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# The Role of pknF and fbpA as a virulence genes with Interleukin4-and 6, in the Pathogenesis of Tuberculosis

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**Abstract:**

Tuberculosis (TB) is a global health problem caused by *Mycobacterium tuberculosis* (MTB), a bacterium that can evade the host immune system and keep at in a latent state. Drug-resistant strains of MTB, such as multi-drug resistant TB (MDR-TB), pose a significant challenge toward the TB control efforts. This study aimed to investigate the role of two MTB virulence genes, *pknF* and *fbpA*, and two host cytokines, Interleukin-4 and Interleukin-6, in the pathogenesis of TB. The expression levels of *pknF* and *fbpA* genes were measured by qPCR in (12) sensitive TB and (12) MDR-TB isolates. IL-4 and IL-6 were measured by ELISA in serum samples from (24) healthy controls, (12) patients having sensitive TB, and 12 patients having MDR-TB. The results revealed a significant difference in *fbpA* gene expression between MDR-TB (2.59± SE 0.36) and sensitive TB (1.01± SE0.037) isolates (P=0.0003), whereas *pknF* gene expression did not vary significantly across the two groups (2.53±0.62 in MDR-TB and 1.72±0.40 in sensitive TB) (p=0.289). IL-4 levels were markedly elevated in patients with MDR-TB (1091.967± SE 108.793 pg/ml) compared to the control group (105.3358 ± SE 5.543 pg/ml) (p<0.0001), but not significantly different from patients with sensitive TB (1054.763 ± SE 71.482 pg/ml). IL-6 levels were significantly higher in both MDR-TB and sensitive TB patients than in the control group (9.253 ± SE 0.456 pg/ml). However, MDR-TB patients showed a non-significant lower ratio of IL-6 (38.5851 ± Se 4.601 pg/ml) than sensitive TB patients (42.458 ± SE 1.809). A significant negative correlation were observed between *fbpA* gene expression and IL-4 levels in both MDR-TB and sensitive TB patients (r = -0.375; p < 0.0001 and r = -0.165; p < 0.0001, respectively), and a positive correlation between *fbpA* gene expression and IL-6 levels in both groups (r = 0.1006; p < 0.0001 and r = 0.466; p < 0.0001, respectively). These findings suggest that *pknF* and *fbpA* genes may play a role in the virulence of MTB, especially in drug-resistant strains, and that IL-4 and IL-6 may be involved in the host immune response to MTB infection. These potential biomarkers could be used to develop targeted therapies for MDR-TB and improve TB control efforts globally.

