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BIOMARKER-BASED ASSESSMENT OF DISEASE SEVERITY IN RIFAMPICIN-RESISTANT TUBERCULOSIS WITH DIABETES ANDCHRONIC KIDNEY DISEASE (CKD)

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ABSTRACT

Background: Tuberculosis (TB) caused by Mycobacterium tuberculosis is a global health issue, particularly with the emergence of MDR-TB. Rifampicin resistance is a sign of drug-resistant TB, although disease severity is difficult to assess. CRP, ESR, and D-dimer biomarkers elucidate inflammation and disease severity.Aim:The GeneXpert MTB/RIF test was used to detect rifampicin resistance in pulmonary tuberculosis patients and to correlate CRP, ESR, and D-dimer levels with disease severity.Materials and Methods: In all, 300 samples (150 sputum and 150 blood) were obtained from pulmonary TB patients. Sputum samples were stained with Ziehl-Neelsen and microscopically examined for acid-fast bacilli (AFB). M. tuberculosis and rifampicin resistance were detected using GeneXpert MTB/RIF. Blood samples were analysed for CRP, ESR and D-dimer levels.Results: All the 150 sputum samples were positive for M. tuberculosis by Ziehl–Neelsen staining and GeneXpert testing. Of them, 26 (13%) were found to be rifampicin-resistant and 124 (87%) rifampicin-sensitive. Biomarker study found that CRP was high in 143 (95.3%) patients, ESR was raised in 119 (79.3%) cases, and D-dimer was discovered in only 7 (4.7%) instances. The results show that CRP and ESR are more related to the severity of TB than D-dimer.Conclusion: GeneXpert MTB/RIF is a powerful tool for quick identification of rifampicin resistance. Elevated CRP and ESR levels were significantly correlated with the severity of pulmonary TB and might be helpful supporting biomarkers in the clinical evaluation of TB patients.

Keywords: *Biomarkers Rifampicin Resistant; Mycobacterium tuberculosis; GeneXpert.*

INTRODUCTION

Tuberculosis (TB) is a contagious disease that is spread by a group of closely related mycobacterial species called *Mycobacterium tuberculosis* (MTB) complex. It is due to waxes and fatty acids for example mycolic acid in their cell wall. It is estimated that 9 million new cases of TB occur worldwide in which 1.7 million death occurs among infected people every year because of MTBC and about 1 million people are infected with Multi-Drug Resistant (MDR) strains. Tuberculosis is one of the most significant contagious disease worldwide, and the incidence of TB has been increasing globally [1]. TB causes serious health problems throughout the world, but this disease is mostly present in the poor countries. Mortality of TB is over 95% in low and middle-income country [2]. Among all age groups TB is the most common causes of death and disease. It is assessed that one 3rdpopulation of the globe is infected with latent TB [3]. Causative agent for TB disease is *Mycobacterium-Tuberculosis-Complex* (MTBC). *Mycobacterium* species mostly infect lungs causing pulmonary TB [4]. TB can infect and spread within the body to different locations and organs, like bones, brain, urinary tract, abdomen, heart and lymphatic system called as extra pulmonary TB [5]. MTB comprises different species which are*M. bovis, M. africanum, M. canetti, M. pennipedii*and *M. microti* [6]. Topographically, more cases of TB in the year 2018 reported in Southeast Asia were

