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DRUGS PROFILING, QUANTIFICATION OF RIF/RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* BY USING AUTOMATED GENEXPERT: A DIAGNOSTIC CHALLENGE

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Abstract
The study was conducted from February 2022 to January 2023 with permission of the National Tuberculosis Control Program and Tertiary care Hospitals, Peshawar. Among a total of 1,390 individuals, 621 (44.6%) were male and 769 (55.5%) were female. Regarding *Mycobacterium tuberculosis* types, there were 1,153 cases (83%) of pulmonary TB, 237 cases (17%) of extra-pulmonary TB, 571 cases of *Mycobacterium tuberculosis* detection with low levels, and no detection of RIF/resistance genes. Additionally, there were 623 cases of *Mycobacterium tuberculosis* detection with medium levels, with 2 cases of RIF/resistance detected, and 196 cases of *Mycobacterium tuberculosis* detection with high levels, with 38 cases of RIF/resistance detected. First-line treatment was recommended for 457 patients (32.8%), with a mean of 244 (±106). Second-line treatment was recommended for 40 patients (2.8%), with a mean of 21.4 (±9.2). New patients accounted for 893 cases (64.2%), with a mean of 478.6 (±207). In recent times, there have been significant advancements in desktop technology, particularly the GeneXpert MTB/RIF system. This system has proven to be effective in detecting *Mycobacterium tuberculosis* in specific population groups, enabling prompt identification of drug resistance in infected patients.

Keywords: GeneXpert MTB/RIF system, Drugs profiling, *Mycobacterium tuberculosis,* RIF/ resistance detected, World Health Organization

Introduction

According to The *World Health Organization* (WHO) report that since 2015 almost one-third of the world's population (2.5 billion people) have been infected with *Mycobacterium tuberculosis* as tuberculosis were surpassed the human immunodeficiency virus infection and attained immunodeficiency syndrome (HIV/AIDS) which causes the death from all around the world. Almost 95% of the cases are reported in developing countries. The maximum number of cases reports in Asia, Africa and as well as in the Eastern Mediterranean area (Cha et al., 2020).

Mycobacterium tuberculosis (MTB) is a highly effective pathogen which can preserve the host tissues for decades without causing disease (Uplekar et al., 2015). The *Mycobacterium tuberculosis* complex is consist of at least nine species in the genus Mycobacterium, family Mycobacteriaceae, and order Actinomycetales which is probably causes of human *Mycobacterium tuberculosis* and zoonotic infection. The *Mycobacterium tuberculosis* complex species consist of 99.9% same sequence and probably develops from a single clonal progenitor. The species *Mycobacterium tuberculosis* sensu stricto is the reason for human *Mycobacterium tuberculosis* in the extensive large number all around the world (Koch and Mizrahi, 2018). *Mycobacterium tuberculosis* spread through the air when an affected patient coughs or even speaks. Someone can get easily affected when breathing close in bacteria air, these bacteria can settle in the lungs and grow (Yates et al., 2016). These can easily move through the blood to the kidney and spine. The propagation of tuberculosis must be stopped if we are to stop the epidemic. Furthermore, high-risk people and environments must be the focus of the measures necessary to stop the spread of tuberculosis (Ullah et al., 2016).

According to the National Institute of Health, the prevalence of *Mycobacterium tuberculosis* in Pakistan is 348 per 100,000. Whereas, the number of new cases is reportedly 276 per 100,000 population. Pakistan ranks 5th amongst the high-burden countries in the world. The prevalence, incidence and mortality per 100,000 population per year from TB in Pakistan are 348, 276 and 34 respectively. The country is said to have the fourth highest prevalence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) globally (Hasan et al., 2006).

Around 56% of the country's *Mycobacterium tuberculosis* cases are detected in Punjab, the most populous province. Of them, 75% fall in the productive age group, i.e. 15-45 years. The number of MDR-*Mycobacterium tuberculosis* patients in Pakistan is difficult to estimate as drug sensitivity testing on internationally-accepted protocols has recently started in the country. Factors causing this are delayed diagnosis, unsupervised, improper drug regimens, lack of follow-up and little or no social support program (Akhtar et al., 2016).

