Original Article

Study of Immunological and Molecular Analysis of Virulence Genes in *Mycobacterium tuberculosis* Isolates from Hilla Teaching Hospital

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Abstract

Background: Despite the widespread use of a live attenuated vaccine and many medications, tuberculosis (TB), remains one of the top killers among infectious diseases. **Objectives:** The objectives of the study were to assess the immunological and molecular aspects of certain virulence genes among *Mycobacterium tuberculosis.* **Materials and Methods:** A total of 155 infected patients and 21 healthy control patients with suspected respiratory tract infections were enrolled from June 2022 to January 2023 in the Chest Unit at Hilla Teaching Hospital. **Results:** Of the 150 sputum samples taken, 93 were culture positive (62%), *Mycobacterium tuberculosis* was responsible for 35(37.6%) of the positive culture isolates, results showed that out of 35 isolates initially diagnosed as *Mycobacterium* by the Vitek II method, only 20 (57.1%) tested positive by PCR. Only 18 isolates tested positive for the presence of the virulence-related *inhA* gene; 16(80%) of them tested positive for the *katG* gene, and 14 (70%) of them tested positive for the *rpoB* gene. Determination of TNF-α, TNF-β, IL-12, and IL-17 in 20 *M. tuberculosis* isolates was investigated, and the results showed that the highest mean of TNF-α level was found among patients with RTI infected with *M. tuberculosis* compared with the control group showed a significant difference between the two groups ($P < 0.0001$). TNF-β, IL-12, and IL-17 levels were found among patients with RTI infected with *M. tuberculosis* compared with the control group. **Conclusion:** Compared to conventional biochemical tests and the automated Vitek 2 system, the identification of *M. tuberculosis* by employing a particular primer gene, was more specific. There were identified virulence genes that were found to be crucial to pathogenicity.

Keywords: *16sRNA*, *katG*, *Mycobacterium tuberculosis*, PCR, *rpoB*

Introduction

Mycobacterium tuberculosis causes TB disease. It spreads through the air with coughs and sneeze. Prevention and treatment are crucial, to ease. Despite the widespread use of a live attenuated vaccine and many medications, tuberculosis (TB), one of the oldest recorded human ailments, remains one of the top killers among infectious diseases.[1]

It is an airborne disease, meaning it spreads through the air when an infected person coughs or sneezes, and another person inhales the bacteria. Once the bacteria enter the body, they can travel to the lungs and settle there, causing an infection. The immune system of the infected person tries to fight off the bacteria, leading to inflammation and the formation of small nodules or tubercles in the lungs. If the infection is not treated, the tubercles can grow and merge, leading to the destruction of lung tissue and the formation of cavities in the lungs. In addition to the lungs, *M. tuberculosis* can also infect other parts of the body, such as the brain, spine, kidneys, and lymph nodes.

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When the bacteria spread to these other parts of the body, it is called extrapulmonary tuberculosis. The symptoms of tuberculosis can vary depending on the severity of the infection and the part of the body, that is, affected. Common symptoms of pulmonary tuberculosis include a persistent cough, chest pain, coughing up blood, fatigue, fever, night sweats, and weight loss. In cases of extrapulmonary tuberculosis, the symptoms can vary depending on which part of the body is affected. Treatment for tuberculosis usually involves a combination of antibiotics taken for several months. The antibiotics work to kill the bacteria and prevent the spread of the infection. It is important to complete the full course of treatment, even if symptoms improve, to prevent the development of drug-resistant strains of the bacteria. Prevention of tuberculosis involves several measures, including vaccination with the Bacillus Calmette-Guerin (BCG) vaccine, which can provide some protection against the disease. Other prevention measures include reducing exposure to infected individuals, such as by wearing a mask or isolating individuals with active tuberculosis.