44%, in Africa were 24% and in the Western-Pacific were 18%, with little rates in the Eastern-Mediterranean were 8%, in USA and Europe were also 3%. Eight nations represented 66% of the worldwide aggregate of tuberculosis. The prevalence in India is 27%, in China 9%, in Indonesia 8%, in the Philippines 6%, in Pakistan it is 6%, in Nigeria 4%, in Bangladesh 4% and in South-Africa 3% as per WHO, Global TB report 2019 [7]. Among 22 countries with high burden of TB Pakistan is on 5th number. Out of 100,000 populations, about 350 cases of TB are reported annually in Pakistan [8]. In the year 2018, new Rifampicin resistant TB cases were about 5,00,000 of which multidrug-resistant were 78%. The 3 nations with the biggest proportion of the worldwide burden i.e. India were with 27%, China with 14% and the Russian-Federation were with 9% prevalence. All around the world, a normal 1.7 billion individuals were infected with TB adherence at risk of developing the infection. With a well-timed determination and management of the disease with antibiotics, many individuals who infect with TB could be relieved and stop spreading [9]. The process of TB infection can be divided into 3 distinct stages. The 1st stage is the transmission of air borne droplets having bacilli from an infected person to a healthy one. Once inside the lungs, bacilli enter and dwell inside alveolar macrophages (AMs) and dendritic cells. Even though AMs ingest bacilli and regularly kill them. Mild inflammation in the lungs started due to the multiplication of MTB in primary infection ([10]. The 2nd stage of illness is described upon rising of the Cell-Mediated-Immunity (CMI) and granulomas' development. TBthat avoids from the killing impacts of AMs, begins growth and as a result AMs is destructed. The blood monocytes and neutrophils are attracted towards the infected site of body [11]. The 3rd stage is the reactivation of latent MTB disease. There are 2 primary reasons depicted for a reactivation occasion to happen, a decrease in the host's inability to create and keep up safe signs. Under these conditions, the granuloma structure upsets and results in lung cavitation and pneumonia [12]. The sign and symptoms of TB including an individual constantly coughing for at least three weeks, or under three weeks, or of unsure span, however some individuals are presumptive cases if they are having hemoptysis fever most probably at night, weight reduction, anorexia, dyspnea, chest pain and close contact of TB patient, especially a smear positive patients can co-exist with other conditions such as Patients with Asthma and HIV may develop TB in addition to their chronic [13]. However, due to misuse of TB drugs administration emergence of drug resistance in MTB were reported. To control the evolution of antibiotics resistance in MTBthere is a need of following strategy like diagnosis on initial infection, effective drug combination awareness and patient compliance on therapy duration [14]. Conventional light microscopy is a highly specific, simple, and quick method for determining the acid-fast bacilli (AFB) which are used for the routine diagnosis of TB in both PTB (Pulmonary-TB) and EPTB (Extrapulmonary-TB). For the growth of MTBC, phenotypic-based analytical equipment is used on various culture media and identification. In clinical samples, 10-100 organisms/ml can be identified, and their sensitivity is greater than microscopy [15]. In *M. tuberculosis* Infectionseveral pro inflammatory responses occur in which CRP, ESR and D-dimer are mainly increased, and we should consider a type of biomarkers in the case TB infection. By using biomarker to further evaluate the diagnosis of *MTB* infection including mainly CRP and ESR. The CRP is a chronic inflammation indicator. Moreover, CRP acts on the surface of already dead cells to trigger sTBinfection. It is also used to find out the efficacy of Anti-TB drugs [16]. CRP test is used for the assessment and prediction of PTB. It can represent the direction of sickness and furthermore the efficiency of medicines. CRP is likely to be positive in individuals with active stage of PTB infection. Due to disease or tissue-inflammation, cytokines stimulate the production of CRP, especially Interleukin 6 and 1, and Tumor Necrosis Factor (TNF). Normal levels of CRP inhale peoples are ordinary under 6 mg/l, anyway in pathological conditions the concentration of CRP raises in the start 6 to 8 hours of infection and increases near 48 hours up to 350 to 400 mg/l [17].

The current research was aimed to analyse the correlation of CRP, ESR and D-dimer biomarkers with rifampicin resistant TB for disease severity evaluation. D-dimer is a clinical marker. CRP and ESR are more typically related with chronic inflammatory infections. The present research was undertaken to find out the presence of rifampicin resistance in Mycobacterium tuberculosis by GeneXpert MTB/RIF test and to determine the severity of pulmonary TB as per biomarker increase. To assess the rifampicin resistance in TB patients on combination treatment. To study the variations in CRP, ESR and D-dimer levels in patients with pulmonary tuberculosis.

MATERIAL AND METHOD

A cross-sectional study was performed with patients diagnosed as having pulmonary *Mycobacterium tuberculosis* infection who are receiving anti-tuberculosis combination therapy. A total of 300 (150 sputum and 150 blood samples) were taken from TB patients at Prime Teaching Hospital Peshawar. All age groups and both sexes were included, and patients who had extrapulmonary TB and those who were not receiving combination therapy were excluded. This was gathered using a structured questionnaire for demographic data (age, gender and clinical information).

