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RESEARCH ARTICLE - MEDICAL TECHNIQUES

Assessment of Haematological Parameters in Drug-Resistant TB

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Abstract

Mycobacterium tuberculosis causes tuberculosis (TB), the world's deadliest infectious illness. In addition to the lungs, bone marrow is also impacted by tuberculosis. Significant haematological abnormalities can be found in TB patients. This means that these haematological markers can be used to assess a patient's diagnosis, prognosis, and treatment outcome. The goals of this study are (a) to assess the hematological characteristics of TB patients, and (b) to examine the impact of rifampin (RIF) resistance on the prevalence of mutations in the whole rpoB gene of Mycobacterium tuberculosis. Fifty people participated in the study after being chosen through a systematic random sample process. From each participant, about 4 milliliters of blood were taken from a vein and estimated for the haematological parameters like Hb, WBC, Lymphocyes, neutrophils, Erythrocyte sedimentation rate, and platelet count. Fifty tuberculosis isolates had their whole rpoB genes sequenced so that we could analyze the positions of the codons and the frequency with which they occurred. When compared to healthy controls, the values of hemoglobin and other blood indices were significantly lower or abnormal ($p<0.05$). ESR values were alarmingly increased in the subjects along with platelets (P value < 0.05). All of our 25 isolates exhibited four types of mutations at four RRDR positions (codons 510, 516, 522, and 526). We found codons 526 showed a high level (92%) of RIF resistance or mutations when compared to other positions. An easy and affordable way to forecast the progression of the illness and keep track of complications in underdeveloped nations is to test the haematological parameters of tuberculosis patients. RIF resistance is linked to certain mutations in the rpoB gene that may impact how RpoB and RIF interact. These results may be used to create new antibiotics and create cutting-edge diagnostic tools for TB medication resistance.

Publisher: Middle Technical University

Keywords: Mutation; Rpob Gene; Drug Resistance; *Mycobacterium Tuberculosis*.

1. Introduction

The most prevalent infectious disease is tuberculosis (TB), which is brought on by mycobacterium tuberculosis. Despite the development of novel diagnostic techniques and treatments, it continues to be one of the major public health issues of the world [1]. Nearly 10 million individuals are affected every year, making it a key factor of death around the globe. During the past five years, it has been the single most important cause of death, even more so than HIV/AIDS [2]. Haematological indicators, including haemoglobin (Hb), packed cell volume (PCV), red blood cell (RBC) count, blood indices, platelet count, white blood cell (WBC) count, and erythrocyte sedimentation rate (ESR), are used for the diagnosis, prognosis, and follow-up of patients [3]. ESR and (C reactive protein) CRP have been shown in a few studies to be sensitive indicators for TB [4].

Measures of blood cell health are fundamental in formulating treatment strategies and gauging their efficacy. The patient's prognosis could be affected [5]. Improving results of treatment, life quality and survival of patients all rely on hematological marker-based infection surveillance and control like tuberculosis. These haematological results can aid in attracting the attention of specialists and primary care support staff quickly, which is crucial for the early diagnosis of alterations in TB patients [6].

Despite gradual declines in both incidence and mortality rates, There are an estimated 10 million new cases of tuberculosis (TB) per year, leading to an estimated 1.2 million fatalities [7]. Drug-resistant tuberculosis has alarmingly increased recently, posing a significant threat to TB control. Drug resistance in TB patients must be quickly and accurately identified if the disease is to be successfully controlled. One of the most significant anti-TB medications is rifampin (RIF).

It is a key medication in the treatment of tuberculosis (TB) because of its potent bactericidal action against Mycobacterium tuberculosis (M. tuberculosis). RIF specifically targets the rpoB gene's DNA-dependent RNA polymerase -subunit [8]. A conformational shift caused by mutated rpoB affects RIF's affinity for interaction with the RNA polymerase-subunit (RNAP), rendering the medicine inert since it was unable to properly attach to the target location [8]. In Mycobacterium TB, alterations to a region of the rpoB gene spanning 426 as well as 452 base pairs in the 81-bp segment are primarily responsible for RIF resistance [9], also, these mutations are present in roughly 90-95% of RIF-resistant isolates [10, 11]. The remaining 5% of RIF-resistant isolates, however, lack a known mechanism of resistance, suggesting that there may be additional mechanisms at play, such as decreased cell wall permeability12 or increased efflux pump activity [12].