**Keys words**: Tuberculosis, *mycobacterium tuberculosis, Pknf* gene , *FbpA* gene , Interleukin 4, Interleukin 6

## 1. Introduction:

Tuberculosis (TB) is a disease caused by bacterium MTB, and has some charters as small, rod shaped, strictly aerobic, acid fast bacillus that resists discoloration by acid and alcohol once stained and grows slowly, leading to a more gradual development of disease compared to other airborne bacterial infections. TB typically affects the lungs but can also involve other body parts, such as the brain, kidneys, or spine (Sharma *et al*, 2021; Torfs *et al*, 2019). Multidrug-resistant TB (MDR-TB) has been identified as a form of TB resistant to as a minimum isoniazid (INH) and rifampin (RIF), as well as more than one anti-TB drug (Sambas *et al*,2020). The World Health Organization (WHO) recently updated its definitions of extensively drug-resistant tuberculosis (XDR-TB) and defined pre-XDR-TB for the first time. Pre-XDR-TB is now defined as TB caused by MTB strains meeting the criteria for multidrug-resistant and rifampicin-resistant TB (MDR/RR-TB) and exhibiting resistance to any fluoroquinolone (Viney *et al*, 2021; Walsh, 2019). Bacterial virulence factors, which typically cause damage to the host, are produced by bacterial cells. They increase adhesion, facilitate colonization and invasion into eukaryotic cells, evade host immune responses, and provide essential nutrients (Land, 2023; Gnanagobal and Santander, 2022). *pknF*, a serine/threonine protein kinase (STPK) in MTB, found to display a vital role in the bacterium's physiology and pathogenesis (Mori *et al*, 2019). This kinase is involved in various cellular processes of physiological importance, such as cell division, arabino synthesis, mycolic acid synthesis, peptidoglycan synthesis, TCA cycle, methionine cycle, signaling, chaperone, and transport (Cabarca *et al*, 2021). Additionally, *pknF* has been demonstrated to play a crucial role in innate immune evasion (Rastogi *et al*, 2021). The Fibronectin-Binding Protein A (*fbpA*) gene is one of three genes encoding the antigen 85 complex in MTB. Composed of three distinct trehalose dimycolyl transferases (Ag85A, Ag85B, and Ag85C), the antigen 85 complex is involved in mycolate deposition (Mehaffy *et al*, 2019). The *FbpA* protein has been shown to elicit an immune response in TB patients (Ernst *et al*, 2019). Interleukin-6 (IL-6), a cytokine with numerous biological roles, belongs to the proinflammatory cytokine group and enhances the expression of various proteins accountable for acute inflammation. IL-6 plays a significant character in the proliferation and differentiation of cells. It has been reported that MTB modulates host IL-6 production to inhibit type I interferon signaling and consequently, disease progression (Aliyu *et al*, 2022). Interleukin-4 (IL-4), another cytokine with multiple biological roles, stimulates the proliferation of activated B cells and T cells and the differentiation of B cells into plasma cells. It is a crucial regulator in humeral and adaptive immunity (Cao *et al*, 2023). The involvement of IL-4, a T-helper type 2 (Th2) cytokine, in the immune-pathogenesis of human tuberculosis remains uncertain. Some studies suggest that IL-4 may contribute to tissue destruction and/or cell death during MTB infection (Atitey and Anchang, 2022; Wu *et al*, 2022).

## 2. Materials and Methods:

**Study population:** 24 Blood samples (12 samples of sensitive TB patients, 12 samples of MDR-TB patients, and 24 healthy control) for measuring interleukins 4 and 6. And 24 confirmed TB patients (12 sensitive TB, 12 MDR-TB) for measuring *pknf, fbpA* gene expressions.

**Total RNA Extraction:** The total RNA was gained from cell lysates by distraction with small glass beads. First, bacteria were lysed with lysozyme (Sigma-Aldrich, 20 mg/mL) and proteinase K (Sigma-Aldrich, 2 mg/mL) solution and incubated for (10) min at (37) ◦C. Then, (600) µL of RLT buffer (Qiagen, Hilden, Germany) was added to guarantee bacterial lysis. The samples were shaken in a Fast Prep Homogenizer (MP Biomedicals, Santa Ana, CA, USA) at a speed of 6.5, 2 cycles of (30) s and were then centrifuged at 8000× g for 1 min (Eppendorf, Hamburg, Germany) to remove cell fragments. Total RNA was gained with the RNeasy system (Qiagen, Hilden, Germany). Total RNA was measured by spectrophotometry and stored at (−70) ◦C.

**Conversion of RNA to cDNA:** LunaScript Reverse Transcriptase/ Biolabs/England RT component Kit is considered to make the reverse transcription optimized for real-time RT-PCR. It uses RTase,


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which features admirable extendibility and makes fast, effective cDNA template synthesis for Real Time PCR.

