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Research article

A Performance Assessment Study of Different Respiratory Samples for the Diagnosis of Pulmonary Tuberculosis (TB) using GeneXpert, Smear Staining, and TB Cultures

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Abstract: Tuberculosis (TB) is one of the major re-emerging diseases. An increase in disease burden and subsequent misuse of anti-tuberculosis drugs has made the situation worse. Early diagnosis followed by antibiotic susceptibility testing can lower the increase in occurrence of multidrug-resistant (MDR). Conventional methods of diagnosing TB are laborious and time-consuming, so the present study was designed to evaluate the relative efficacy of conventional methods, including Ziehl-Neelsen (ZN) stain, fluorescent stain, BD Mycobacteria growth indicator tube (MGIT) liquid culture system, and molecular diagnosis via GeneXpert, using different respiratory samples. Results of the present study showed that A total of 642 samples were found positive via the BD MGIT Liquid cultures method, 483 using the ZN staining technique, 507 with the fluorescent staining technique, and 663 for GeneXpert, respectively. It was evident from the results that out of the samples tested, GeneXpert has a higher sensitivity than AFB smear microscopy and liquid culture methods in respiratory samples. GeneXpert can be a useful tool for early diagnosis of patients with high clinical suspicion of pulmonary TB. Positive GeneXpert, but culture-negative results should be read cautiously and well correlated with the clinical and treatment history of the patient. The other major advantage of GeneXpert is that it simultaneously detects Rifampicin (Rif) resistance and is especially beneficial in patients with MDR tuberculosis, and should be studied further. As False negative or false positive cases cause overuse of anti-TB drugs, leading to the emergence of MDR-TB. So, timely and accurate diagnosis is the key factor in the management of the disease, especially in countries like Pakistan with limited resources.

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Introduction

The disease tuberculosis stands amongst top ten causes of deaths worldwide. Pakistan ranks 5th, amongst high burden countries, globally. The biggest issue in the treatment, prevention and control of tuberculosis is drug resistance. Unfortunately, Pakistan lies at number 6th high burden country in drug-resistance tuberculosis (Malik et al., 2018). About 5,10,000 people, including 15,000 children acquire this infection leading to more than 70,000 deaths in Pakistan, annually. Occurrence of multidrug resistant (MDR) TB is a serious issue which

needs to be addressed. It is a type of TB in which the isolate of *Mycobacterium tuberculosis* is resistant to rifampicin and isoniazid (Abubakar et al., 2021).

According to surveillance report of World Health Organization (WHO) in 2017, around 6,00,000 people have rifampicin resistance TB while about 4,90,000 patients develop MDR. Out of which, 47% of the patients belong to China, Pakistan, India and Russia. During 2016, in Pakistan 518000 new cases of TB were reported with an incidence rate of 268/1,00,000 and the death rate was 23/1,00,000 (Saleem et al., 2018). Co-infection rate of patients with human immunodeficiency virus were reported to be 3.5/1,00,000 in patients with TB while it was 14/1,00,000 in patients with MDR TB. New patients of TB have MDR and rifampicin resistance rate of up to 4.2% and 16% among those who previously received treatment. There is another issue of emergence of extensive drug resistance (XDR); which is a more complicated form of MDR TB as it also shows resistance to fluoroquinolones and at least one of the three injectable second line anti-TB drugs i.e. Capreomycin, Kanamycin and Amikacin (Gautam et al., 2021).

Culture for TB is the gold standard in diagnosing TB although it is unable to provide results as promptly as compared to other routine bacterial cultures and usually takes about 4-6 weeks for ultimate diagnosis. Other methods including radiometric (BACTEC) and non-radiometric methods like fluorescent labeled mycobacterium growth indicator test (MGIT) are relatively faster to get the diagnosis within 7-10 days but these methods are still time consuming as more time efficient tests can aid in early diagnosis (Jabbar et al., 2006). Serological tests were also found less efficient for clinicians in diagnosing TB. The rapid identification and diagnosis, which is crucial for early treatment, effective public health interventions and improved patient outcomes, relies on nucleic acid hybridization techniques (Qureshi et al., 2008).