Nomenclature & Symbols			
TB	Tuberculosis	CRP	C-Reactive Protein
MTB	Mycobacterium Tuberculosis	RNAP	Ribonucleic Acid Polymerase
rpoB	Rna Polymerase Beta Subunit	LJ	Lowenstein-Jensen
RIF	Rifampicin	MCV	Mean Corpuscular Volume
MIC	Minimum Inhibitory Concentration	MCH	Mean Corpuscular Haemoglobin
Hb	Haemoglobin	MCHC	Mean Corpuscular Haemoglobin Concentration
PCV	Packed Cell Volume	TLC	Total Leukocyte Count
RBC	Red Blood Cell	DLC	Differential Leukocyte Count
WBC	White Blood Cell	HCT	Hematocrit
ESR	Erythrocyte Sedimentation Rate	EMB	Ethambutol
RNA	Ribonucleic Acid	DNA	Deoxyribonucleic Acid

In the current study, the quantitative Rifampin resistance phenotyping with MIC measuring has been systematically done for the clinical isolates of 50 Mycobacterium tuberculosis, and analysis of the complete mutation of the rpoB gene with its impacts on Rifampin resistances has been carried out. Our study aimed to determine the mutation that occurs in the rpoB gene with drug resistance.

2. Materials and Methods

2.1. Sample collection and Species identification

Sputum culture-positive specimens were gathered for this prospective investigation between February 1 and July 2022. Subjects were chosen randomly from the patients who attended the TB centre, in Baghdad. This study included clinical stored isolates of Mycobacterium tuberculosis obtained from (50) patients. The isolates were taken from (50) epidemiologically-unrelated adult pulmonary tuberculosis patients. Venous blood samples were taken from these patients and from (n=50) healthy individuals who served as a control group. Hematological analysis was performed on approximately 2.5 ml of blood taken from each individual. For 3–4 weeks at 37°C, cultures were observed weekly for observable colony growth as p-nitrobenzoic acid, 2-thiophene-carboxylic acid hydrazide, and DST was used to identify species on Lowenstein–Jensen (LJ) media. The standard isolate H37Rv (Mycobacterium tuberculosis ssp. tuberculosis ATCC 27294) and sterile deionized water (ddH2O) were used as positive and negative controls, respectively, in all of the experiments shown here.

The research was approved by the institution's ethics board. According to (Ref: NDDFMSR/IEC/04/21). All patients provided their written informed permission.

2.2. Inclusion criteria

Patients with both positive and negative sputum smears for tuberculosis (and age above 18 years).

2.3. Exclusion criteria

Women who are pregnant, children, people who have been diagnosed with extrapulmonary TB or TB-diagnosed subjects with multi-drug resistance MDR, and people who have chronic diseases (kidney, liver, leukemia, and HIV).

2.4. Hematological evaluation

All of the study participants (n=50) had their venous blood taken in an amount of about 4ml under strict aseptic conditions. The Siemens Genetix haematology analyzer was used to evaluate approximately 2ml of EDTA tube blood for haematological purposes and an additional 2ml to estimate ESR. The Wintrobe method was used to estimate ESR. In this study, we assessed haematological parameters such as haemoglobin (Hb), Blood indicators including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) are measured alongside a total leukocyte count (TLC), differential leukocyte count (DLC), hematocrit (HCT), and platelet count.

2.5. Drug susceptibility testing

The in vitro drug sensitivity of all the M. tuberculosis (MTB) isolates was determined. Rifampicin (RIF), as well as ethambutol (EMB), were used in the study All of the isolates were examined for their level of resistance to RIF and EMB at IC50 doses of μ40 g/ml and 2.0 μg/ml, respectively. In brief, triplicates of the Isolate dilutions were inoculated into an LJ medium, and the plates were placed in a 37°C incubator. with and without treatment. Every day during the first 42 days of incubation, the results were read. When growth was shown in the drug-containing media, an isolate was deemed resistant to that particular antibiotic. As a control, we used the MTBC H37Rv wild-type strain (ATCC 27294) [13].

