Molecular Approaches for Detection of Rifampin-Resistant *Mycobacterium tuberculosis* and its Associated Risk Factors among General Population

ABSTRACT

GeneXpert MTB/RIF is a molecular technique for the diagnosis of tuberculosis. This method not only detects *M. tuberculosis* but also identifies the absence or presence of different drug resistant mutations in DNA fragments like rifampicin resistance. In this study, about 200 septum samples were collected from tuberculosis suspected people of Isfandyar Bukhari District Head Quarter Hospital Attock for screening and determination of Mycobacterium tuberculosis and multi-drug-resistant tuberculosis. The samples were screened for the presence of *M. tuberculosis* using Fluorescence Microscopy staining followed by GeneXpert MTB/RIF assay. The results were further confirmed by Line Probe Assay and Mycobacterium Growth Indicator Tube 960 techniques. In a total of 200 samples, 45(22.5%) were found positive, 155 (77.5%) were classified negative on culture while 38(19%) were positive and 162(81%) were negative on FM, 33(16.5%) were positive and 167(83.5%) were negative on ZN staining microscopy and 47(23.5%) were noted positive and 153 (76.5%) were negative on GeneXpert. The overall sensitivity of GeneXpert was 07% higher than that of smear microscopy and 4.2 % higher than culture.Different risk factors reported for TB are; malnutrition (23.40%), health care workers (14.89%), immunosuppression (12.76%), unimmunized with Bacille Calmette-Guérin (BCG) vaccine and contact with infected persons (10.63%), diabetes mellitus (8.51%), poverty and foreign-born children are (4.2%). It is concluded that the most common risk factor for TB was malnutrition (23.40%) and the foreign-born children (4.2%) were least affected.GeneXpert assay for identification of tuberculosis was found more sensitive than fluorescence microscopy and for isolation of MTB and rifampicin resistance,GeneXpert RIF assay can efficiently be usedwhich is simple, robust and efficient with minimum handling technique.

Keywords:*Mycobacterium tuberculosis*, Rifampicin, Microscopy, GeneXpert, Multidrug-resistant tuberculosis

INTRODUCTION

Mycobacterium tuberculosis is responsible for an air-borne infection which is also a health problem occurring globally i.e. Tuberculosis (TB). Worldwide It is the second primary death cause after Acquired Immunodeficiency Syndrome (AIDS). It is a global health problem common in developing countries and its early detection and proper treatment are important to save human life [1-3]. Although GeneXpert *Mycobacterium tuberculosis*/Rifampicin (MTB/RIF) assay is a widely used diagnostic procedure for tuberculosis but still incidence of tuberculosis is 4.1 million people worldwide. Therefore, more efficient and cheap tests are needed for screening tuberculosis. World Health Organization (WHO) and Foundation for Innovative New Diagnostic (FIND) have made a

global report on tuberculosis in May 2018. The report elaborated, latest laboratory parameters for Drug Susceptibility Testing (DST) of respiratory medicines used to treat drug-resistant tuberculosis. DST is a culture-based procedure for testing the susceptibility of TB drugs to suggest anti-tuberculosis drugs and depends on the Minimum Inhibitory Concentration (MIC) of a drug that is enough to inhibit the growth of 99.1% wild type strains of *M. tuberculosis*.

In Asian countries, more than one-fourth of the World's tuberculosis cases and deaths occur, among which the recorded incidence in India is 10.4 million annually. Pakistan is among the top in 20 countries with high tuberculosis and high multi-drug resistant tuberculosis (MDR-TB), as 0.51 million new cases of tuberculosis occurred in 2015 with 4.2% drug-resistant tuberculosis cases [4]. In the case of tuberculosis, a strain is considered to be multi-drug resistant if it develops resistance against at least 2 first-line antibiotics i.e. Isoniazid and Rifampicin [5]. In 2018, the treatment was successful in 54% MDR-TB and 30% extensively drug-resistant tuberculosis (XDR-TB) cases. According to WHO, automated real-time PCR (RT-PCR) assay with an integrated semi-automated instrument is the rapid diagnostic procedure to detect tuberculosis and Rifampicin resistance in *M. tuberculosis*. In addition, a semi-automated device to diagnose tuberculosis and MDR-TB is GeneXpert Rifampicin resistance assay [6].