The *Mycobacterium tuberculosis* complex (MTBC) comprises firmly related Mycobacterium species, including *Mycobacterium tuberculosis*, M. Bovis, M. africanum, M. microti, and M. caprae. These species are the essential driver of tuberculosis in people, and they likewise taint wild animals. The nearby interrelatedness of these species has been exhibited by DNA hybridization, multilocus compound electrophoresis, and sequencing of the 16S rRNA qualities and the 16S-23S rRNA quality inside interpreted spacers (Riojas et al., 2018).

Multidrug-resistant strains of *Mycobacterium tuberculosis* are a serious threat to tuberculosis (TB) treatment and control (Hoagland et al., 2016). Drug resistance in *Mycobacterium tuberculosis* is mostly related to the development of drug target gene mutations; these mutations affect the drug's titration or cause the target such as RNA polymerase and catalase-peroxidase in rifampicin and isoniazid resistance, respectively to change (Walter et al., 2015). Genetic and molecular analysis of drug resistance in *Mycobacterium tuberculosis* suggests that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation or by titration of the drug through overproduction of the target. The probability of resistance is very high for less effective antitubercular

drugs such as thiacetazone, ethionamide, capreomycin, cycloserine, and viomycin; intermediate for drugs such as INH, SM, EMB, kanamycin, and p-amino salicylic acid ; and lowest for RIF. Consequently, the probability of a mutation is directly proportional to the bacterial load (Hameed et al., 2018), (Aftab et al., 2021).

Methodology

Study area

The study was conducted at the Tertiary care Hospitals, Peshawar

Study calendar

The study was conducted from February 2022 to January 2023 with permission of National Tuberculosis Control Program and Tertiary care Hospitals, Peshawar.

Study design

We used a convenient study design for patient demographic, medical history, sample collection and management.

Clinical examination and Medical history

The Sum of n=1831 patients was fixed appointment based on the availability of patients and consultants. All the study patients were examined by a concerned consultant at Tertiary care Hospitals, Peshawar. During the examination of patients, we noted the history of patients with this regard it is an asset of patients and will not share on any platforms.

Recommended diagnostic Tests

The patient was examined by the concerned consultant, they were recommended *Mycobacterium tuberculosis* diagnostic test based on the clinical examination, infected organs, and severity of the infection. We followed the prescription of the consultant for laboratory diagnoses as they recommended 2 major diagnostic procedures: AFB smear, and MTB GeneXpert.

Data collection and processing

The data was collected from patients under the supervision of the concerned consultant and pathologist of the National Tuberculosis control program. All the procedures, analysis, sample management, and sample processing, were employed at the Tertiary care Hospitals, Peshawar**.**

Sample storage and transportation

We used special sterilize containers for the specimen collection such as 40ml of containers used for sputum, and 20ml of sterilised screw-cape containers used for fluid collection. Before the study, all the containers of samples were transported via sample transport box (with ice bag) as per described (Tagliani et al., 2017), and stored samples at the laboratory fridge.

Sample inoculation and preparation

All the study samples were gathered into biosafety. Inoculation, smear preparation and GeneXpert sample preparation were conducted inside the biosafety cabinet due to safety issues of lab personnel.

Discarding Positive samples

After processing and examining positive samples, we properly discarded them as well as handed them over to the biosafety and hazard waste management department.

Inclusion criteria

We included patients already on Anti-*mycobacterium tuberculosis* treatment, active signs & symptoms, and males and females.

Exclusion criteria
Negative smears, coinfected patients, and less than 18 years.

Ethical Approval
For this study ethical approval was obtained from the Ethical Committee member of the Tertiary care Hospitals, Peshawar from where the samples were processed and analyzed for the *Mycobacterium* tuberculosis examination of the study patient. Before proceeding with the study the aim and objectives of the study were described in the local language and were understood by them very easily.

Ziehl-Neelsen (ZN) Staining
The Ziehl-Neelsen (ZN) stain was developed to exploit the mycobacterial genus acid fastness including *M. tuberculosis, M. ulcerans, M. leprae,* and nontuberculous mycobacteria (NTM**)**. Mycobacteria are stained with carbul fuchsin combined with phenol in the cold technique. The stain binds to the mycolic acid in the mycobacterial cell wall. An acid decolorizing solution removes the red dye from the background cells, tissue fibres, and any organisms in the smear except mycobacteria which retain (hold fast to) the dye and are therefore referred to as acid-fast bacilli (AFB). Malachite green or methylene blue stain is mostly used as a counterstain.