Sputum samples were taken in sterile labeled bottles and sent under proper biosafety measures to the Microbiology Department of Abasyn University for analysis. The acid-fast bacilli (AFB) were identified by microscopic examination with Ziehl–Neelsen (ZN) staining. Smears were made and stained with carbolfuchsin and then decolorized with acid alcohol and counterstained with methylene blue. The stained slides were then looked at under oil immersion microscopy and positive

samples were grouped by AFB grading (1+, 2+ and 3+). Identification of *M. tuberculosis* and rifampicin resistance was done by molecular method using GeneXpert MTB/RIF. Sample reagent was used in 2:1 ratio and then loaded into cartridges that were then subjected to automated real-time PCR detection. The Ct values were used to classify the MTB load as either high, medium, low, or very low.

Venous blood (6 ml) was collected in gel/clot activator tubes, sodium citrate tubes and ESR tubes and sent to the laboratory in a sterile container for the biomarker analysis. The inflammatory and coagulation biomarkers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and D-dimer were measured, especially in rifampicin-resistant TB patients. CRP levels were measured using latex agglutination and automated analysis and a cut-off of 6mg/L was used to identify significant levels. ESR was done by Westergren method and D-dimer by an automatic MD PACIFIC analyzer. Association between diabetes mellitus, chronic kidney disease, biomarker levels, and severity of rifampicin-resistant tuberculosis was assessed by performing statistical analysis using Z-test, chi-square test, ANOVA, binomial test and one sample median test, respectively.

RESULTS

All individuals who presented with symptoms were suspected of having pulmonary TB. Sputum samples were collected for PTB illness testing. A total of 150 first-morning sputum specimens were collected from patients attending Prime hospital Peshawar. The collected sample was further processed for Zn Staining for M. tuberculosis. All 150 samples received were Zn positive. After Zn Staining the samples were further send for GeneXpert assay. Among 150 positive sputum smear AFB 1+ are 47, AFB 2+ 23 and AFB 3+ 47 as presented in table 01.

Table 01 Distribution of AFB Grading Among Ziehl–Neelsen Positive Sputum Samples

AFB Grading (ZN Staining)	Number of Samples (n)	Percentage (%)
AFB 1+	47	31.3
AFB 2+	23	15.3
AFB 3+	47	31.3
Total Positive Samples	150	100

In the table below, the severity of 79 male MTB patients was examined using GeneXpert, and the findings of high, medium, low, very low and trace were 10, 17, 35, 07, and 10 accordingly. The results from a total of 71 female MTB patients were 10, 13, 28, 08, and 12 with high, medium, low, very low, and trace respectively. With a P-value of 0.004, the total number of MTB patients with high, medium, low, very low, and trace was 20, 30, 63, 15, and 02 correspondingly as presented in Table 02.

Table 02 Gender wise Table of MTB patients with severity via GeneXpert Assay

Gender	High	Medium	Low	Very Low	Trace	P-value
Male	10	17	35	8	10	0.004
Female	10	13	25	9	13	
Total	20	30	60	17	23	

In given table total of 79 MTB male patients were assessed for Rifampicin Resistance in which ratio of Detected, not detected, and intermediate resistance were 01, 73, 05 respectively. While in 71 female MTB patients Rifampicin Resistance was detected in 4 patients not detected in 51 and intermediate resistance was in 16 patients. In total 150 MTB patient's resistance was detected in 5 while intermediate resistance was 21 and resistance was not detected 124 MTB Patients with P-value 0.004.Moreover, Rifampicin Resistance data for further sensitive is presented in Table 3.

Table 3 Rifampicin Resistance (Sputum samples further for sensitivity)

Gender	Detected	Not detected	Intermediate	Total	P-value
Male	1	73	5	79	0.004
Female	4	51	16	71	
Total	5	124	21	150	

Demographic parameters of the 150 confirmed MTB patients showed a slightly higher prevalence of MTB among males, 79 (52.7%) versus 71 (47.3%) among females. As for age-wise distribution, the largest group of MTB patients was the 21 to 30 years age group (63; 42.0%) followed by the 31 to 40 years age group (32; 21.3%). The age group with the lowest percentage (9; 6.0%) was the 51-60 age group. The average age of the study population was 35.12 years; indicating that young and middle-aged population were more affected by MTB infection show in the table (4).