2.6. Extraction, amplification and sequencing of DNA

DNA was extracted from all 25 isolates resistant to RIF. A loopful of culture was powdered, mixed with 500μl of ddH2O, and then incubated in a heating block at 95°C for 20min. Once the supernatant was collected via centrifugation at 10,000g for 15 minutes, it was poured into a clean tube. With the utilizing of an MTB DNA extraction kit (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA), The genomic DNA of MTB that had been rendered inactive was isolated and preserved for future use. 5μL of the collected DNA was then utilized as a model for the amplification of rpoB.

2.7. PCR amplification

The DNA extracted in the previous section was used for amplification. The primers rpoB were: (FW 5′-GCGGCTCAGCGGTTTAGTTG-3′), rpoB (RV 5′-ACAGCGGGTTGTTCTGGTCC-3′) (product length ~304bp). The PCR reaction's parameters were for a 12min initial denaturation step at 94°C, then 29 cycles of 15s at 94°C, 15s at 56°C, and 30s at 72°C, and finally a 7min extension step at 72°C. PCR products following quality check with UV-Vis spectrophotometry were then sent for sequencing. 2µl of DNA template, 1µl of each (reverse and forward) primer, and 12.5µl of the 10xTaq Mix (DNA polymerase (1 U/50µl reaction) was used in the amplification.

2.8. rpoB – sequencing

Primer pairs (both FW and RV) were employed for the 308bp rpoB fragment, and Sanger sequencing and assembly were carried out by the previous study. Sangon Biotech (Iraq) performed the synthesis and sequencing of the primers. According to established protocols, all areas with variations were amplified by PCR and sequenced using the cycle sequencing kit of the ABI Prism Big Dye Terminators [14].

All the 25 PCR products were analysed through DNA sequencing and sequences obtained were aligned manually and the polymorphisms were detected with the control sequence (rpoB gene of MTB H37Rv sequence (Acc.No: NC_000962.3). The mutations observed were then analyzed with clustal Omega programs (Clustal Omega < Multiple Sequence Alignment < EMBL-EBI).

2.9. Statistical analyses

Statistical analysis was carried out by the use of Statistical Package for the Social Sciences SPSS (ver 2.0). Student T test was applied for comparison between the diseased and control group [15]. All the values are averages of triplicates and expressed as value ±SD. P value < 0.05 was considered statistically significant in our study.

3. Results

In this study, according to the age distribution results, the highest infection rate (48%) was found among the age group (20–40) years, while the number and percentage of females was 29 (58%) compared to males 21 (42%) with no significant difference (P>0.05) as shown in Table 1.

Table 1. Showing the Demographics of patient groups and the controls from TB centre as represented according to Age group and gender

Demographics			Studied groups		P – value
			Control (n= 50)	Patient (n=50)	
Age groups / Year	**< 20**	No (%)	2 (4%)	3 (6%)	Non sign. (P>0.05)
	20 – 40	No (%)	25 (50%)	24 (48%)	
	41 – 60	No (%)	19 (38%)	17 (34%)	
	61 – 80	No (%)	4 (8%)	6 (12%)	
Gender	**Male**	No (%)	22 (44%)	21 (42%)	(P>0.05)
	Female	No (%)	28 (56%)	29 (58%)	

From our hematological tests among TB patients, 31(62%) showed normal WBC count while 30 and 8% showed leukocytosis and leucopenia respectively (p<0.05). About 12 (24%) and 15(30) were found to be suffering from severe to moderate anemia respectively (Fig. 1). The lymphocyte count was normal in 25 (50%) of the TB patients while 17 (34%) and 8 (16%) showed symptoms of lymphocytosis and lymphopenia respectively in comparison with the healthy controls (100%), with a highly significant difference (P<0.01). Also, there was a highly significant increase in Neutrophil % level among patients 26 (52%) with neutrophilia. While 6 (12%) showed neutropenia (P<0.05) (Fig. 2).

