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Diagnostic approaches and prevalence of Rifampicin resistant *Mycobacterium tuberculosis* in District Mardan

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Abstract

The disease tuberculosis (TB) caused by *Mycobacterium Tuberculosis* (MTB) is most common infectious disease in developing countries. The disease is fatal if not treated during the early stages of infection, thereby early and precise detection is a decisive step in curing the disease. The aim of this study was to analyze the prevalence of tuberculosis in patients reporting to Mardan Medical Complex (MMC), located in the district Mardan, KPK, Pakistan. The sputum of patients was analysed by Ziehl-Nilsen (ZN) staining technique followed by light microscopy called Acid-Fast Bacillus (AFB) staining. The sputum samples were collected from the patients and analysed by special PCR method called GeneXpert *MTB*/RIF assay, for genomic detection and resistance assay for rifampicin antibiotic were used, are the commonly used medicine for the treatment of MTB infection. Total 121 patients reported to MMC, represented 74 % patients from Mardan, 12% from Nowshera and 14% from Swabi. These patients were screened for the aim to evaluate the techniques for the detection of MTB. The light microscopy method confirmed 66 (55%) of the patients positive for MTB, whereas the same samples reported 78 (68%) patients positive for MTB through GeneXpert *MTB*/RIF assay, The Positive Predictive Value (PPV) and Negative Predictive Value (NPV) found for light microscopy were 99% and 78.1% respectively. The most used drug rifampicin was found ineffective in 9 patients (7%). Additionally, 83% of the patients when interviewed had a folk history of tuberculosis.

Key words: Microscopy, PCR, Mycobacterium tuberculosis, Rifampicin

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1. INTRODUCTION

Tuberculosis (TB) is a disease known since antiquity and still the major cause of mortality (1). It kills two million people each year around the world. TB is a pulmonary disease which is caused by the accumulation of *Mycobacterium tuberculosis* (MBT) in the lungs. The strains of MBT are highly virulent, rapidly transmissible and highly mortal in patients.(2).Pakistan ranks fifth among TB patients. Pulmonary embolism (EPTB) has increased steadily, which now accounts for 20% of all confirmed TB cases making very limited information about EPTB disease. The study was conducted to describe the demographic characteristics, clinical manifestations and treatment outcomes of EPTB patients in Pakistan (3). Extra pulmonary Tuberculosis (EPTB) represents 15 % of the world's reported TB cases. The World Health Organization differs from 8 in the Western Pacific, 17 in East Asia and 24 in the Crisis East. (1,2*)* MBT is an acid fast obligate human pathogen, slow-growing, intracellular and curved shape bacilli(3). The generation time of MBT over growth medium is about 20 hours (4). MBT is poor Gram positive bacterium because it does not stain with conventional Gram staining because of their unique cell wall composition(5).The rate of mortality from this disease per year is two millions, while each year about eight million new cases of tuberculosis are reported (6).Pakistan ranked sixth amongst highest tuberculosis reported countries. In Pakistan the annual reporting patients infected by MBT are 420 thousand, with an estimate of 231 infected patients of MBT in every 100,000 population(7).he commonly used detection techniques for MBT are collection of specimen, its digestion and liquefaction followed by the staining technique and light microscopy (8). The bacterium is also cultured specially for the purpose of its identification and isolation from other closely related bacterium like *Mycobacterium Bovis* and *M. Africanum* (9). The colonies of MBT could be tested for different drugs response. There are also nucleic acid based identification using real time PCR (GeneXpert), these techniques not only shorten the detection time by weeks but also promise more accuracy and high sensitivity (10). GeneXpert *MTB*/RIF assay is fast method (2 hours detection time) detecting DNA fragments of MBT in specimen as well as identify the mutations developed in DNA which leads to the drug resistance against rifampicin medicine(11). he most commonly used drugs against tuberculosis are rifampicin, isoniazid and streptomycin (12). Rifamycin, a chemical compound having antimicrobial activity was extracted from *Amycolatopsis mediterrane* by the Lepetit research laboratory in Milan in 1957 (13). This compound showed activity against MBT and the modified form proved increase activity against MBT. This oral medicine can be absorbed by gastrointestinal tract and is used as first line drug in anti-tuberculosis therapy (14).The aim of this study was to analyze the prevalence of tuberculosis in patients reporting to Mardan Medical Complex (MMC), located in the district Mardan, KPK, Pakistan.

2. MATERIALS AND METHODS

Sample Collection

The sample was collected from T.B laboratory of MMC, during the period December 2014 to July 2015. Total 121 Extra pulmonary tuberculosis samples were collected from individuals of three different regions i.e., Mardan, Swabi and Nowshera. From these samples 90 were from Mardan, 17 patients from Swabi and 14 patients from Nowshera. In these samples 69 are male patients and 52 are female patients. The age of 36 patients is from 10 to 25 years, 39 patients is from 25 to 40 years, 25patients is 40-55 years and 21 patients 55-70 years. Signs and symptoms of TB were present in all patients.