In 2011, WHO endorsed the use of a fully automated molecular test utilizing the real-time polymerase chain reaction (PCR) and is called Xpert MTB/RIF assay. It has the ability to simultaneously detect MTB and resistance mutations in the *rpoB* gene against rifampicin. It can provide results within 2 hours (Lun et al., 2012). The GeneXpert MTB/RIF assay is a novel integrated diagnostic device that performs sample processing and hemi nested real-time PCR analysis in a single hands-free step for the diagnosis of tuberculosis and rapid detection of RIF resistance in clinical specimens. Xpert has minimal requirements of technologist time in terms of sample processing and laboratory equipment and provides rapid identification of rifampin resistance, allowing earlier treatment of drug-resistant TB (Maori et al., 2021).

The most important first line drug used to treat tuberculosis is rifampicin and is considered as a key in determining the efficacy of different treatment regimens adopted for tuberculosis. Around 90% of strains resistant to RIF are also resistant to isoniazid, this can be further used as marker for determining MDR-TB (He et al., 2012). RIF works by stopping the DNA-directed RNA synthesis of MTB by working in relation with RNA polymerase subunit (RNAP). According previous researches, 95% strains mutation (*rpoB*) of RIF resistance are located in 81-bp region, which are called as RIF resistance determining region (RRDR). In RRDR, mutations within codon 516,531,526 are responsible for about 90% of RIF-resistant strains (Chung et al., 2013). With increase in prevalence of tuberculosis and higher antibiotic resistance rate in many districts of Punjab at alarmingly high level, the present study was conducted among the population of district Narowal to study the prevalence of pulmonary TB in relation to risk factors like age, gender, educational level, residential area, family history and health condition of partner. As of these factors are related to the tuberculosis infection and important in determining the frequency of rifampicin resistance in *Mycobacterium Tuberculosis.*

Materials and Methods

The elaborative descriptive study was conducted on the patients of pulmonary tuberculosis, who visited the TB clinics of tertiary care hospital in Lahore, Pakistan. Broncho-alveolar lavage (BAL) and sputum samples were collected from the patients. To avoid contamination, sterile containers were provided for sample collection. After collection, samples were immediately transported to biosafety level-III laboratory, by following standard protocols. A total of 2862 samples from the suspected pulmonary tuberculosis patients. From the total samples 171 were BAL while other 2691 were sputum. The socio demographic characteristics of patients were also noted before the collection of samples. All of the samples were proceeded for BD MGIT liquid culture system, fluorescent microscopy, ZN staining and CBNAAT GeneXpert (Cartridge Based Nucleic Acid Amplification Test). Samples from the Extra pulmonary TB patients were excluded from the study.

Processing of samples for staining and TB cultures

The samples were proceeded for TB culture using BD MGIT liquid culture media. After inoculation of samples in the liquid culture system the tubes were incubated and the remaining samples were processed for ZN and fluorescent staining. ZN staining technique was used to differentiate the acid-fast bacteria from other bacteria. Acid fast bacilli (AFB) which is a member of genus *Mycobacterium* is a type of bacteria which remains unstained by gram stains used in gram staining, and they can only be visualized by using acid fast or Ziehl-Neelsen (ZN) staining. Due to the presence of unique component in their cell wall called mycolic acid, they develop the acid fastness property (Kumar et al., 2021).

For fluorescent staining, an equally distributed 2×4 cm smear was made in the middle of a glass slide. Sample must be dry during smear preparation or leave it for air dry. Heat fix at 40-60°**C**. Place the slides on staining rack with 1cm apart from each other. Pour auramine rhodamine for 20 minutes. Wash with tap water. Pour decolorizer (acid-alcohol) for 2-3 minutes. Use tap water to wash the smear. Pour the smear with methylene blue for 2-3 minutes. Lastly, air dry the slide (Maori et al., 2021).

Testing on CBNAAT GeneXpert (Cartridge based nucleic acid amplification test)

The collected samples were treated with lysing buffer and shaking well on vortex mixer and was allowed to liquefy for 10-20 minutes. Buffered sample was shifted to MTB/RIF Assay cartridge by dropper. Cartridge was loaded on GeneXpert instrument by scanning the barcode present on cartridge. This procedure takes less than two hours for one test (Maori et al., 2021).

Data presentation and statistical analysis

For statistical analysis different statistical tools were used including the Microsoft Excel (version 2017) and SPSS (version 21). The data was entered in MS excel and SPSS separately. Descriptive statistics were applied to get five parameter summaries. Chi square test was applied find association between variables. *P*-value of < 0.05 was considered as significant.