MDR-TB shows resistance to Rifampicin and Isoniazid drug and may have resistance or sensitivity to other anti-TB antibiotics as well. Rifampicin resistance is because of point mutations in the *rpoB* gene in the beta subunit of DNA-dependent RNA polymerase while resistance to Isoniazid is because of mutations at one of two main sites, in the *katG* and *inhA* genes of MTB. The appropriate detection of MDR-TB needs a positive culture growth of *M. tuberculosis* and drug susceptibility testing [7]. Multi Drug resistanttuberculosis are more common in countries like Pakistan and there is little information about theMolecular Approaches for Detection of Rifampin-Resistant Mycobacterium tuberculosis and Risk Factors associated with Tuberculosis in District Attock, Punjab province. Therefore, this study was planned to compare the diagnosis techniques used for the detection of multidrug resistant tuberculosis and different risk factors associated with tuberculosis.

MATERIALS AND METHODS

Samples Collection and Processing

Informed consent was taken from all participating patients as approved by the ethical committee of the Department of Biotechnology and Microbiology, Abasyn University Peshawar. About 200 samples were collected from tuberculosis suspected patients visiting Isfandyar Bukhari District Head Quarter (IYB-DHQ) hospital, Attock. Standard procedure was followed for collecting a sputum sample from tuberculosis patients [8]. Patients diagnosed with tuberculosisandDeep lungs specimen will be included in the study and Bloodstained and Watery or bubbled samples excluded from the study.

Determining and Confirmation of Samples

The collected samples were screened for the presence of *M. tuberculosis* using ZN and FM staining followed by GeneXpert MTB/RIF assay. For FM staininga standard protocol was followed to prepare slides for each sample [21]. The results were further confirmed by LPA and Mycobacteria Growth Indicator Tube 960 (MGIT 960-DST) techniques.BACTEC MGIT 960 system was used for culturing of MTBC and DST as per recommended procedures [22].

GeneXpert (MTB/RIF) Assay

A sputum sample was mixed with decontamination reagent in a 2:1 (v/v) ratio for 15 min at ambient temperature with intermittent mixing. The mixture was then loaded into the cartridge and placed in GeneXpert instrument (GX). Results were ready within two hours. Sensitivity and specificity of the fluorescence microscopy and GeneXpert assay were calculated as follows,

$$Sensitivity = \frac{TP}{TP + FN} \times 100$$

where TP represents True Positive and FN represents False Negative

$$Specificity = \frac{TN}{TN + FP} \times 100$$

where TN represents True Negative and FP represents False Positive.

Risk Factors

Data were collected from the participants using questionnaires that had different factors and data were collected from the patients.

Data Analysis

All the microscopy, culture, GeneXpert (MTB/RIF assay) [9], and MGIT 960-DST data were maintained on Microsoft Excel 2007. The LPA and GeneXpert (MTB/RIF assay) results were statistically calculated and compared. The overall accuracies of results for LPA and GeneXpert (MTB/RIF) assay, gold standard Mycobacteria Growth Indicator Tube-Drug susceptibility testing (MGIT-DST) and sequencing results were compared equally [10].

RESULTS

Samples were collected from children and adults of both genders, among the 200 samples, 47(23.5%) were found positive and 153(76.5%) were negative for TB. Out of 200 collected samples, 112(56 %) were male and 88(44 %) were female. The positive male patient was 28(14.0%) and 19(9.5%) were female.The 47 positive cases were analyzed among different age groups; 01-10 accounts for2.1%, 11-20 for 8.5%, 21-30 for17.0%, 31-40 for 19.1%, 41-50 for 21.2%, 51-60 for14.8%, 61-70 for 10.6%, 71-80 for 4.2% and 81-90 for 2.1%, respectively. Gender and age-based distribution of positive and negative isolates are shown in **Figure 1** and **2**.

Microscopy Results

Pulmonary sputum samples from all 200 samples were processed for fluorescent microscopy technique and ZN microscopy technique.