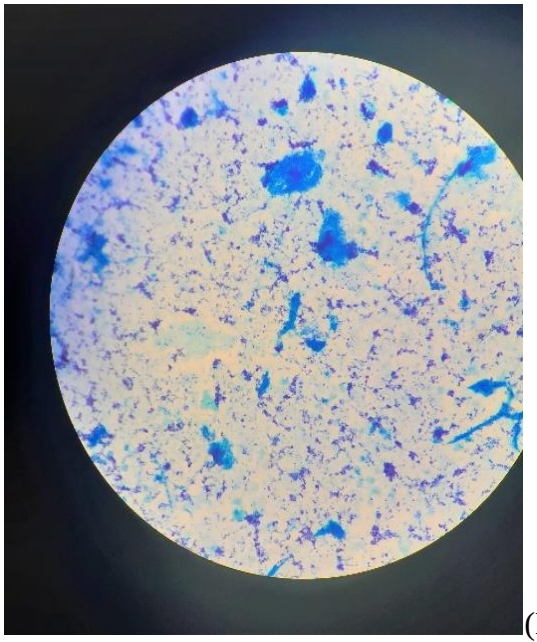
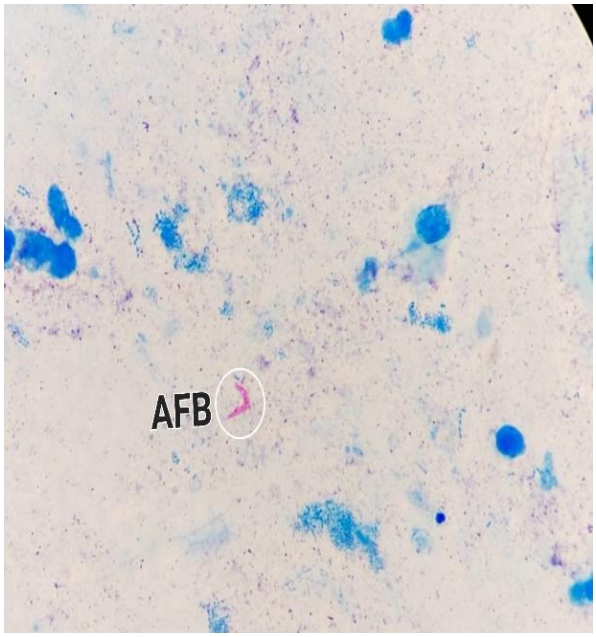
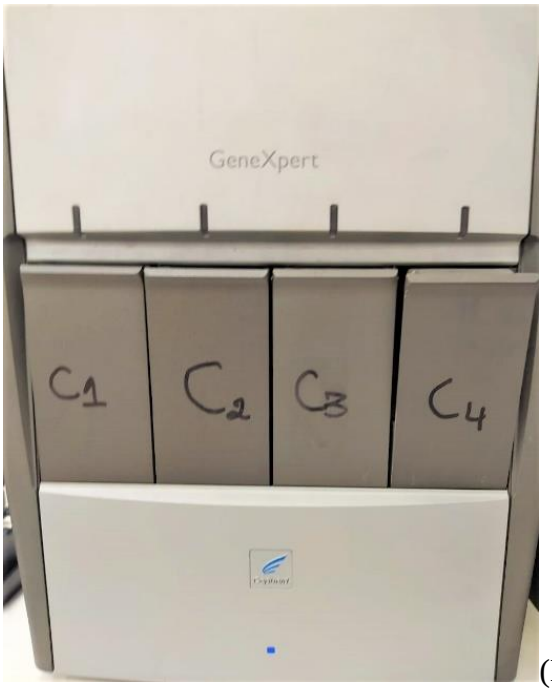
(A)  (B) 

Figure 1: Microscopic examination of AFB smear of Mycobacterium tuberculosis (A) Negative Smear (B) Positive smear

