrs	4.8 (2)	5.5 (2)	5.2 (4)	Upper	(0.9) 3	1.3 (1)	1 (4)
21-40 yrs	26.8 (11)	27.8 (10)	27.3 (21)	Upper Middle	14.3 (46)	10.4 (8)	13.5 (54)
41-50yrs	26.8 (11)	13.9 (5)	20.8 (16)	Lower Middle	16.7 (54)	19.5 (15)	17.3 (69)
>50 yrs	41.6 (17)	52.8 (19)	46.7 (36)	Upper lower	29.4 (95)	31.2 (24)	29.7 (119)
				Lower	38.7 (125)	37.6 (29)	38.5 (154)

Auramine phenol staining and ZN staining identified 15.5% (n=62/400) and 12% (n=48/400) patients respectively as positive for *Mycobacterium tuberculosis*. Auramine phenol staining demonstrates higher sensitivity 79.2% as compared to ZN staining: 59.74%. The number of false negative cases was comparatively lower for FM 20.8% (n=16/77) than ZN staining 42.2% (n=31/77); $p<0.001$, prioritizing FM techniques for improved detection efficiency. The observed fluorescence intensities in FM-positive cases were as follows; Scanty: 3%, FM1+: 5.25%, FM2+: 4.55%, and FM3+=2.75% respectively. Predominant cases of ZN staining yielded negative results followed by mild staining (1+): 4.25%, moderate (2+): 1.75% and strong staining 3+: 1% as shown in Table II. The observed grading for both techniques prioritizes FM over conventional staining. Ziehl Neelsen staining and fluorescence microscopy exhibited concordance with GeneXpert ($\kappa=0.129$, $p<0.001$ versus $\kappa=0.832$, $p<0.001$, respectively). The GeneXpert have a slight agreement with ZN and almost perfect agreement with FM. All GeneXpert-negative samples remained negative across both microscopic methods. ROC analysis showed fluorescence microscopy superiority (AUC=0.986) over Ziehl Neelsen staining (AUC=0.821). Overall, the GeneXpert remains the most accurate modality with 95% sensitivity, 98% specificity, 96% positive predictive value, and 97% negative predictive value in comparison to other techniques.

Table II. Comparison and correlation of different Diagnostic tests used % (n) of MTB suspected patients attending Khalifa Gul Nawaz Medical Teaching Hospital, Bannu, from July to November 2023

Attributes	FM %(n)	ZN %(n)	GeneXpert %(n)	Staining Techniques	GeneXpert		Kappa	*p* value	PPV(%)	NPV (%)
Positive	15.5 (62)	12 (48)	19.3 (77)	FM	Positive			Negative		
Negative	84.5(338)	88(352)	80.7 (323)	Positive	61	1	0.129	0.001	98.38	97.04
Scanty	19.4(12)	39.6(19)		Negative	16			328		
1+	33.8(21)	33.3(16)				ZN Staining				
2+	27.4(17)	16.7(8)		Positive	46	2	0.832	0.001	95.83	92.89
3+	19.4(12)	10.4(5)		Negative	31	327				

Note: FM: Fluorescence Microscopy, ZN: Ziehl Neelsen , PPV: Positive predictive value, NPV: Negative predictive value,n: Number, %: Percentage

SOCIOECONOMIC STATUS AND MTB POSITIVE CASES

A strong relation was observed between MTB-positive patients and their socioeconomic status as shown in Table 1. The highest GeneXpert positive cases were observed in the upper lower 31.2% (n=24/77), and lower 37.7% (n=29/77) socioeconomic groups and decreased consistently among higher socioeconomic tiers. Overall a significant association was found between socioeconomic status and MTB positive cases; $X^2(4) = 29.29$, $p<0.001$. Among the upper lower and lower socioeconomic groups an estimated 1 out of 4 individuals tested MTB positive.

DISCUSSION

Mycobacterial culture represents the gold standard for tuberculosis diagnosis; however, resource constraints, technical expertise limitations, and prolonged incubation periods preclude routine implementation in district healthcare facilities. Consequently, Ziehl-Neelsen microscopy, fluorescence microscopy, and GeneXpert serve as rapid diagnostic alternatives in a limited number of facilities. This investigation evaluated Ziehl-Neelsen staining and fluorescence microscopy performance against GeneXpert as the reference standard for sputum-based tuberculosis detection.