Grading Positivity of Acid Fast bacilli on ZN and FM Microscopy

All the 200 samples have proceeded on ZN and FM microscopy in which 33(16.5%) were recorded positive and 167(83.5%) were negative using ZN Microscopy. Based onthe numbers of Acid Fast Bacilli (AFB) present in the slides the results were given

as scanty, 1+, 2+ and 3+ on 100 X objective. All the 200 samples havealso proceeded on FM microscopy in which 38(19%) were recorded positive and 162(81%) were negative. Based onthe numbers of AFB present in the slides the results were given as scanty, 1+, 2+ and 3+ on 20X and 40 X objectives. The microscopy results of the 40X objective were more prominent than 20X.

GeneXpert Assay

All the 200 pulmonary samples were also processed on GeneXpert assay in which 47(23.5%) were recorded positive and 153(76.5%) were noted negative. Out of 47 (23.5%) positive, 28(59.5%) were male and 19(40.5%) were female, out of 47 positive 4(8.5%) were noted rifampicin resistance. It means out of 200 pulmonary samples, 153(76.5%) samples produced negative results, while 47(23.5%) were observed as positive on GeneXpert (MTB/RIFF) assay. Frequency distribution of positive (MTB/Rifampin resistant) isolates on GeneXpert (MTB/RIFF) assay and MGIT culture are shown in **Table 1**.

MGIT-DST Culture Results

All the GeneXpert positive samples were also cultured on MGIT media for further confirmation in which 45 (22.5%) were noted positive and 2(4.2%) were negative. Out of 47 samples, 45 were *M. tuberculosis* and the remaining 2 were negative while4 showed rifampicin resistance TB. It means out of 47 pulmonary GeneXpert positive samples, 2(4.2%) samples produced negative results on MGIT culture, while 45 (22.5%) were observed as culture positive.

Common Risk factor for TB

Different risk factors for TB were also reported including poverty (4.2%), malnutrition (23.40%), immune suppression (12.76%), diabetes mellitus (8.51%), unimmunized with BCG (10.63%), contact with infected persons (10.63%), foreign-born children (4.2%) and health care workers (14.89%), respectively as shown in **Table 2**. It was concluded that the most common risk factor for TB was malnutrition (23.40%) and the foreign-born children and poverty (4.2%), were the least affected.

Table 1: Frequency distribution of positive (MTB/Rifampin resistant) isolates on GeneXpert assay and MGIT culture

Samples	Gene Expert positive	MTB culture positive	Non MTB positive	*M. tuberculosis* Rifampicin resistance culture positive
200	47 (23.5%)	45 (22.5)	2(4.2%)	4 (8.5%)

Table 2: list of Risk Factors for *Mycobacterium Tuberculosis*

S.No	Risk factors	Positive isolates	Percentage (%)
1	Unimmunized with BCG	05/47	10.63
2	Malnutrition	11/47	23.40
3	Age (< 20 years)	05/47	10.63
4	Immune suppression	06/47	12.76
5	Foreign born children	2/47	4.2
6	Contact with infected persons	05/47	10.63
7	Health care workers	0747	14.89
8	Poverty	02/47	4.2
9	Diabetes mellitus	4/47	8.51

Figure 1: Gender based distribution of positive and negative isolates

Figure 2: Frequency distribution of positive isolates among different age groups

DISCUSSION

Drug resistance cases are increasing day by day due to the misuse of antibiotics. MDR-TB, internationally, is the focus of both society and health care organizations. Drug resistance and accurate investigation of MTB is a serious problem today in developing countries such as Pakistan.In our results, the positive male patient were 28(59.5%) and female 19 (40.5%). A similar study was also conducted by Patel *et al.* [11] who diagnose 1200 EPTB samples in which the positive male samples were 177 and females were 73. Their study showed that the male wasmore prevalent for the disease as compared to female patients detected as positive for MTB. The age range 41-50, 10(21.2 %) out of a total of 47 positive samples, showed the highest frequency of pleural TB. Similarly in the study of Nisar *et al*. [12] conducted on morbidity in the hospitals of KP, Pakistan. Total 6549 suspects patients were included consist of 77 cases treated previously and 2318(2241 new cases). The age group ≥15 was highly affected with 1702(75.94 %) followed morbidity by age group 4-15 years with 373(16.64 %) and age group 0-4 with 166(7.40%), respectively.