A significant increase in the platelet count leading to thrombocytosis and thrombocytopenia was seen in 27 (54%) and 8 (16%) while 30% showed normal levels (p<0.05). ESR was also found to be significantly elevated p<0.05). 42 (84%) of them showed elevated levels (>40mm) while only 3 (6%) showed normal or low levels (<20mm) (Fig. 3). This is highly significant when compared to the control group (p<0.05).

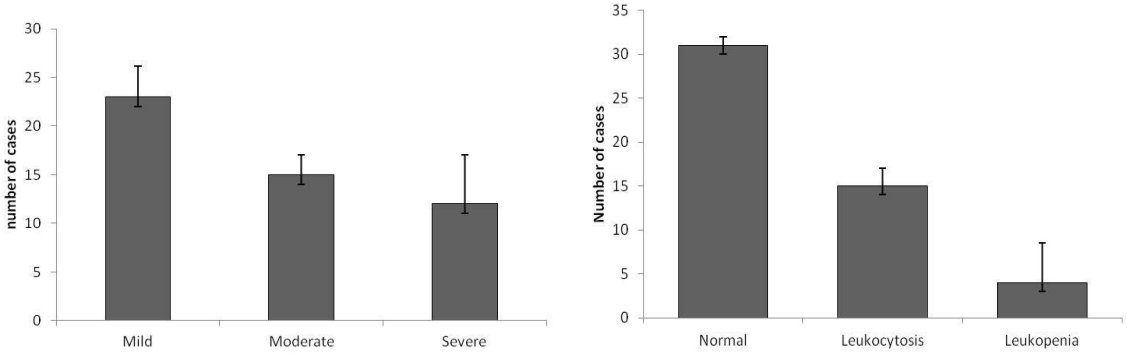

Fig. 1. Graphs shwoing the hematological parameters (Hemoglobin & WBC) among the subjects (n=50), All the values are average of triplicates P<0.05

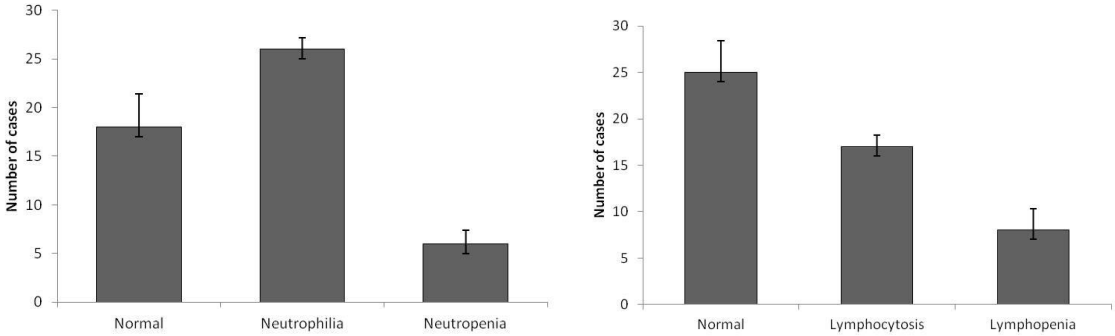

Fig. 2. Graphs shwoing the hematological parameters (Neutrophils & Lymphocytes) among the subjects (n=50), All the values are average of triplicates P<0.05

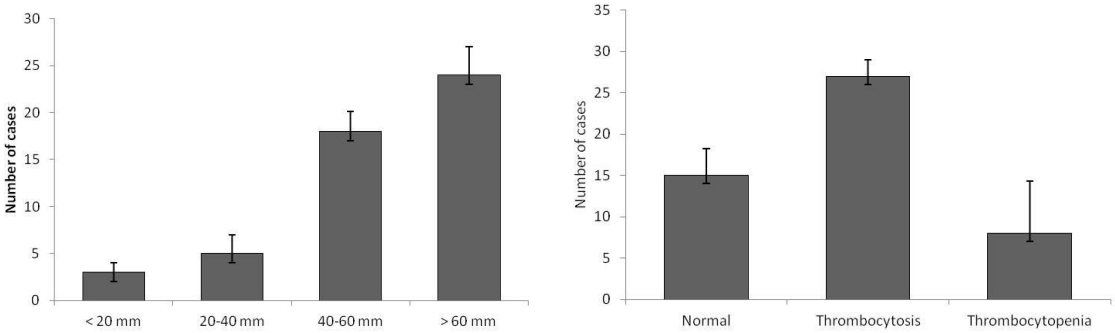

Fig. 3. Graphs shwoing the hematological parameters (ESR & Platelets) among the subjects (n=50), All the values are average of triplicates. P<0.05