The global TB epidemic, which claims the lives of two million people annually, can only be stopped with the development of new vaccinations and treatments. Researching the genetics and physiology of *M. tuberculosis* and similar mycobacteria is crucial for the rational development of novel antitubercular medicines.[2] Exploring the *M. tuberculosis*-host interaction is crucial for learning the pathogen's mechanisms of evading the host's defenses and causing disease.[3] Several of these genes and the proteins they encode, together with any future ones that are identified, should give novel bacterial targets for use in the development of vaccines, medicines, and more selective diagnostic reagents in the future.[4] Classical virulence factors, such as those generated by *Corynebacterium diphtheriae*, *Escherichia coli* O157:H7, *Shigella dysenteriae*, or *Vibrio cholerae*, are the main causes of disease caused by other bacterial pathogens, whereas M. tuberculosis lacks these.[5] There are over 4000 genes in the 4.4 106 kb *M. tuberculosis* H37Rv genome. The genomic annotation of *M. tuberculosis* reveals distinctive characteristics of this bacterium.[6] There are around 200 genes that have been identified as encoding enzymes involved in fatty acid metabolism, which accounts for about 6% of all genes. There are about 100 of these enzymes that are believed to be involved in the oxidation of fatty acids, whereas *E. coli* only contains about 50 enzymes engaged in fatty acid metabolism. The capacity of *M. tuberculosis* to develop in the tissues of the infected host, where fatty acids may be the predominant carbon source, may be related to the high number of enzymes that putatively utilize fatty acids. An integral part of *M. tuberculosis* physiology during infection.[7] The development of *M. tuberculosis* triggers inflammatory host responses that are vital for infection management but can have devastating effects on the body's tissues.[8] Proteases like cathepsin D are expected to play a significant role in granuloma lysis and are among the many cellular agents engaged in tissue degradation. Moreover, absorption of *M. tuberculosis* might result in macrophage death, which may contribute to harm to the surrounding tissue.[9] Tumor necrosis factor beta (TNF-α) is a critical cytokine in the inflammatory or Th-1 response of the cellular immune system, which is required for infection management.[10] An aerosol model of mouse infection shows that when this cytokine is present in high concentrations, significant pulmonary inflammation and premature death result in the mice. The levels of TNF-α in the cerebrospinal fluid are directly correlated with the severity of disease induced by numerous *M. bovis* and *M. tuberculosis* strains, suggesting that TNF-α is a primary predictor of disease in TB meningitis.[11] Studies of cytokine responses and virulence in patients infected with different M. tuberculosis strains, however, show that TNF-α is not the only determinant in TB development.[12]

Materials and Methods

Patients and sample collection

At Hilla Teaching Hospitals chest unit, who were suspected of having respiratory tract infections that were enrolled in the study between July 2022 and March 2023; the total sample size was 155 infected patients and 21 healthy control patients. The ages of our patients covered the gamut from 25 to 70. Data on the severity of the cough, the color of the sputum, and the temperature were also collected.

Sputum specimens were obtained in advance of the antibiotic treatment (two replicates in 2 days for all patients). Morning sputum was collected and placed in sterile receptacles.[13] For blood sampling, blood was drawn from every single patient, both infected and uninfected. The aseptic heart puncture blood draw used a disposable syringe to collect 5 mL of blood. After letting it coagulate at room temperature for 5 min, it was centrifuged at 3000 rpm to remove the clot. After removing the serum from the vial, it was stored at a temperature of -25° C until needed.[14] Ziehl-Neelsen stain method was performed according to.[15]

Each specimen was subjected to quantitative sputum culture based on sputum Gram stain for *M. tuberculosis* infections. Using a vortex mixer, sputum specimens were mixed with the same volume of normal saline until they were completely uniform. Gentamicin and blood agar using a sterile swab, 0.1 mL of homogenized specimen was dispersed across blood agar plates as an inoculum. Overnight, plates were placed in a 5-10% CO_2 incubator.[16]

Mycobacterium tuberculosis identification

Mycobacterium tuberculosis identifications according to morphology staining, culture characteristics, and biochemical reactions.[15] The identification of *M. tuberculosis* was confirmed by Vitek II System

DNA extraction

This procedure was developed using components from a genomic DNA purification Kit, as supplied by the company that makes genomic (USA). Macrogene Corporation of Korea supplied the primer [Table 1].