Table 04: Demographic Characteristics of MTB Patients (Gender and Age-wise Distribution)

Variable	Category	Number of Patients (n)	Percentage (%)
Gender	Male	79	52.7
	Female	71	47.3
Age Group (Years)	10–20	17	11.3
	21–30	63	42.0
	31–40	32	21.3
	41–50	16	10.7
	51–60	9	6.0
	>61	13	8.7
Mean Age		**35.12 years**	

According to the blood biomarkers analysis the mean CRP level in MTB patients is noted as 40.41 mg/L while ESR and D-dimer 40.39 mm/hr and 0.511 mg/L FEU respectively. Total 150 samples were analyzed for CRP level the number of CRP positive male were 78 (98.73%) and CRP negative were 1 (1.26%) while CRP positive female was 65 (91.54%) and negative were 6 (08.46%). Our results indicated that the level of CRP could be used for the assessment of TB presumes with negative results of their smears. CRP can possibly be added to future calculations for the essential consideration finding of tuberculosis in combination with clinical evaluation. Out of 150 samples the number of ESR positive male were 63 (79.74%) and ESR negative were 16 (20.26%) while ESR positive female was 56 (78.8%) and negative were 15 (21.12%). Total 150 samples were analyzed for D-dimer levels in MTB patients the number of D-dimer positive male were 05 (6.32%) and D-dimer negative were 74 (93.68%) while D-dimer positive female was 02 (2.81and negative were 69 (97.18%).The Biomarkers of MTB patients are tabulated in Table 05.

Table 05 Biomarkers of MTB patients

Name of Biomarkers	Unit	n	Mean	P-Value
D-dimer levels in MTB patients	mg/L FEU	150	.511	0.001
C- reactive Protein (crp) level in MTB patients	mg/L	150	40.41	0.001
ESR level in MTB patients	mm/hr	150	40.39	0.001

According to bivariate analysis there is weak positive correlation (r= 0.006) between D-dimer and Rifampicin Resistance. Similarly, significantly (0.001) intermediate negative correlation (r= -.271) was found with CRP. Our study also noted weak negative correlation (r= -240) between Rifampicin Resistance and ESR level in MTB patients as mentioned in Table 06.

Table 06 Correlation between blood biomarkers

Correlations of Rifampicin Resistance with biomarkers		Rifampicin Resistance (Sputum samples Further for Sensitivity)	D-dimer (mg/L FEU) levels in MTB patients	C- reactive Protein level (mg/L) in MTB patients	ESR level in (mm/hr) in MTB patients
Rifampicin Resistance (Sputum samples Further for Sensitivity)	Correlation	1	0.006	-.271**	-.240**
	P-Value		0.945	.001	.289**
	Samples size	150			150
D-dimer (mg/L FEU) levels in MTB patients	Correlation	1	0.006	.311**	.289**
	Correlation	0.945		.000	.000
	Samples size	150		150	150
C-reactive Protein level (mg/L) in MTB patients	Correlation	-.271**	.311**	1	.868**
	Significant	.001	.000		.000
	Samples size	150	150	150	150
ESR level in (mm/hr) in MTB patients	Correlation	-.240**	.289**	.868**	1
	Significant	.003	.000	.000	
	Samples Size	150	150	150	150

DISCUSSION

In this study the prevalence of TB was determined to provide the baseline epidemiological information for population living in District Peshawar, KPK, Pakistan who come for clinical examination to Prime Hospital Warsak road Peshawar. The consequences of the present study will be useful to manage and root out the TB infection. The epidemiological study is significant for the planning to stop tuberculosis ([18].

In the current research overall 150 samples were positive for Ziehl–Neelsen and GeneXpert, Furthermore in MDR and XDR in which out of 150, 26 were detected as rifampicin resistant and 124 were none detected for rifampicin resistant gene. Additionally blood samples of positive cases were analyzed for CRP, ESR and D-dimer. In CRP test out of 150, 143 were detected as CRP positive and 7 were detected as a CRP negative. In ESR test out of 150,119 samples were positive for ESR while in 31 ESR were not raised, moreover in D –Dimer test 7 samples out of 150 , were positive while in 143 samples in D –Dimer were not detected.