**Performing RT-PCR:** A 2X reaction mix that can be used for real-time qPCR to detect and quantify target DNA sequences is the NEB Luna Universal qPCR Master Mix. It is compatible with the SYBR/FAM channel of most real-time qPCR instruments. Hot Start Taq DNA Polymerase is contained in it and a unique passive reference dye that is compatible across different instrument platforms (including those that require a high or low ROX reference signal) is formulated with it. It also features dUTP for carryover prevention and a non-fluorescent, visible dye to monitor reaction setup. This dye does not spectrally overlap with fluorescent dyes used for qPCR and will not interfere with real-time detection. The master mix formulation is supplied at 2X concentration and contains all PCR components required for amplification and quantitation of DNA except primers and DNA template.. Primer used in this study for amplifying *PknF* and *FbpA* genes are (F-GTGGTGATCAGCCAGCATCT), (R- AATCTCCTCGCGACATTCCC). (F-GCTTCATAGCGTTGAGCTGC), (R- AGCTTGTTGACAGGGTTCGT) respectively.

**IL-4 and IL-6 measurement**: They were measured by using sandwich enzyme linked immunosorbent assay (ELISA) kits (Elabscience, Swedish).

**Statistical analysis:** The data were analyzed using SPSS software (version 23.0) . Continuous variables were expressed as mean ± standard Errors compared using t-test. Correlations between IL-4 and IL-6 levels and clinical variables were evaluated using Pearson's correlation coefficient. A p-value <0.05 was considered statistically significant.

## 3. Results and Discussion:

### 3.1 Gene expression levels of *PknF* and *FbpA* in sensitive TB and MDR-TB patients :

Relative expression level of *pknF* and *fbpA* were using the RNA 16S as internal control for normalization of RNA quantities in both two groups (sensitive and resistance TB)(Figure 1). Expression level measured in vitro for sensitive MTB and MDR-TB (Table 1,2). In (Figure 2) there is non-Significant up-regulation of the *pknF* gene in MDR-TB isolates (Mean 2.53±SE 0.62), compared to drug-susceptible TB isolates (Mean 1.72±SE 0.40 ) (P-value 0.2891 ). Additionally, *fbpA* also measured for both groups and there is a significant up-regulation in MDR-TB ( Mean 2.59±SE 0.36), compared to drug susceptible TB (Mean 1.01±SE 0.037) (P-value <0.05) (Table 3). These findings are consistent with previous studies that have reported increased expression of drug resistance genes in MDR-TB patients for example, Nguyen *et al.*(2005) showed the disruption of *fbpA* gene has a low rate 45% in pathogenesis of mycobacterium tuberculosis.

**Table(1):** Presents gene expression levels of 16s rRNA, *FbpA*, and *PknF*

| N-MDR | 16SRNA | *fbpA* | Fold (2^-ΔΔCT) | *pknF* | Fold (2^-ΔΔCT) |
|---|---|---|---|---|---|
| 1 | 22.6 | 28.6 | 0.65975 | 38.4 | 0.21764 |
| 2 | 25.6 | 29.1 | 3.73213 | 37 | 4.59479 |
| 3 | 24.8 | 28.5 | 3.24901 | 38.2 | 1.1487 |
| 4 | 25.4 | 29 | 3.4822 | 36.8 | 4.59479 |
| 5 | 24.6 | 28.4 | 3.03143 | 39.9 | 0.30779 |
| 6 | 25.2 | 30.4 | 1.1487 | 36.6 | 4.59479 |
| 7 | 24.4 | 28.3 | 2.82843 | 39.7 | 0.30779 |
| 8 | 25 | 31.6 | 0.43528 | 36.4 | 4.59479 |
| 9 | 24.2 | 28.2 | 2.63902 | 39.2 | 0.37893 |
| 10 | 24.8 | 28.7 | 2.82843 | 36.2 | 4.59479 |
| 11 | 24 | 28.1 | 2.46229 | 39 | 0.37893 |
| 12 | 24.6 | 27.8 | 4.59479 | 36 | 4.59479 |