Sample Preparation

For sample preparation standard procedures were followed for the analysis of the samples. Sputum samples were collected from both males and female patients having different ages. An early morning deep cough sputum were collected from each patient, volume of each specimen was at least 2 ml.

ZN Staining Technique:

The ZN staining was performed for each sample for the purpose of light microscopy. First the slides were covered with carbon fuchion and heated till the steam aroused from slides and were left for 4-5 minutes allowing bacteria to absorb color. Then decolorization were performed by applying 25% alcohol to each slide. After about three minutes slides were rinsed with distilled water. Smears were flooded with methylene blue solution for one minute. Slides were tilted with forceps to drain of methylene blue. Then slides were cleaned with tap water. At last slides were taken from the rack using forceps and placed on the edge letting the water drain off.

Light Microscopy (AFB)

The stained slides were observed under light microscope. Two drops of immersion oil were put on the left edge of the smear. Smears were observed under 100 X magnification lens. Positive slides AFB were counted for quantification and recordings were categorized as follows.

Table. 1. Light microscopy post AFB staining of patient's sputum

Number of AFB	Category
No AFB found in at least 100 fields	Negative
10–99 AFB per 100 fields	+ (low)
1–10 AFB per field (count at least 50 fields)	++ (medium)
More than 10 AFB per field (count at least 20 fields)	+++ (High)

GeneXpert *MTB*/RIF Assay (Real Time PCR):

The GeneXpert *MTB*/RIF were performed for each specimen. Sample reagents were mixed with the clinical sample at a ratio of 3:1. The closed container of specimen was vigorously agitated three times and was kept for 15-20 minutes at room temperature. 2ml of sample were transferred into test cartridge and were introduced into GeneXpert machine as advised by the manufacturer. All those specimens which were AFB smear positive and *MTB*/RIF assay negative and specimens which were smear negative and *MTB*/RIF assay positive were tested twice and results were considered for analysis.

3. RESULTS AND DISCUSSIONS

AFB Staining/Microscopy Results

After Acid fast bacillus staining, slides were subjected to light microscope using 1000 X magnification, with emulsion oil. Pink rod-shaped AFB were observed and counted with blue background. The case was reported as positive where at least one red purple bacillus was seen with bluish background. Approximately 54.5% samples out of 121 reported to MMC were AFB positive, whereas 55 (45.4%) samples were found AFB negative. Most of the AFB positive specimens were observed with medium concentration of the tubercle bacterium i.e AFB++ (5-10 AFB counted under microscope), while the remaining sample had lower concentration i.e AFB+ (less than 5 AFB counted under microscope) and very few samples were noted to have high concentration of the bacterium i.e AFB+++ (more than 10 AFB counted under microscope).

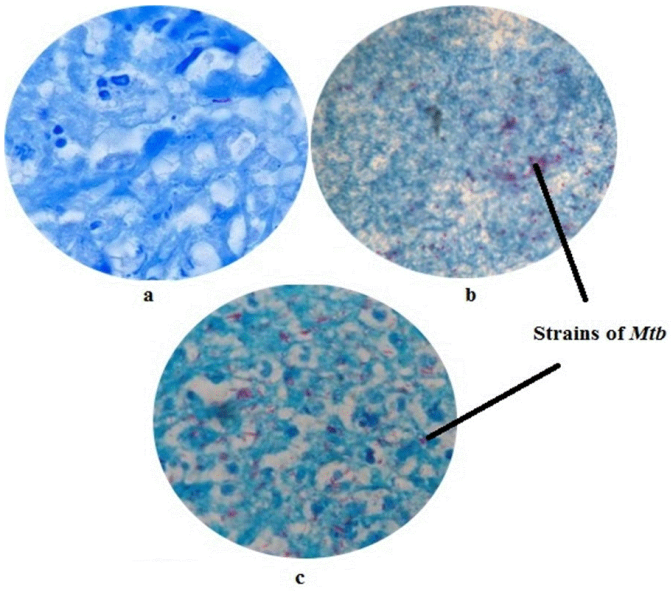

Figure.1. Light microscopy of MBTfollowed by Z-N staining. This figure shows a presentation of the AFB staining of sputum samples for distingwishing between non-infection, low inefction and high infection sputem. Here (a) is AFB- shows no strain of MBT, (b) is AFB++ showing few stains of MBT while (c) is AFB+++ showing high number of MBT.