Cytokines detection

The tests were designed to determine how much interleukin (TNF-α, TNF-β, IL-12, and IL-17) was present in the serum of healthy and infected individuals. This is a simple sandwich ELISA experiment (Enzyme-Linked Immunosorbent Assay).

Statistical analysis

For this statistical work, we used SPSS 25. Categorical variables were represented by frequencies and percentages. Statistics on continuous variables were displayed as (means SD). A Student *t* test was performed to compare the two groups' mean scores. *P* value under 0.05 was considered statistically significant. It has been shown that.[17]

Ethical approval

Ethical consent from the hospital's ethical review board, patients, and their families is required. In addition, all participants are verbally informed, and consent for testing and publication of results is obtained before samples are collected.

Results

A total of 150 infected patients and 21 healthy control patients with a suspected respiratory tract infection were enrolled from June 2022 to January 2023 in the chest unit at Hilla Teaching Hospital. Figure 1 shows that 93 (62%) of the 150 sputum samples were positive for culture, while only 57 (38%) tested negative.

Table 1: Detection of *16sRNS*, *inhA*, *katG*, and *rpoB* genes in *Mycobacterium tuberculosis*

Genes	Primer sequence (5′- 3′)	Size BP	Condition	References
16sRNA specific primer	F: ACCAACGATGGTGTGTGTCAT R: GGCAAGGTCACCCCGAAGGG	549	94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with the last cycle concluding with 72°C for 5 min and 4°C for 5 min.	Domenech *et al.*[17]
inhA	F: GTCACACCGACAAACGTCAC R: TCGCTGTCGGTGACGTCA	810	94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with the last cycle concluding with 72°C for 5 min and 4°C for 5 min.	Domenech *et al.*[17]
katG	F: AT CTGGAGAACCCGCTGGC R: ACCCATGTCTCGGTGGATCAG	580	94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with the last cycle concluding with 72°C for 5 min and 4°C for 5 min.	Domenech *et al.*[17]
rpoB	F: TGGTCCG CTTGCACGAGGGTCAGA R: CTCAGGGGTTTCGATCGGGCACAT	280	94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with the last cycle concluding with 72°C for 5 min and 4°C for 5 min.	Domenech *et al.*[17]

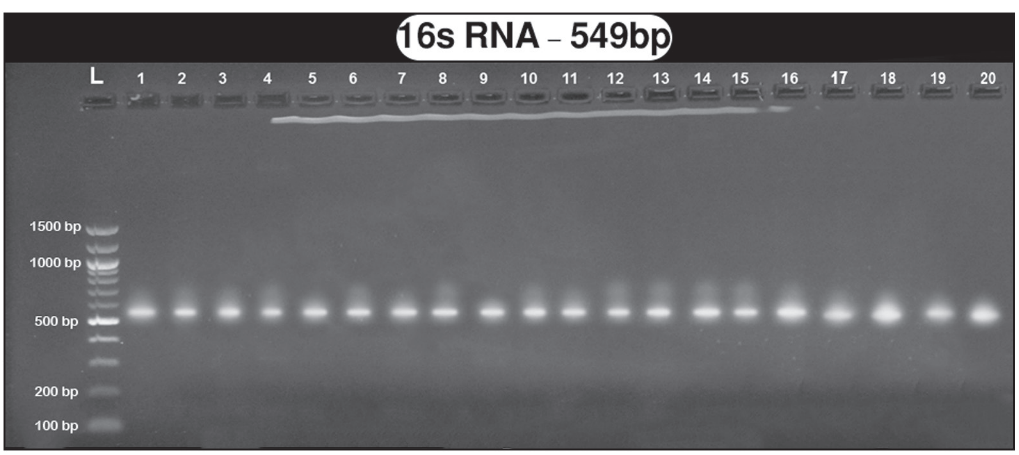

Figure 1: Agarose gel electrophoresis (1.5%) of PCR amplified of *16sRNA* specific primer gene at (548 bp) of *Mycobacterium tuberculosis* for (55) min at 5 V/cm^2. 1× TBE buffer for 1:30 h. L: DNA ladder (100)