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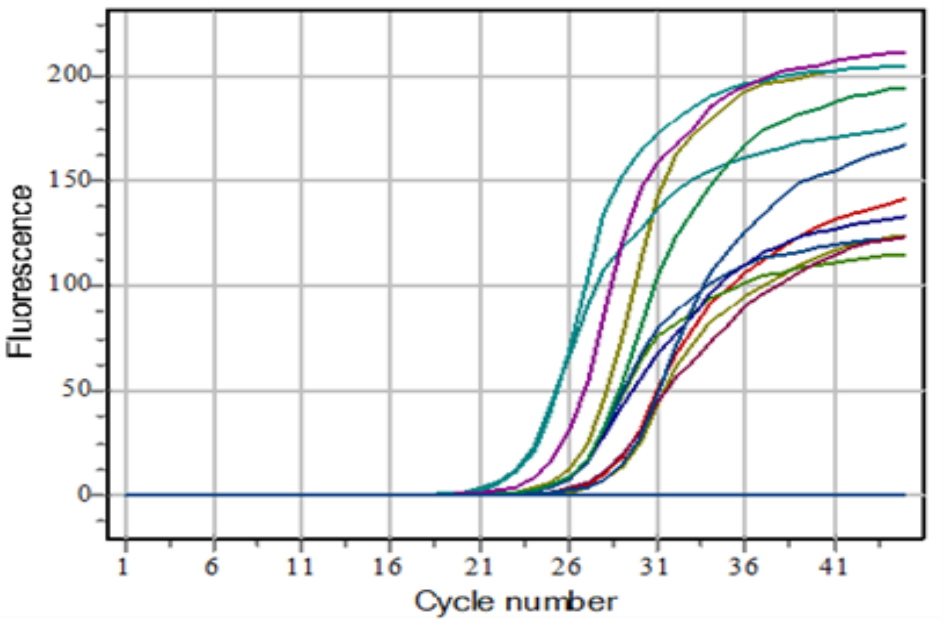
**Table(2):** Gene expression levels of 16srRNA, *fbpA*, and *pknF* genes in MDR-TB genes in sensitive MTB

| N-Sensitive | 16SRNA | FbpA. | Fold (2^-ΔΔCT) | pknf | Fold (2^-ΔΔCT) |
|---|---|---|---|---|---|
| 1 | 25.8 | 31 | 1.1487 | 39.8 | 0.75786 |
| 2 | 26.4 | 31.5 | 1.23114 | 38.4 | 3.03143 |
| 3 | 25.6 | 30.9 | 1.07177 | 39.6 | 0.75786 |
| 4 | 26.2 | 31.4 | 1.1487 | 38.2 | 3.03143 |
| 5 | 25.4 | 30.8 | 1 | 41.3 | 0.20306 |
| 6 | 26 | 31.3 | 1.07177 | 38 | 3.03143 |
| 7 | 25.2 | 30.7 | 0.93303 | 41.1 | 0.20306 |
| 8 | 25.8 | 31.2 | 1 | 37.8 | 3.03143 |
| 9 | 25 | 30.6 | 0.87055 | 40.6 | 0.25 |
| 10 | 25.6 | 31.1 | 0.93303 | 37.6 | 3.03143 |
| 11 | 24.8 | 30.5 | 0.81225 | 40.4 | 0.25 |
| 12 | 25.4 | 31 | 0.87055 | 37.4 | 3.03143 |

**Table(3):** Expression of *fbpA* and *pknF* Genes in Sensitive and MDR-TB: Analysis of ΔCT , ΔΔCT, and Fold

| Genes | Groups | ΔCT (Mean ± SE) | P value | ΔΔCT (Mean ±SE) | P value | Fold Change ($2^{-\Delta\Delta CT}$) Mean ±SE | P value |
|---|---|---|---|---|---|---|---|
| ***FbpA*** | **N-Sensitive** | **5.40±0.05** | **0.0017** | **0.00± 0.05** | **0.0017** | **1.01±0.037** | **0.0003** |
| | **N-MDR** | **.30±0.314** | | **-1.11±0.31** | | **2.59±0.36** | |
| ***PknF*** | **N-Sensitive** | **13.58± 0.51** | **0.6029** | **-0.02±0. 51** | **0.6029** | **1.72±0.40** | **0.2891** |
| | **N-MDR** | **13.18±0.56** | | **.560-0.42±** | | **2.53±0.62** | |