GeneXpert of *Mycobacterium tuberculosis*
The GeneXpert MTB/RIF is a cartridge-based nucleic acid amplification test (NAAT) for simultaneous rapid tuberculosis diagnosis and rapid antibiotic sensitivity test (Agrawal et al., 2016). It is an automated diagnostic test that can identify *Mycobacterium tuberculosis* (MTB) DNA and resistance to rifampicin (RIF). A sputum sample is collected from the patient with suspected TB. The sputum is mixed with the reagent that is provided with the assay, and a cartridge containing this mixture is placed in the GeneXpert machine. All the processing was fully automated and performed by the GeneXpert machine, the GeneXpert MTB/RIF assay should be interpreted along with clinical, and radiographic. Results from the GeneXpert MTB/RIF assay indicate whether or not MTBC was detected in the sample. In some instances, the result is “invalid," whereby the test was repeated.

(A) 

(B) 

Figure 2: Equipment used in study (A) GeneXpert automated system (B) MTB/RIF Ultra cartridge.

Statistical Analysis

All the statistical analyses of the variables were calculated by SPSS 2.0. We used Frequency, percentage, Mean and standard deviation as a statistical test.

Results

Demographics

The sample and supportive data were collected from a patient with a different residential background. All the procedures and analyses were conducted at Tertiary care Hospitals, Peshawar. A Sum of n=1831 was tested for *Mycobacterium tuberculosis based* on the concern consultant's recommendation. n=1390 was tested positive for *Mycobacterium tuberculosis* based on signs and symptoms, clinical manifestations, and lab diagnostic examination out of n=1831. We included only positive patients in the study while excluding negative patients. Out of n=1390, male tested 621 (44.6%) and female tested positive as 769 (55.5%) (Fig no.3).

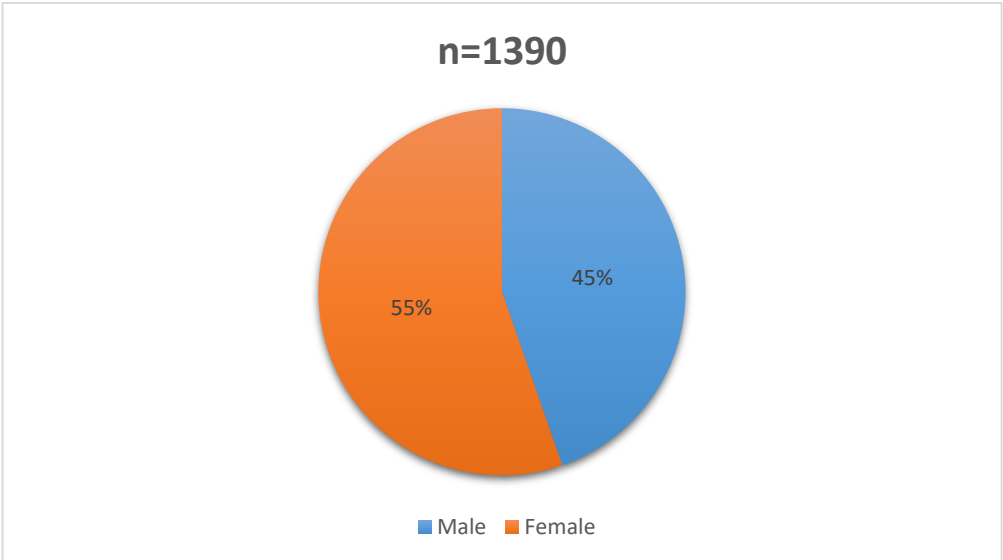


Figure 3: Gender status of the patients

The age scale of the infected patients was designed as 18-25 years 435(31.2%), 25-32 years 357(25.6%), 32-39 years 145(10.4%), 39-46 years 178(12.8%), and above 46 years 275(19.7%).

Specimen Management

The specimen was collected based on consultant recommendation, clinical examination and previous medical history such as Sputum 732 (52.6%), Mean ± SD was calculated like 339.6(143.5), Tracheal aspirates 264 (18.9%), Mean ± SD was calculated like 122.5(51.7), Bronchi alveolar lavage 157 (11.2%), Mean ± SD was calculated like 72.8(30.7), Pleural fluid 154 (11%), Mean ± SD was calculated like 71.4(30.1), and Ascetic fluid 83 (5.9%), Mean ± SD was calculated like 38.5(16.2) (fig no.4).