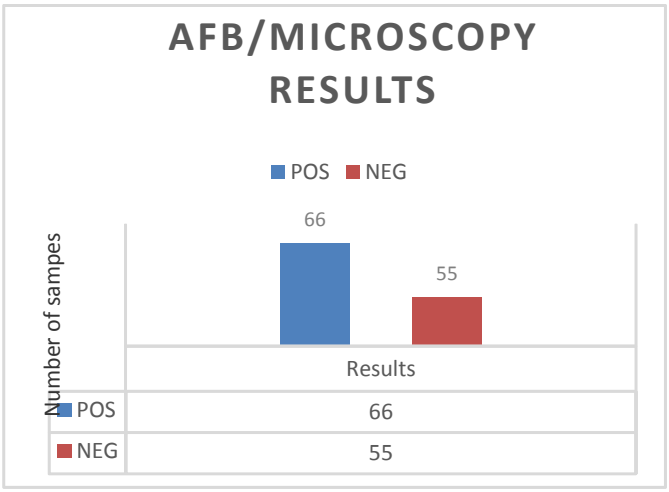

Figure.2. The AFB light microscopy. The green bar represents the number of patients found positive with MBT infection, whereas the blue bare represents the number of patients found negative for MBT infection.

GeneXpert *MTB*/RIF Assay

For GeneXpert *MTB*/RIF assay the samples collected in cube were transferred to cartridges, buffer was added to each cartridge, and cartridges were left to liquefy the samples. After ten minutes cartridges were transferred to GeneXpert machine wells. In each batch of PCR 4 cartridges were processed.

GeneXpert MTB/RIF assay showed 64% of samples were positive for mycobacterium tuberculosis, 28% of them were detected for low *M. Tuberculosis* concentration, 42% for medium *M. Tuberculosis* concentration, and 30% for high *M. Tuberculosis* concentration. 35% of all the samples were not detected by GeneXpert *MTB*/RIF assay.

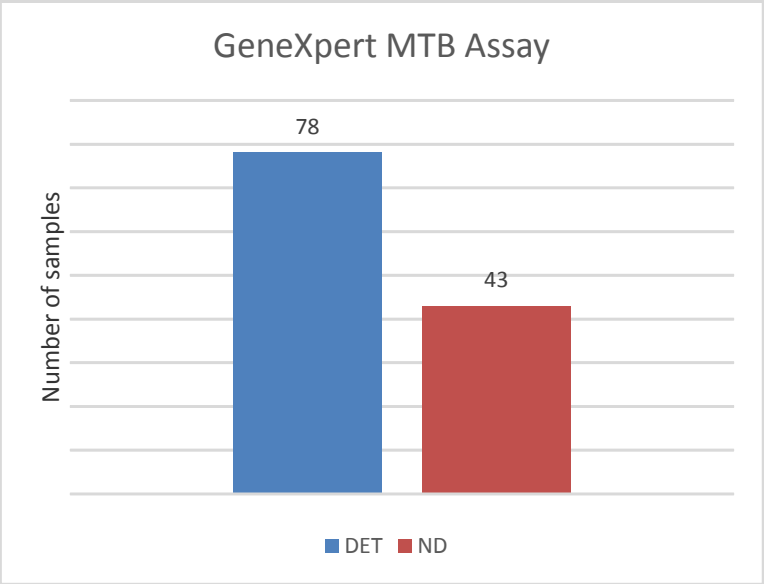

Figure.3. The gene Xpert assay. The green bar shows those patients, where MBT infection was found positive by gene Xpert assay, whereas the blue bare shows those patients where MBT infection was found negative by gene Xpert assay.

Rifampicin Resistance

GeneXpert results showed MBT detection as well as rifampicin resistance in samples. 7% samples were detected as resistant to rifampicin by GeneXpert *MTB*/RIF assay, while the remaining 92% samples were not resistant to rifampicin.