**(Figure 1)** represent the gene expression of 16s rRNA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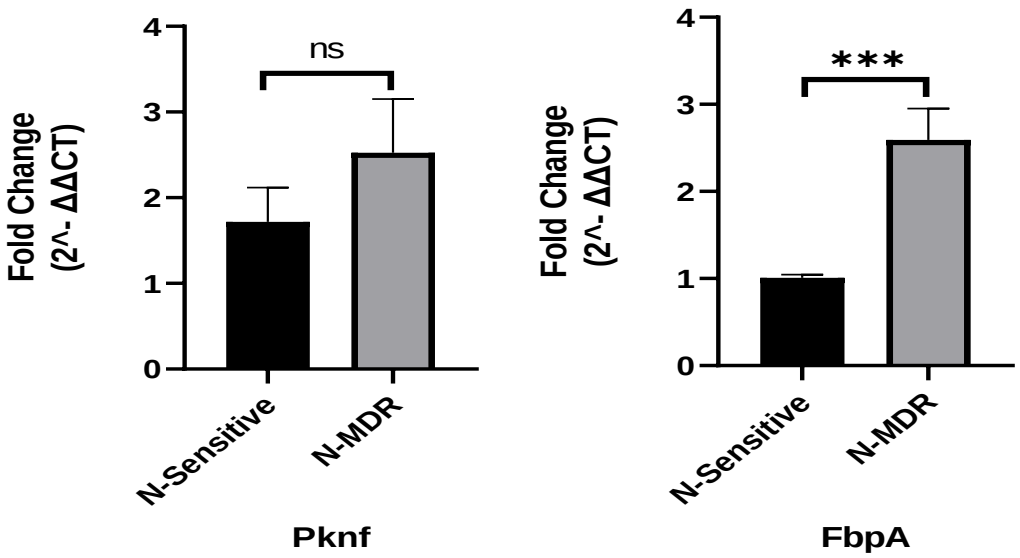


Figure 2. The mean level of *pknF* and *fbpA* gene expressions of MTB in Sensitive TB and MDR-TB

### 3.2 Interleukins levels (4 and 6) in sensitive TB and MDR-TB patients:

As shown in table (4), the study reported a highly significant difference in IL-4 levels between active TB and control group (p< 0.0001). Moreover, the study results revealed a significant difference in IL-4 levels between MDR-TB and control group (p< 0.0001), with no statistically significant difference observed between active and MDR-TB cases (P= 0.388). This elevation between MDR-TB and healthy controls relative to active TB and healthy individuals suggests that high IL4 levels correlated with disease progression. A study from India investigated serum concentration of IL-4 revealed a significant rising in MDR-TB cases compared to control group (p<0.001), as observed in present study and other studies by Rook *et al*, 2004 and Smith *et al*, 2002 showed that IL-4 was higher in all TB patient groups compared with healthy control, these changes imply a decreased Th1-lymphocyte activity in these groups (active TB and MDR-TB).

The study also reported a highly significant difference in IL-6 levels between active TB and healthy controls (P< 0.0001), with no statistically significant difference observed between active and MDR-TB cases (P= 0.2209). These findings are agreed with studies by Correia *et al*, 2009. The robust increase observed in this study indicates that IL-6 contributes to the inflammatory activity in TB patients, in accordance with its pro-inflammatory potential in experimental models of acute infection (Poveda *et al*, 1999