Table 2: Determination of cytokines in *Mycobacterium tuberculosis*

Parameter	Sample	N	Mean ± S.E	P value
TNF-α (pg/mL)	Patients	20	620.53 ± 18.455	0.0001
	Control	20	280.69 ± 108.80	
TNF-β (pg/mL)	Patients	20	630.32 ± 25.150	0.0001
	Control	20	230.13 ± 20.818	
IL-12 (pg/mL)	Patients	20	690.82 ± 34.669	0.0001
	Control	20	249.22 ± 6.2316	
IL-17 (pg/mL)	Patients	20	650.13 ± 34.460	0.0001
	Control	20	220.24 ± 12.078	

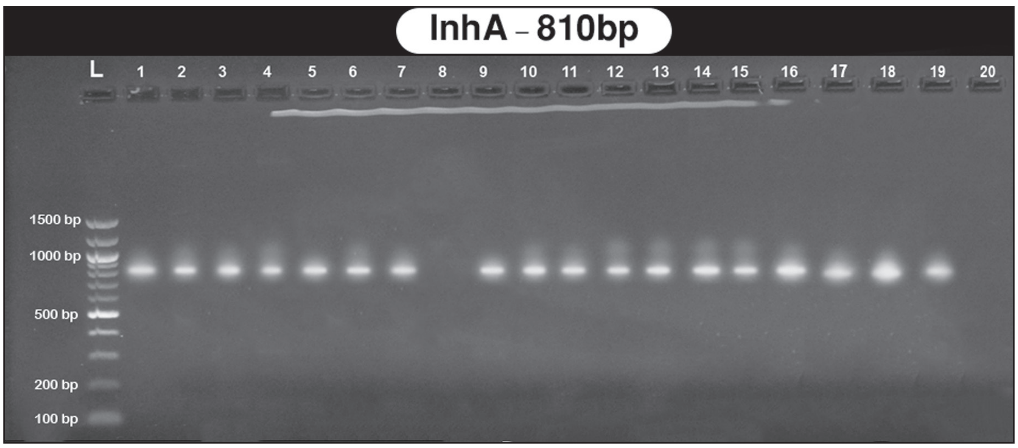

Figure 2: Agarose gel electrophoresis (1.5%) of PCR amplified of *inhA* gene at 810 bp of *Mycobacterium tuberculosis* for (55) min at 5 V/cm^2. 1× TBE buffer for 1:30 h. L: DNA ladder (100).