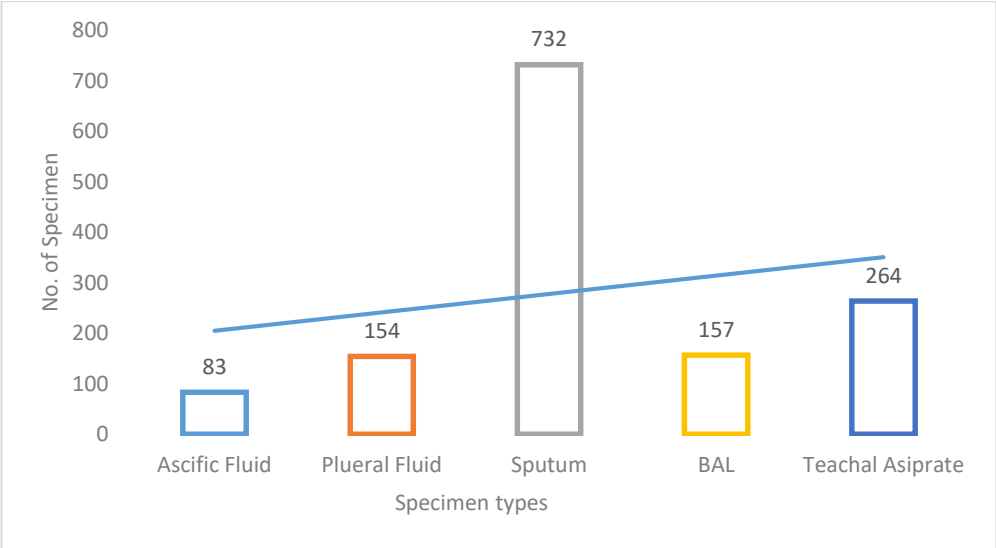


Figure 4: specimen collection of patients

Diagnostic profile of positive patients

***Acid fast-bacilli* detection through the ZN-hot method**

We used the Ziehl-Neelsen hot staining method and identification of *Mycobacterium tuberculosis organism* observed under the 100x magnification of a microscope brand called Olympus (CX-21). Sum of n=1390 in which 893 were documented as newly positive patients, 381 confirmed positive less than 1 month ago, 78 confirmed positive less than 2 months ago, and 38 confirmed positive less than 3 months ago (fig no.5).

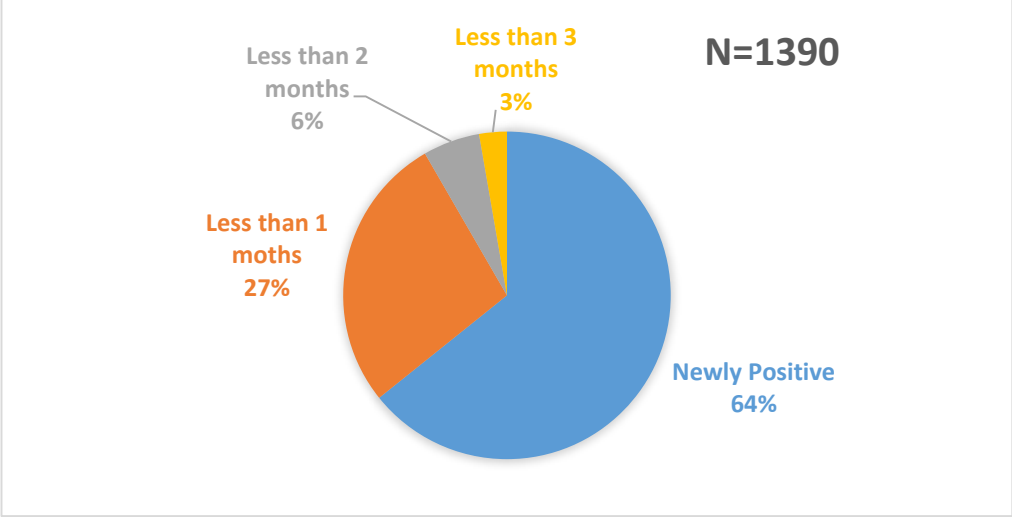


Figure 5: Diagnostic profile of positive patients

Table 1: Reporting criteria for Microscopic examination

No. organism/field	No. of affected patients	Remarks
>10 organisms/field	893	++++
6-8 organisms/field	381	+++
5-6 organism/field	78	++
2-4 organism/field	38	+