**Table (4):** Comparison of IL-4 and IL-6 expression levels in control group, sensitive TB and MDR-TB patients

| Parameter | Groups | N | Mean | Std. Error | P-value |
|---|---|---|---|---|---|
| IL-4 (Pg/ml) | Control | 24 | 105.358 | 5.543 | 0.0001* |
| | G1 | 12 | 1054.763 | 71.482 | |
| | G2 | 12 | 1091.967 | 108.793 | 0.388 |
| IL-6 (Pg/ml) | Control | 24 | 9.253 | 0.456 | 0.0001* |
| | G1 | 12 | 42.458 | 1.809 | |
| | G2 | 12 | 38.585 | 4.601 | 0.2209 |

**GI:** patients with sensitive TB
**G2**: patients with MDR-TB
P-value ≥0.05

### 3.3 .Correlation between gene expression levels and interleukins levels:

In tables (5) and (6) the correlation analyses has been done to investigated the relationship between gene expression levels and cytokines levels in the study population. The results showed a


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significant weak negative correlation between the expression levels of *fbpA* and the levels of interleukins 4 in MDR-TB patients (r = -0.375). While in drug-susceptible TB patients, *fbpA* vs. IL-4 was (r = -0.165). The study showed a positive relationship between *fbpA* gene expression levels and IL-6 levels in both groups, in MDR-TB patients ( r = 0.514). In drug-susceptible patients *fbpA* vs. IL-6 (r = 0.46)(P< 0.05). It was shown that virulence attenuation in mouse models was induced by disruption of the *fbpA* gene in MTB, indicating the essential role of *fbpA* in pathogenicity. A strong immune response was also elicited by the *ΔfbpA* mutant in vaccinated mice, which is consistent with the potent immunogenicity and vaccinogenicity of Ag85 as a complex proteins. The alterations in cytokine levels in infected mice were observed, with increased levels of IFN-γ, IL-6, and TNF-α in mice infected with the ΔfbpA mutant. This suggests that the host immune response modulated by *fbpA*, possibly by suppressing the production of pro-inflammatory cytokines (Peeridogaheh *et al*, 2019; Mukhopadhyay *et al*, 2012), This is consistent with previous studies showing that Ag85 complex proteins can modulate the host immune response in various ways, including by inhibiting the production of cytokines and chemokines (Layre, 2020). Further studies are needed to fully understand the interactions between *fbpA* and the host immune system in TB pathogenesis.

The results of expression levels of *pknF* showed no significant differences in MDR-TB vs. IL-4 (r= 0.088) and drug-susceptible TB patients vs. IL-4 (r= -0.380). The study also showed no significant differences in MDR-TB vs. IL-4 (r = 0.110). And in drug-susceptible patients *pknF* vs. IL-6 (r = 0.1809) (P>0.05).

*pknF*, a serine/threonine kinase, is a critical player in the pathogenicity of MTB (Pal *et al*, 2022). It is involved in the regulation of cell wall biosynthesis and has been shown to interact with *Rv1747*, an ABC-transporter protein, in a phosphorylation-dependent manner (Hui, 2021). The absence of *Rv1747* results in a reduced growth rate in macrophages, highlighting its significance for the normal multiplication phase of the bacterium within these hosts (Li, 2021). Bonne Køhler *et al.*(2020) showed that *PknF's* involvement in the pathogenicity of MTB extends beyond its role in regulating cell wall biosynthesis. The kinase also interacts with other proteins that are critical for the growth and survival of the bacterium within the host. Also Narayan *et al.*(2007) reported that the *pknF* is one of the STPKs plays important roles in regulating various cellular processes such as stress response, cell cycle regulation, and development. They have been revealed to be a vital virulence factors in various pathogenic bacteria, including mycobacteria. These findings suggest that there may be a weak relationship between the expression of specific genes and the levels of pro-inflammatory and inhibitory cytokines in TB patients.