3.1. Drug Susceptibility patterns

From the total of 43 MTB-positive isolates (n=50), 31 (72%) were mono-resistant (18 isolates resistant to RIF and 13 resistant to EMB). A total of 25 RIF isolates were found to be resistant to RIF ($p<0.05$) (Fig. 4).

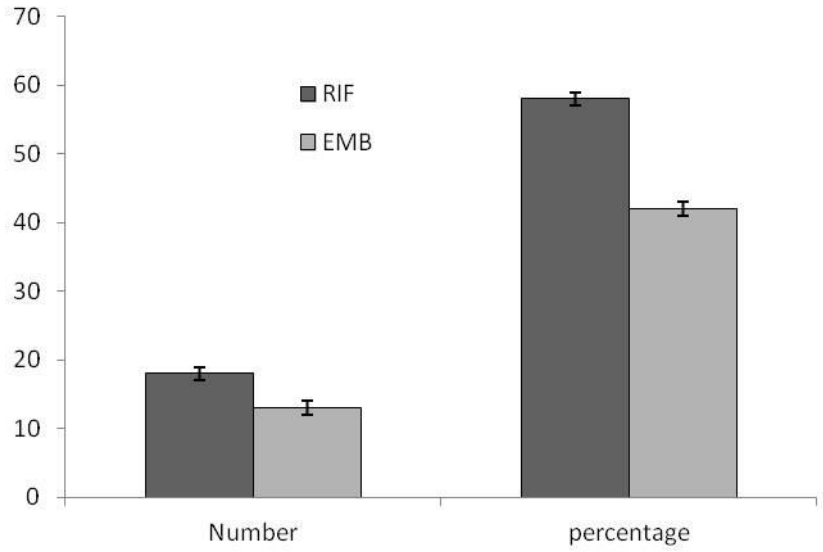

Fig. 4 Graph showing the possible isolates resistant to RIF and EMB. All the experiments are done in triplicates. (n=50)

3.2. Molecular validation of the rpoB gene

A 304bp band was seen on 1.5% agarose gel confirming the positive amplification (not shown). All 25 isolates had one of four mutations in the rpoB gene. Codons 510, 516, 522, and 526 within the RRDR region were found to be mutated (Table 2). This includes [Gln-510Leu (CAG-CTG), Asp-516Val and Tyr (GAC-GTC and GAC-TAC), Ser-522Leu (TCG-TTG), and His-526Asp (CAC -GAC)]. Isolates with a mutation at codon 526 demonstrated a high level of RIF resistance (92%; 23/25), followed by those with a mutation at codon 516 (60%; 15/23). Codon

510 showed the least rate of mutations with 8% (2/25) (Fig. 5). As seen from the phylogeny tree constructed with MEGA 7, the samples were in line with the outlier or control (Acc: NC_000962.3) (Fig. 6).

Table 2. Showing the types of mutations at the hot spot regions of the 4 codon positions (n=25)

Codon	Mutations	Amino acid change	Number of resistant strains (%)
510	CAG ➔ CTG	Gln ➔ Leu	2 (8%)
516	GAC ➔ TAC	Asp ➔ Val	15 (60%)
522	TCG ➔ TTG	Ser ➔ Leu	11 (44%)
526	CAC ➔ GAC	His ➔ Asp	23 92%)