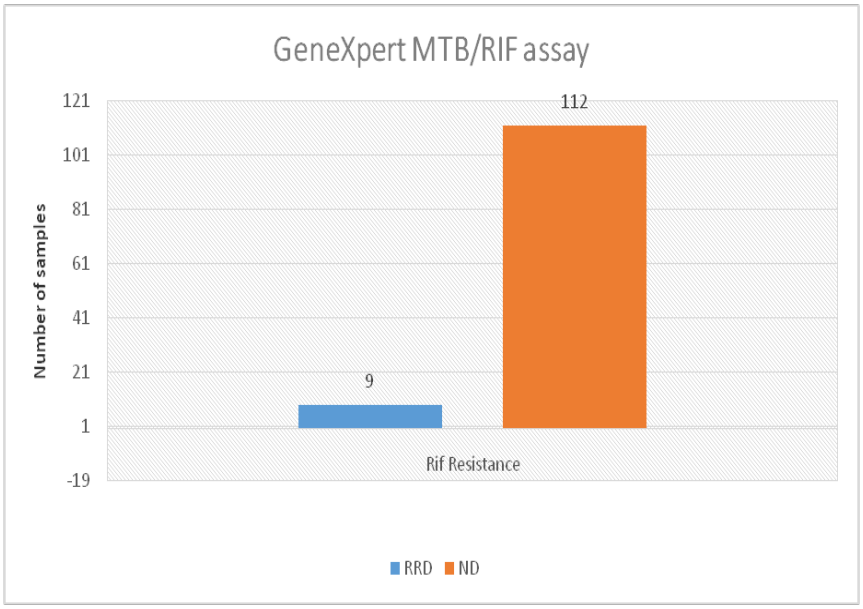

Figure. 4. The gene Xpert/RIF assay. The blue bar shows those patients, where MBT infection was found resistant to rifampicin antibiotic, whereas the blue bare shows those patients where MBT infection was found sensitive to rifampicin antibiotic.

Prevalence data table

(Ahmad et al *Egyptian Journal of Chest Diseases and Tuberculosis*, *65*(2), 461-464.)

Table 1 Gender wise distribution of registered suspected PTB patients at Dargai, n (%).

Year	Frequency distribution of PTB-SS + ve cases				PTB-SS−ve cases		
	Total cases	PTB SS + ve	Male	Female	PTB SS−ve	Male	Female
2011	174	69 (37.5)	27 (39.13)	42 (60.87)	29 (16.67)	18 (62.07)	11 (37.93)
2012	187	73 (39.04)	32 (43.84)	41 (56.16)	33 (17.65)	15 (45.45)	18 (54.55)
2013	191	78 (40.84)	27 (34.62)	51 (65.38)	28 (14.66)	15 (53.57)	13 (46.43)
2014	184	75 (40.76)	39 (52)	36 (48)	25 (13.59)	13 (52)	12 (48)
Total	736	295 (40.08)	125 (42.37)	170 (57.63)	115 (15.63)	61 (53.04)	54 (46.96)

The present study showed the high prevalence of tuberculosis in the three closely located cities/districts of KPK. The diagnosis of MBT in patients' sputum samples were conducted by AFB staining as well as Gene Xpert assay. The GeneXpert assay was found highly specific method for MBT detection in comparison to AFB staining. Some of the patients were diagnosed resistant to the first line drug, rifampicin by GeneXpert MTB/RIF assay. Prevalence of Tuberculosis reported from different parts of KPK shows high prevalence rate in district Mardan as compared to Swabi and Nowshera. The occurrence ratio of tuberculosis in Mardan, Swabi and Nowshera is 45:8:7 respectively. The fact that MMC is located in District Mardan, thereby most patients reporting to MMC are from the local areas. Due to the availability and the accurate results of the GeneXpert *MTB*/RIF assay patients from the nearby cities move towards the MMC. The high prevalence of tuberculosis in KPK might be attributed to poor hygiene, less awareness about tuberculosis, incomplete ATT, and lack of interest in proper care and prevention of TB disease by the clinical institutes. In this study we reported 57% male patients and 47% female patients. Similar gender-based prevalence of TB patients have been reported in resent studies (15). These results indicated similar prevalence ratios of TB disease in both genders over the time. The light microscopy showed 84.6% sensitivity and almost 100% specificity for MBT infection, although the handling errors could not be omitted for AFB staining procedures. The sensitivity and specificity of GeneXpert *MTB*/RIF assay for detection of MBT was found almost 100% nearly 16% sensitive compared to AFB light microscopy. Approximately 83% of patients had family background of tuberculosis, thereby indicating poor hygiene's and non-serious behaviour of the general population specially in rural areas. These findings showed the stability and resistant of MBT against commonly home used detergent and antiseptics. Tuberculosis is not a genetic disease but once it introduces itself in a family it haunts their generations for decades. Thereby extreme care in preservation of this deadly infective disease crucial for the reduction in the high prevalence in the developing countries like Pakistan.

4. CONCLUSIONS

The current study provides an overview of TB, clinical manifestations, and treatment outcomes. Further studies are needed to clarify the key differences between the provinces and their specific risk factors. From a public health perspective, attention needs to be paid to EPTB, which carries 20 burdens of TB disease, and to identify the quality and treatment challenges of identifying patients with abnormally exposed TB.

5. FUTURE PRESPECTIVE

The resistance of *Mycobacterium tuberculosis* could be explored further to understand the pattern of resistivity within the genome of MBT as well as development of resistant strains to one or several drugs. Thereby effective drug and /or vaccines could be design for the prohibition and treatment of tuberculosis disease. Procedures could be optimized for PCR detection of genetic resistivity to multiple antibiotics in MBT. To control the prevalence of MBT in the rural areas of the developing country like Pakistan, huge efforts are needed for the awareness among the population. Thereby specified seminars and orchestrating courses could be useful.

6. ACKNOWLEDGMENT

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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