RIF/resistance profile of positive patients by GeneXpert system

We used the GeneXpert system for the detection of Rifampicin resistance (RIF/resistance) and the burden of *Mycobacterium tuberculosis in* different body specimens recommended by the concerned consultant. The results of GeneXpert were recorded as MTB detected (low) 571, the RIF/resistance genes were detected as 0, they were not started anti-mycobacterium therapy, MTB detected (medium) 623, RIF/resistance was detected as 2, they already started anti-mycobacterium first-line therapy and MTB detected (high) 196, RIF/resistance was recorded as 38, they started anti-mycobacterium second-line therapy (figure no.6).

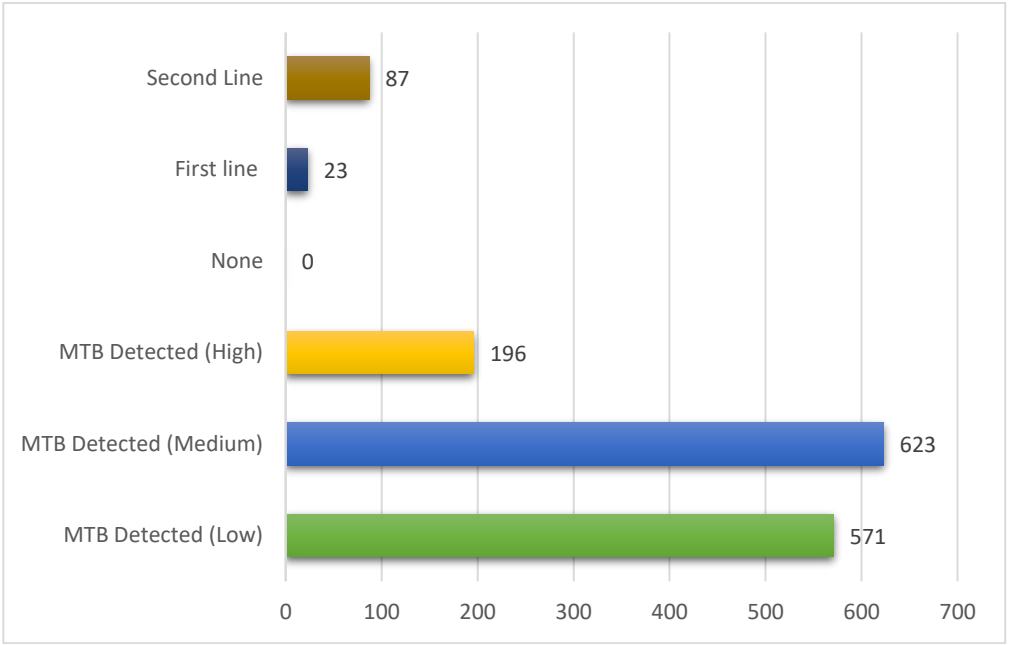


Figure 6: detection of Mycobacterium tuberculosis and RIF/resistance in a specimen of patients by GeneXpert

Table 2: GeneXpert Analytical results of the specimen

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
SPC	25.0	128	N/A	PASS
IS1081-IS6110	19.2	598	N/A	PASS
rpoB1	30.8	213	POS	PASS
rpoB2	29.0	175	POS	PASS
rpoB3	30.9	103	POS	PASS
rpoB4	32.8	91	POS	PASS

***SPS** (Sample Processing Control), **Ct** (Conc. of DNA template), **NA** (Negative).

***Mycobacterium tuberculosis* category**

After confirmation of *Mycobacterium tuberculosis* via ZN hot method and GeneXpert automated system. They were classified into 2 major categories such as pulmonary 1153(83%), Extra pulmonary noted as 237(17%) based on their specimen type, specimen identification and confirmation and organ specification.

Follow-up treatment recommended by the concerned consultant

Already the follow-up treatment recommended to patients, they were classified as first-line treatment patents recorded as 457(32.8%), Mean ± SD was calculated like 244(106), second-line treatment recorded as 40(2.8), Mean ± SD was calculated like 21.4(9.2), and new patients 893(64.2%) (No treatment recommended), whereas Mean ± SD was calculated like 478.6(207).