Results

A total of 2862 samples were collected from patients. Overall, out of 2862 collected samples 663 (23%) were found positive for TB. While sample wise distribution showed 23% (621/2691) and 24% (42/171) from sputum and BAL respectively. The prevalence of samples collected in relation to positive cases is showed in **Table 1**.

Table 1. Prevalence of positive TB (GeneXpert) cases from different samples.

Type of sample	**Total number of samples**	**Positive Samples for TB**	***P*-value**
Sputum	2691	21.69 % (*n* = 621)	> 0.05
Broncho-alveolar Lavage (BAL)	171	1.46 % (*n* = 42)	
Total	2862	23.16% (*n* = 663)	

TB infection was most prevalent among individuals aged 31–49 years, accounting for 48.41% of cases, followed closely by those aged 20–30 years (44.34%), while the lowest occurrence was observed in the 10–19 years (3.16%) and >50 years (4.07%) groups **(Table 2)**. Males constituted the majority of TB cases (93.66%), compared to females (6.33%). Similarly, married individuals represented a higher proportion of TB cases (91.40%) compared to unmarried participants (5.59%). Urban residents showed a greater frequency of TB infection (66.06%) than rural residents (33.93%). Educational status also showed a marked difference, with uneducated individuals exhibiting a higher burden of TB infection (58.37%) relative to those who were educated (42.98%).

Table 2. Socio demographic characteristics of TB patients (GeneXpert).

Characteristics		**Total (*n* = 2862)**	**TB Infection (*n* = 663)**
Age (Yrs.)	10-19	123	21 (3.16%)
	20-30	1536	294 (44.34%)
	31-49	942	321 (48.41%)
	>50	261	27 (4.07%)
Gender	Male	2526	621 (93.66%)
	Female	336	42 (6.33 %)
Marital Status	Married	2253	606 (91.40%)
	Unmarried	609	57 (5.59%)
Residence area	Urban	1511	438 (66.06%)
	Rural	1351	225 (33.93%)
Education status	Educated	1668	285 (42.98%)
	Uneducated	1194	387 (58.37%)

Staining and liquid culture media

Out of 2862 samples, a total of 483 samples were found to be positive for ZN-staining **(Table 3)**. Fluorescent stain is little more specific as compared to conventional ZN-staining. Out of 2862 samples, 507 samples were found positive for fluorescent staining. While results of the MGIT liquid culture system showed that a total of 642 samples were positive for TB.

Table 3. Positive samples showing the comparison of various techniques used to detect TB in various sample sources.

Testing techniques	**Sputum (*n*/%)**	**Broncho-alveolar lavage (*n*/%)**	**Total (*n*/%)**
BD MGIT Liquid cultures	624 (94.11)	18 (2.71)	642 (96.83)
ZN staining	468 (70.58)	15 (2.26)	483 (72.85)
Fluorescent staining	483 (72.85)	24 (3.61)	507 (76.47)
GeneXpert	621 (93.66)	42 (6.33)	663

GeneXpert detection

Results of GeneXpert assay showed that, a total of 663 patients were positive for tuberculosis via GeneXpert assays. From these 663 cases, 42 cases were found resistant against Rifampicin **(Table 4)**.

Table 4. Prevalence of Rifampicin resistant cases by GeneXpert.

Type of sample	**MTB detected in GeneXpert**	**Rif. resistant cases**	***P*-value**
Sputum	621 (93.66%)	39 (5.88%)	> 0.05
Broncho-alveolar Lavage (BAL)	42 (6.33%)	03 (0.45%)	
Total	663	42 (6.33%)	

Discussion

Tuberculosis has turned out to be the most common re-emerging disease worldwide, especially in third world countries. According to WHO the main reason of re-emergence of it is pandemic nature of HIV as poor living standards and increase in multi drug resistance in tubercle bacilli, aids in spreading the disease (Wada et al., 2014; Yaqoob et al., 2021). The causative agent of TB was discovered hundreds of years ago, and with advancement and development of the new drugs and treatments, it is now possible to successfully treat and cure the disease. It remains as the major cause of morbidity and mortality worldwide because of its solitarily

irresistible nature on this planet (Tahseen et al., 2020). The present study was conducted in the TB clinic at a tertiary care hospital in Lahore, Pakistan on the total 2862 samples. From the total 2862 samples, 2691 were sputum and 171 were Bronco-Alveolar lavage. Results of the present study showed that 663 sample were positive for tuberculosis via GeneXpert.