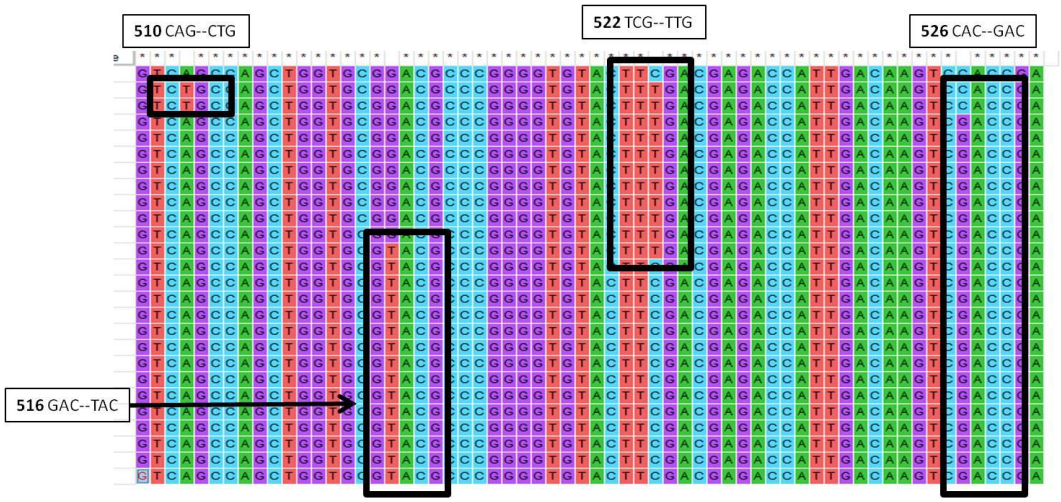

Fig. 5. Image showing the 4 types of rare mutations observed among 25 samples within the *rpoB* gene of RIF resistant MTB isolates

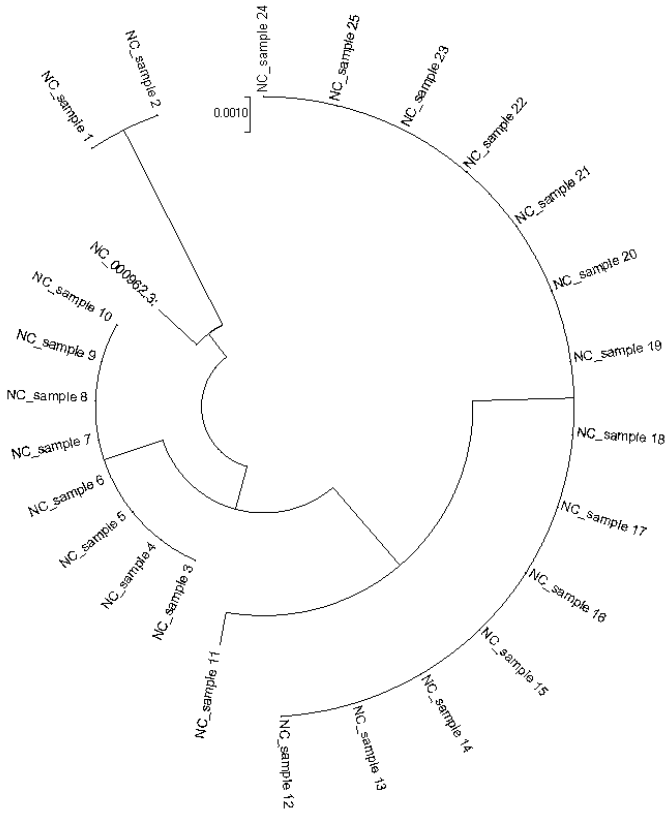

Fig. 6. Phylogeny tree constructed using Neighbor-Joining method [16]. The optimal tree branch length = 0.01819876. The analysis involved over 26 nucleotide sequences (including outlier/control). All positions with gaps were eliminated. There were 263 locations included in the final dataset. Phylogenetic examinations were assayed with MEGA7 [17]

4. Discussion

In addition to being a major health issue across the globe, tuberculosis is still one of the most significant communicable illnesses. Chest pain, systemic symptoms, and a productive cough lasting longer than three weeks are all tuberculosis signs. An X-ray of the chest and blood or sputum culture are the main diagnostic tools used to identify tuberculosis (TB). There are also several alternative techniques, including skin examinations, IGRAs (interferon-gamma release assays), in vitro blood examinations, and PCR tests. However, a quick and affordable diagnostic test might be useful in developing nations. One of these economical approaches in such a situation is the assessment of haematological parameters.