Prescription of anti-*Mycobacterium tuberculosis* drugs

After detection and confirmation of *Mycobacterium tuberculosis* in a specimen of patients. They proceeded with anti-tuberculosis therapy. So, we documented the anti-*mycobacterium tuberculosis* drugs prescribed by the concerned consultant. For the first line of therapy, the concerned consultant followed the WHO anti-tuberculosis drug panel such as Isoniazid, Rifampicin, Pyrazinamide, and Ethambutol. All of these drugs were recommended to patients who were infected for the first time such as n=893 and patients already on treatment such as n=457. For second-line therapy, the consultant recommended Bedaquiline, Kanamycin, Capreomycin, and Amikacin. All of these drugs were recommended to patients who were reinfected or failed in Rifampicin efficacy such as n=40. The routine of administration of the drug was oral and the nature of the drug was Tablets or pills.

Anti-*Mycobacterium tuberculosis* drugs Sorting, dosage based on weight

All those patients who tested positive for *Mycobacterium tuberculosis* organism. They were recommended drugs dosage by a concerned consultant based on their weight. For the first line therapy, the anti-tuberculosis drug panel for daily dose before breakfast such as Isoniazid 5mg/kg/day, 200mg/28-38kg/day, 300mg/39-48kg/day 300mg/49-59kg/day, 300mg/60-72kg/day Rifampicin 10mg/kg/day, 200mg/28-38kg/day, 450mg/39-48kg/day 450mg/49-59kg/day, 600mg/60-72kg/day Pyrazinamide 25mg/kg/day, 800mg/28-38kg/day, 1000mg/39-48kg/day 1200mg/49-59kg/day, 1600mg/60-72kg/day, and Ethambutol 20mg/kg/day, 600mg/28-38kg/day, 800mg/39-48kg/day 1000mg/49-59kg/day, 1200mg/60-72kg/day. All of these drugs were recommended to patients. For second-line therapy, consultant recommended Bedaquiline 400mg/day for 2 week consecutive, 200mg three times/day for a week, Kanamycin 20mg/kg/day, 500mg/28-38kg/day, 625mg/39-48kg/day 750mg/49-59kg/day, 875mg/60-72kg/day, Capreomycin 20mg/kg/day, 500mg/28-38kg/day, 600mg/39-48kg/day 750mg/49-59kg/day, 800mg/60-72kg/day, and Amikacin 20mg/kg/day, 500mg/28-38kg/day, 600mg/39-48kg/day 750mg/49-59kg/day, 800mg/60-72kg/day.

Table 3: consultant recommended drug dosage, distribution and administration based on the patient's weight

Anti-mycobacterium Therapy							
Rifampicin	Dosage	Administration	mg/kg	28-38kg/day	39-48kg/day	49-59kg/day	60-72kg/day
Isoniazid	300mg	Oral	5mg/kg	200mg	300mg	300mg	300mg
Pyrazinamide	500mg	Oral	25mg/kg	800mg	1000mg	1200mg	1200mg
Ethambutol	100mg/400mg	Oral	20mg/kg	600mg	800mg	1000mg	1200mg
Bedaquiline	100mg	Injectable	400mg/kg				
Kanamycin	15mg/kg	Injectable	20mg/kg	500mg	625mg	750mg	875mg
Capreomycin	20mg/kg	Injectable	20mg/kg	500mg	600mg	750mg	800mg
Amikacin	250mg	Injectable	20mg/kg	500mg	600mg	750mg	800mg

Route of administration of anti-*Mycobacterium tuberculosis* drugs

Tablets or pills such as Isoniazid, Rifampicin, Pyrazinamide, and Ethambutol were documented from the patient's prescription (n=1350) who was infected for the first time and already on treatment. For second-line therapy, a consultant recommended drugs to those who developed resistance against Rifampicin or failure of any anti-tuberculosis drug as well as reinfected patients such as Bedaquiline, Kanamycin, Capreomycin, and Amikacin. All of these drugs avail to the patients in injectable form.