In our study, GeneXpert identified 19.3% of the patients' positive for Mycobacterium tuberculosis followed by Auramine phenol staining at 15.5% and ZN staining at 12% respectively. GeneXpert,s superior performance over FM and ZN staining for the diagnosis of pulmonary tuberculosis has been previously observed by different studies (9-13). The introduction of GeneXpert for the diagnosis of MTB, especially in developing countries is considered a vital assay for the rapid diagnosis of smear-negative MTB cases and plays a crucial role in TB control programs. Also, the higher diagnostic yield for FM was observed as compared to ZN staining in our study and is in compliance with the previous reports where FM has superior performance over conventional ZN staining for the detection of MTB (9, 14-16). The introduction of FM for the MTB diagnosis would allow quality microscopy and prompt diagnosis.

Ziehl-Neelsen microscopy showed significantly higher false-negative rates (7.75%, n=31) relative to fluorescence microscopy (4%, n=16), which is in consistence with the previous studies (9, 17). Higher diagnostic sensitivity of fluorescence microscopy has been substantiated across numerous investigations (9, 18). Both methodologies demonstrated statistical concordance with GeneXpert; nevertheless, fluorescence microscopy exhibited markedly stronger correlation ($\kappa=0.86$, $p<0.001$) compared with Ziehl Neelsen microscopy ($\kappa=0.60$, $p<0.001$). Similar findings had been reported previously (9).

We observed similar specificity (99.7%) for both FM and ZN staining techniques when compared to that of GeneXpert. However, FM has more sensitivity 79.2% than ZN staining 59.7%. Ideally, the diagnostic techniques should be 100% specific and sensitive. Previously it has been observed that FM has better sensitivity and specificity for diagnosis of MTB in comparison to ZN staining techniques (9, 19). A high percentage of males were GeneXpert positive (51.3% male vs 48.7% female). These considerable differences in the MTB occurrence among gender might be due to socioeconomic, cultural as well as immunological

factors (20). Patients in the age groups 21-40 years and >50 years were likely more infected with MTB in comparison to other age groups. The same patron had been observed in previous studies (20, 21). The former is the productive age group, more active, frequent mobility, and the exposure chances are also high, these may contribute to an increase of MTB infection in this specific age group whereas the latter age group is aged, usually immunocompromised, and is more prone to such kind of infections.

The GeneXpert showed 95% sensitivity and 98% specificity, which is parallel to the previous report from Pakistan (22). However, studies from other regions have demonstrated comparatively low sensitivity and specificity (20, 23). The variation in sensitivity and specificity may be due to the genetic variation of the study participants. Furthermore, technical expertise and assay conditions might also have impacted findings.

The inverse relation of MTB-positive cases was observed with socioeconomic status groups. Interestingly among these lower socioeconomic groups, an estimated 1 out of 4 individual's tested positive which highlights that socioeconomic disadvantage is a major risk factor for TB prevalence and recognizes poverty as a core drive for MTB transmission. Previously, higher MTB infections had been reported among poor physical condition and underprivileged groups (24, 25). Furthermore, the presence of MTB infections among higher economic tires highlights the transcapacity beyond economic classes and emphasizes on need for universal access to prevention and screening.

CONCLUSION

Our findings suggest that the FM technique is superior for the detection of MTB compared with the ZN technique. The FM technique offers a robust, cost-effective and should be scaled up in resource-limited and high-burden settings as a practical alternative. It offers reasonable specificity, greater sensitivity, and faster screening.

Conflict of interest:

The authors declare no conflict of interest.

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Authors' contribution:

AU & MU contributed equally to this work; MA, MWK & HU assisted in data collection and laboratory analysis; SU & JU participated in statistical analysis and interpretation of results; AK manuscript formatting and reference management. IA supervised the study, reviewed and finalized the manuscript. All authors read and approved the final version of the manuscript.

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