Our study showed that 48% of the cases belong to the age group 20–50 years. These findings were by reports from Rohini K et al., [18] and Jagielski et al., [19]. Rajesh H et al. [20] reported that the lowest prevalence was seen in the old age groups (> 60 years). Our results are again in line with this statement wherein we found 6% of them belong to an age group greater than 60 ($p<0.05$). We also found of the positive subjects, 58% of them are females as compared to 42% of cases in women. Similar findings were seen by Jagielski et al. [19]. But some reports were seen wherein men were more prone to attack [5].

In terms of hematological parameters, anemia is the most often reported complication among tuberculosis patients and is associated with an increased risk of death [21]. The degree of anemia was evaluated in our study by measuring hemoglobin levels. We discovered only 12% incidences of severe anemia and 37% incidences of mild anemia. In addition, normocytic normochromic anemia was observed in 38% of cases, followed by microcytic hypochromic anemia (42%). nonetheless, twenty percent of the people had a macrocytic hypochromic pattern. Our findings are highly comparable to the reports of Bashir et al. [22].

Even though 50% of them showed a normal lymphocyte count, it was found 34% and 16% showed lymphocytosis and lymphopenia respectively ($p<0.05$). In addition, we also show a significant rise in neutrophilia (54%) and neutropenia (12%) cases which might be due to an immune response from the host against the disease [23],[5]. On the other hand, neutropenia was found to be the most common condition in a study by Thatoi PK. [23] this condition may be brought on by hypersplenism, excessive neutrophil margination, or T lymphocyte activity that inhibits granulopoiesis.

Abnormal platelet count was seen in 70% of the subjects in this study which was by reports from Jagielskiet al. [19] and [5]. Numerous cytokines, including IL 6, which are involved in the creation of granulomas and increased platelet synthesis, may be the cause of these abnormal levels.

Erythrocyte sedimentation rate (ESR) values consistently rise in tuberculosis patients relative to normal controls, according to our findings. In our study, ESR was significantly elevated in 84% of the subjects. Our results are in line with reports from Rohini et al., [18], Jagielskiet al., [19], and Thatoiet al. [23]. ESR is a capable indicator of the inflammatory response. It is employed to gather data on the progression and regression of disease.

We could isolate a 308bp band from our amplification of the rpoB gene. On sequencing we found all of the 25 isolates exhibited four different types of mutations on the rpoB gene.

The four more frequent mutations were seen at four RRDR positions (codons 510, 516, 522, and 526). The most frequent mutations worldwide are those at codons 531, 526, 516, and 511, in that order. The predominant mutant alleles are TTG at codon 531 and CCG at codon 511, however, there are several allelic variants at codons 526 and 516 [24]. We found the mutations at codons 526 showed a high level (92%) of RIF resistance of the isolates followed by codons 516 (60%). Codon 510 showed the least rate of mutations with 8% (2/25). According to Billingtonet al., [25] the mean relative fitness of mutants isolated more frequently in clinical practice is higher, and the prevalence of each mutant type relies on how well it can live. This could be the cause of the higher percentages of global isolates with the mutations TCG to TTG at codon 531 and CTG to CCG at codon 511 [26]. As a result, mutations continue to occur in codons 526 and 516, likely as a result of M. tuberculosis' capacity to adapt to drug exposure [27], which has been proven to cause significant levels of RIF resistance in this pathogen.

5. Conclusion

One of the major public health issues facing the entire world continues to be tuberculosis. There are many markers for diagnosing and tracking the condition on the market, but they are expensive and cannot be used frequently. Along with ESR, the complete blood count (CBC) is a straightforward test that can be used in underdeveloped nations like India to forecast the progression of the illness and track the consequences. Anaemia was the most prevalent symptom reported by patients with pulmonary tuberculosis, and a microcytic hypochromic pattern was the most common haematological sign. Patients with tuberculosis had lower PCV, MCV, MCH, and MCHC than healthy controls, but had significantly higher WBC count, absolute neutrophil count, platelet count, and ESR values.

The key point is that early diagnosis of TB may be aided by knowledge of clinical characteristics, laboratory results (haematological parameters), and demographic information of patients. Any such aberrant haematological findings that are unexplained should prompt a high suspicion of tuberculosis.

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