We also study different risk factors for TB which are: malnutrition (23.40%), poverty (4.2%) immune suppression (12.76%), diabetes mellitus (8.51%), unimmunized with BCG (10.63%),contact with infected persons (10.63%), foreign-born children (4.2%) and health care workers (14.89%), respectively. It was concluded that the most common risk factor for TB was malnutrition (23.40%) and the foreign-born children and poverty

(4.2%), were least affected. The most common signs and symptoms in our study for pleural TB were weight loss 13(30.23%), dry cough 10(21.27%), chest pain 10(21.27%), night sweating 08(17.02%), blood in sputum 4(8.51%) and body aches 2(4.65%), respectively. American thoracic society and CDC (2000) also described some common signs and symptoms for TB that person with latent TB infection does not feel sick. The patient may become sick if the infectious bacteria become active in her/his body while a person with active TB disease may feel sick and may also have signs/symptoms like fever, cough, and weight loss [13-16].

We performed culture, in which 45(22.5%) were MTB positive, 4(8.5) were rifampicin-resistant. These findings were also reported by Promod *et al.* (2012) who suggested that culture is the gold standard. Tortoli *et al.* (2012) evaluated the use of GeneXpert assay in 1476 PTB samples and reported that Xpert assay is 81.3% sensitive and 99.8% specific. Out of 47 GeneXpert positive samples, our culture results as, 45(22.5%) were noted positive and 2 (4.2%) were negative. Goyal *et al.* [17] conducted a similar study to diagnose early-stage human clinical material TB mycobacterial infections with rapidity and accuracy to decrease the incidence. The important techniques for diagnosing TB are generally admired in microscopy and culture. This study also correlates with our results.In total samples, 45(22.5%) were found positive, 155 (77.5%) were classified a negative on culture while 38(19%) were positive and 162(81%) were negative on FM, 33(16.5%) were positive 167(83.5%) were negative on ZN staining microscopy and 47(23.5%) were noted positive and 153 (76.5%) were negative on GeneXpert. The overall sensitivity of GeneXpert was 07% higher than that of smear microscopy and 4.2 % higher than culture. In 2010 December [4] approved the use of MTB/RIF assay which is the initial diagnostic test. Diagnosis of PTB is challenging due lack of rapid diagnostic instruments, especially in limited-resources areas. For the suspected individuals having MDR-TB or HIV-associated TB.

Our study showed that the overall GeneXpert sensitivity was 07% higher as compared to FM microscopy. The study conducted by Jeon *et al.* [18] determined the specificity of GeneXpert and a study was carried out for the analysis of 20 samples which proved that the use of Xpert MTB/RIF test for pleural fluid having reasonable specific

results. The 97% specificity was noted for commercial and 91% was for in-house tests, respectively. Several other studies have also evaluated the performance of GeneXpert for pleural fluid and overall the test showedthe best accuracy with sensitivity ranging from 70% to 90%. The study of Tortoli *et al.* [19] also confirms our findings. In their study, both the pediatric and adult patients were included for a large number of TB samples. The overall sensitivity and specificity of GeneXpert were 81.3% and 99.8% while the sensitivity of microscopy was 48%. For the diagnosis of Pulmonary tuberculosis, the role of culturing of MTBC remains central. The GeneXpert is rapidly diagnosing assay for disease detection which makes it the best investigation method as compared to smear microscopy [20, 23-25].

Our GeneXpert results showed 47(23.5%) positive and 153(76.5%) negative samples. The study of Boehme *et al.* [26] also correlates with our GeneXpert results. They identified 551 of 561 culture-positive patients by MTB/RIF test. The smear-positive TB was 98.29% and smear-negative TB was 72.5%, respectively. The performing of second MTB/RIF test increased the sensitivity in culture-positive and smears negative inpatients by 12.6% and a third by 5.1 percentage points, to a total of 90.2%

CONCLUSIONS

The study concludes that detection of both MTB and rifampicin resistance can efficiently be determined using the new technology of GeneXpert MTB/RIF assay which is simple, robust and efficient with minimum handling technique. Beside this the most common risk factors for this disease is malnutrition (23.40%) and the foreign-born children and poverity are least affected.

Ethical Approval:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

Consent

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

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