Discussion

Tuberculosis is one of the serious and dangerous diseases that is killing numerous people globally. Pakistan is ranked among the sixth country having a high burden of MTB. One-quarter of the people globally are known to be a victim of *Mycobacterium tuberculosis* bacteria. The causative agent of TB is *Mycobacterium tuberculosis*, the lungs being the main target of it.

Females were recorded in a high ratio of about 55.5% due to close contact, poor sanitation, weak immunity, lack of education, culture restriction, no social mobilization, lack of knowledge of *Mycobacterium tuberculosis*, shared foodstuff and utensils, living in a crowded area and no healthcare facilities. Males were recorded in less number, they travelled from one location to another for business matters, weak immunity poor sanitation, less education status, and lack of knowledge of *Mycobacterium tuberculosis* same as females.

The rate of prevalence of *Mycobacterium tuberculosis* in Pakistan is 420, 000 while the incidence rate is 231 per 100, 000 population. It has been found that there is an increasing rate of resistance developed against the most commonly used drugs for *Mycobacterium tuberculosis* such as rifampicin and isoniazid. It was estimated in 2008 that among 48,000 cases of *Mycobacterium tuberculosis* from different countries of the world Pakistan is among those 27 countries having a high burden of the disease (Tahseen et al., 2020). In a survey done in 2008, almost 15000 MDR-*Mycobacterium tuberculosis* patients have been diagnosed in Pakistan (Ahmad et al., 2017). The treatment cost for MDR-*Mycobacterium tuberculosis* patients is 50-200 times more than the cost required for treating drug-susceptible *Mycobacterium tuberculosis* patients. It is quite surprising that almost 26% of *Mycobacterium tuberculosis* patients have not even any idea about this disease before diagnosis but along with this 10% of the healthy population have no idea about this disease (Ali et al., 2020).

We recorded the most exposed and prevalent age group such as 18-25 years 435(31.2%). The highest prevalence of *Mycobacterium tuberculosis* was observed in the rural population of Peshawar such as 530 (38.1%), Mean ± SD was calculated as 284(122.9), due to crowd area, and closed meetup as per their culture scenario.

The ratio of the specimen was verified for Sputum as 732 (52.6%), Mean ± SD was calculated at 339.6(143.5), due to most of the patient suspected of pulmonary *Mycobacterium tuberculosis* with active signs and symptoms such as weight loss, night-sweating, the fluid found in X-ray examination, and high-grade fever.

The results of GeneXpert were recorded as MTB detected (low) 571, the RIF/resistance genes were detected as 0, they were not started anti-mycobacterium therapy, MTB detected (medium) 623, RIF/resistance was detected as 2, they already started anti-mycobacterium first-line therapy and MTB detected (high) 196, RIF/resistance was recorded as 38, they started anti-mycobacterium second-line therapy.

In Pakistan, alternative treatments are being used due to their ease of availability, being less costly and having no harmful effects on therapy. The rate of multi-drug resistance is increasing in Pakistan. The patients are of different view regarding the treatment as some of them thinks that treatment should be stopped once symptoms are resolved while other think that treatment should be carried out till the prescribed date or at least for 6-12 months. From the survey, it has been found that there is a lack of knowledge among the people of Pakistan about this deadly disease (Ullah et al., 2019).

Conclusion and Recommendation

In recent times, advancements in the desktop have been taking place renowned as GeneXpert MTB/RIF. It has been found that the MTB/RIF is an effective test for the case finding of *Mycobacterium tuberculosis* in some specific groups. It also provides quick detection of drug resistance in patients infected with *Mycobacterium tuberculosis*. The test has increasing specificity as it quickly and accurately diagnoses the infected patient, so promoting a positive step towards the treatment of the disease. There are some drawbacks of smear microscopy as it does not give any idea

about resistance in the patients as it is important to detect to provide an alternative way of treatment to prevent the spread of extensively drug-resistant tuberculosis (XDR-TB) and multi-drug resistant tuberculosis (MDR-TB).

Authors Contributions

Awais Khan: Data collection, Data curation, Editing, Writing the first draft, supervision; **Sumbal Nosheen**: Study design, Data analysis, Editing; **Aamna Shah**: Data analysis, Data curation, Editing; **Muhammad Zahid Khan**: Data analysis, Editing. **Tasneem Noor Mohammad**: Study design, Data analysis; **Awais Khan**: Data collection, Data curation

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