This study shows that the Gene-Xpert MTB/RIF testing system can quickly recognizes the existence of MTB and diagnoses the alterations related with the resistance of rifampicin immediately from smear positive and negative clinical specimens of sputum [19]. Our results recommended a direct relationship with the former investigations. This elevated distinction in female/male proportion of tuberculosis is due to the absence of female education, hard work, inattention, and substandard-health facilities offered to them (Hoa*et al.,* 2003). The individuals of the region like other regions are terrified to expose the results because of the fear of separation by the society and believe that once this disease happens then it causes death. The fundamental reason of high predominance is lack of education. Despite these all our study includes total of 150 samples for the prevalence of TB in male & female in which 35% were MDR Isolates and 64.5% Non-MDR Isolates. Among these all-Rifampicin resistances were found in the age of 35-55 (19%) which is followed by age 55-65 (13%). Past researches indicated that the specificity of Gene-Xpert was 79.0%, while sensitivity of Gene-Xpert was 97.3%. Overall, the combined sensitivity and specificity were calculated to be 77.3% and 98.2%. Another study demonstrated that the specificity of Gene-Xpert was

95% and sensitivity of Gene-Xpert was 100%,for the Xpert MTB/RIF assay kit in 340 positive samples. In a study of Agrawal the overall sensitivity, specificity of Gene-Xpert was 86.8% and 96% respectively. Past researches indicated that the specificity of Gene-Xpert was 79.0%, while sensitivity of Gene-Xpert was 97.3% (Tortoli*et al.,* 2012). Overall, the combined sensitivity and specificity were calculated to be 77.3% and 98.2%. Another study demonstrated that the specificity of Gene-Xpert was 95% and sensitivity of Gene-Xpert was 100%, for the Xpert MTB/RIF assay kit in 340 positive samples [26]. In our study the overall prevalence of rifampicin resistance was 3.89%. Sensitivity of Gene-Xpert was 96.88% and the specificity Gene-Xpert was 95.78%.

A study showed that it was striking that high CRP correlated with much more frequent and rapid detection of MTB in clinical samples. A total of 15 patients had direct evidence of disseminated TB in both sputum and urine samples using culture and Xpert MTB/RIF. Of these 80% had a CRP concentration ⩾50 mg /l [21]. Another study showed that the C-reactive protein levels were found to be significantly higher in smear positive group as compared with the smear negative group. Among the smear positive patients, CRP levels were highest in smear 3+ group as compared with the smear 2+ patients. Correlation of CRP levels with extent of disease also revealed that these values were significantly higher in stage III disease as compared with stage II and stage I disease (Rao *et al.,* 2009). In a study the serum concentration of CRP was determined in 60 PTB patients, 30 individuals previously treated with TB treatment and healthy volunteers. The level of CRP in serum was altogether higher i.e 43.65 ± 23.68mg/Lin AFB smear positive individuals as contrasted with patients were already on anti-tuberculosis treatment was9.88 ± 5.23mg/L and the level in smear negative control patients was 4.04 ± 3.85 mg/L, While P value was P< 0.0001. The levels of CRP were elevated i.e 65.28 ± 10.32 in smear positive (3+) patients, in smear positive (2+) patients were 35.93 ± 7.22, in smear positive (1+) patients were 16.37 ± 2.62 and in smear positive scanty patients were 10.92 ± 2.97. Statistically the difference was significant. There was a positive correlation between the serum CRP level and smear positive patients (Shameem *et al.,* 2012). In our study the elevated levels of CRP were found in 97.06% smear positive patients while the elevated levels of CRP were also found in 39.13% smear negative patients. According to the blood biomarkers analysis the mean CRP level in MTB patients is noted as 40.41 mg/L while ESR and D-dimer 40.39 mm/hr and 0.511 mg/L FEU respectively. The analysis revealed that the MDR positive sample is highly co related with ESR and CRP rather than D-Dimer as there is no increase in D-dimer

CONCLUSION

GeneXpert MTB/RIF revealed successful identification of rifampicin resistance and may be utilised as a dependable diagnostic tool for MDR-TB screening. The research showed a larger number of non-MDR (87%) as opposed to MDR (13%) instances. There was also a correlation between resistance and age, with older persons being more resistant, perhaps because of lower immunity. Biomarkers such as CRP, ESR, and D-dimer have been associated with the severity and pattern of resistance of TB illness.

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