In a previously published study, authors analyzed the Recurrence of broadly MDR TB in Pakistan and found that expanded from 1.5% in 2006 to 4.5% in 2009 (p<0.01) (Atif et al., 2018a; Niaz et al., 2020). To understand the mechanism of disease transmission, the chosen strains were genotyped by using spoligo-composing, mycobacterial mixed dreary units–variable number of pair rehashes, and IS6110 limitation part length polymorphism examination. In the present study we have found that 42/663 cases were resistant against rifampicin. TB has become a major medical issue in under developing countries with poor standard of living, with 60% of adult population getting infected by it. In Nepal the prevalence rate of disease is 40% (Mehta et al., 2020). consistently 40,000 patients are suffering from active TB, while 20,000 have irresistible aspiratory infections. According to international evolution, Nepal is positioned 27th for TB cases (Yadav et al., 2020). According to present study, a total of 23.1% of samples were positive for pulmonary tuberculosis. From these total samples confirmed by GeneXpert MTB/RIF method, only 483 samples were found positive for AFBs using ZN staining. A total of 507 samples were positive for AFBs using fluorescent staining.

The major objective of the previous study conducted, was to check the resistance patterns of anti-TB drugs in tuberculosis patients. From 11-2018 to 01-2019, more than 385 cases were studied. From 385 patients of TB, 225 were found sensitive and 130 were resistance to INH (Tweed, 2019). These results were in accordance with results from other countries who utilized comparative study method (Andama et al., 2021; Guo et al., 2021; Mukhtar and Butt, 2018; Nagu et al., 2021). In Italy, out of 90 cases of dynamic TB, 17.8% were females and 82.2% were males (Sweeney et al., 2016). On the other hand, results of the present study showed that a total of 93.66% of positive cases were male, and 6.33% cases were found positive in female. Similarly, 66.06% of the patients belongs to urban areas and the rest 33.93% belongs to rural areas.

Results of the present study showed that the positive case ration was greater in male then females. Results showed by the study conducted in Atlanta USA by Blumberg et al (1991-1997), rate of TB cases among 1536 is 26% in females and 74% in males. Similarly in Korea (Sotgiu et al., 2009) and Russia (Toungoussova et al., 2002), studies showed that the percentage of TB cases among male and female was 66.49 to 34.31%. Both Isoniazid and streptomycin drugs showed higher level of resistance against TB (Atif et al., 2018b). These strains showed resistance to both drugs and accounts for 13-14% resistance percentage, individually. While strains isolated from new and recently treated patients showed 6.6% and 6.4% resistant to streptomycin individually (Maori et al., 2021). The reason behind increase in cases of MDR-TB might be the missed diagnosed or negative smear for TB and subsequently leading it to remain undiscovered which ultimately causes the increase in the cases of MDR-TB (He et al., 2012). In case of positive smear of TB, there might be a chance that anti-TB drugs show different response and it might be difficult to discover MDR-TB (Abubakar et al., 2021).

According to a previous study, conducted in Nepal (Shrestha et al., 2018) analyzed the difficulties in the conclusion of medication safe tuberculosis by the GeneXpert MTB/Rif examine in Nepal and investigate the potential arrangements. They gathered the information from the GeneXpert administrators, clinicians and program directors from 16 focuses the nation over and investigated by SPSS. They confirmed that significant difficulties were deficient preparing, regular force disappointment, trouble in keeping up suitable consistent temperature, module disappointment which is frequently not supplanted in time, issues with alignment and convenient accessibility of cartridges just as proper approaches to store the new cartridges and safe removal of the pre-owned cartridges.

Conclusion

Occurrence of drug resistance TB (MDR-TB) is a serious problem in Pakistan as well as throughout the world. Timely diagnosis of such lethal infection is very important. To prevent the misuse or over-use of anti-TB drugs, it is important to the check the efficacy of different drugs which are being used for the treatment of tuberculosis. Appropriate and precise use of drugs play a vital role in the treatment of these types of invasive infections while keeping the issue of resistance to drugs, minimal.

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