**Table(5):Correlation between *PknF* gene and IL- 4 and IL- 6**

| No | ***pknF DS-TB*** | **IL-4 DS-TB** | **IL-6 DS-TB** | ***pknF* MDR** | **IL-4 MDR** | **IL-6 MDR** | **Correlation (r) DS-TB** |
|---|---|---|---|---|---|---|---|
| **1** | 0.75786 | 1442.14 | 44.29 | 0.21764 | 1503.57 | 36.5 | Il-4 -0.38 |
| **2** | 3.03143 | 950.71 | 50.83 | 4.59479 | 893.57 | 53.57 | Il-6 0.18 |
| **3** | 0.75786 | 977.86 | 43.83 | 1.1487 | 861.43 | 49.92 | |
| **4** | 3.03143 | 957.86 | 40.92 | 4.59479 | 861.43 | 48.96 | |
| **5** | 0.20306 | 977.86 | 38.08 | 0.30779 | 1539.29 | 46.70 | **Correlation (r) MDR TB** |
| **6** | 3.03143 | 978.57 | 48.88 | 4.59479 | 1785.467 | 44.63 | |
| **7** | 0.20306 | 932.86 | 49.46 | 0.30779 | 789.14 | 11.08 | IL-4 0.088 |
| **8** | 3.03143 | 978.57 | 41.54 | 4.59479 | 827.14 | 17.43 | Il-6 0.21 |
| **9** | 0.25 | 878.57 | 39.25 | 0.37893 | 798.57 | 49.92 | |
| **10** | 3.03143 | 954.29 | 30.67 | 4.59479 | 1539.29 | 53.57 | |
| **11** | 0.25 | 1695 | 34.17 | 0.37893 | 811.14 | 44.54 | |
| **12** | 3.03143 | 932.86 | 47.58 | 4.59479 | 893.57 | 50.79 | |


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**Table(6):**Correlation between *fbpA* gene and IL- 4 and IL- 6

| No | ***fbpA DS-TB*** | **IL-4 DS-TB** | **IL-6 DS-TB** | ***fbpA* MDR** | **IL-4 MDR** | **IL-6 MDR** | **Correlation (r) DS-TB** |
|---|---|---|---|---|---|---|---|
| **1** | 1.1487 | 1442.14 | 44.29 | 0.65975 | 1503.57 | 36.5 | IL-4= -0.16 |
| **2** | 1.23114 | 950.71 | 50.83 | 3.73213 | 893.57 | 53.57 | IL-6= 0.46 |
| **3** | 1.07177 | 977.86 | 43.83 | 3.24901 | 861.43 | 49.92 | |
| **4** | 1.1487 | 957.86 | 40.92 | 3.4822 | 861.43 | 48.96 | |
| **5** | 1 | 977.86 | 38.08 | 3.03143 | 1539.29 | 46.70 | **Correlation (r)** |
| **6** | 1.07177 | 978.57 | 48.88 | 1.1487 | 1785.467 | 44.63 | **MDR TB** |
| **7** | 0.93303 | 932.86 | 49.46 | 2.82843 | 789.14 | 11.08 | IL-4= -0.37 |
| **8** | 1 | 978.57 | 41.54 | 0.43528 | 827.14 | 17.43 | Il-6= 0.514 |
| **9** | 0.87055 | 878.57 | 39.25 | 2.63902 | 798.57 | 49.92 | |
| **10** | 0.93303 | 954.29 | 30.67 | 2.82843 | 1539.29 | 53.57 | |
| **11** | 0.81225 | 1695 | 34.17 | 2.46229 | 811.14 | 44.54 | |
| **12** | 0.87055 | 932.86 | 47.58 | 4.59479 | 893.57 | 50.79 | |

## 4. Conclusions:

In conclusion, The study found significant differences in gene expression levels of *FbpA* and non-significant levels in pknF. Cytokine levels of interleukins (4 and 6) were statistically significant between control group compared to sensitive TB and MDR-TB patients. However, there wasn't a significant difference between sensitive TB and MDR-TB. These findings provide further evidence for the complex mechanisms underlying TB pathogenesis, also have important implications for future research and clinical practice. Further research is required to fully comprehend the strategies of the role of specific genes and cytokines implicated in this process.

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