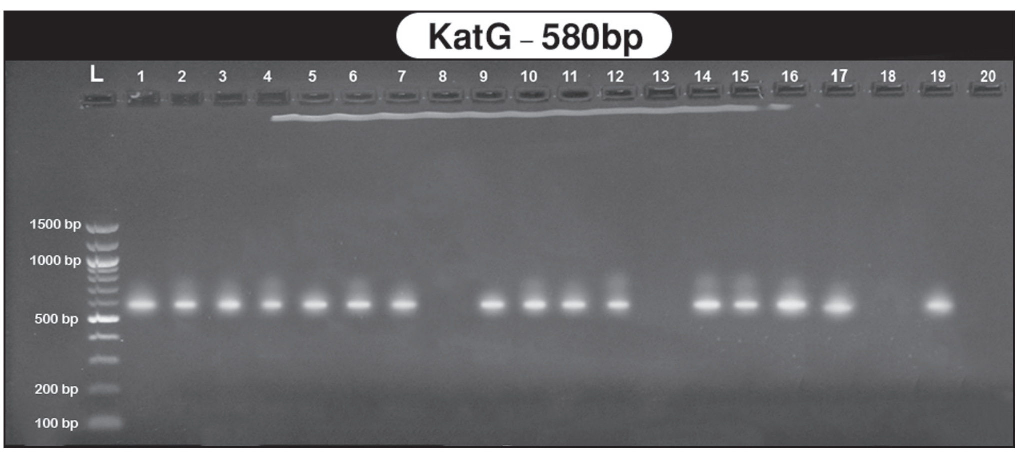

Figure 3: Agarose gel electrophoresis (1.5%) of PCR amplified of *pspC* gene at 580bp of *Mycobacterium tuberculosis* for (55) min at 5 V/cm^2. 1× TBE buffer for 1:30 h. L: DNA ladder (100)

From a total of 93 positive culture isolates, 35(36.6%) were linked to *M. tuberculosis* and 58 (62.4%) were linked to other types of bacteria, as indicated in Table 2. To verify that the isolates were actually *M. tuberculosis*, it was used an automated Vitek 2 system equipped with GN-IP cards containing 64 biochemical tests. All 35 (100%) isolates had their identities confirmed with ID messages scoring in the excellent range of confidence (probability percentages from 94% to 99.7%).

Out of 35 isolates initially diagnosed as *Mycobacterium* TB using the Vitek II system, only 20 (57.1%) were found to be positive for the presence of these bacteria when tested with the PCR approach utilized in this investigation, which relied on a *16sRNA* specific primer gene, PCR products had a molecular length of (549) bp as shown in Figure 1.

Twenty strains of *Mycobacterium* TB were studied for the presence of the virulence-related *inhA* gene. Just 18

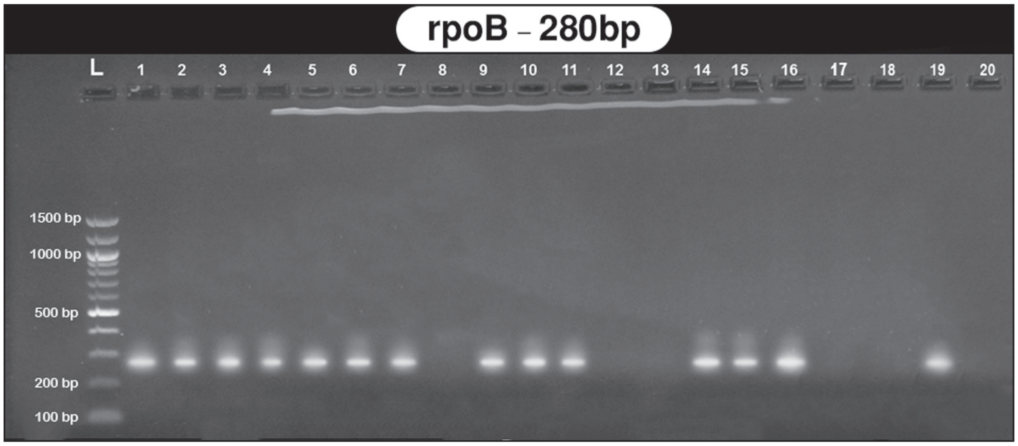

Figure 4: Agarose gel electrophoresis (1.5%) of PCR amplified of *rpoB* gene at 280 bp of *Mycobacterium tuberculosis* for 55 min at 5 V/cm^2. 1× TBE buffer for 1:30 h. L: DNA ladder (100)

(90%) of 20 samples were positive for this gene, and their molecular weight was (810) bp [Figure 2].

The virulence gene *katG* is produced as a consequence. The amplified positive samples were matched to an allelic ladder, and the PCR results were regarded as a match (580bp). There were 20 different *Mycobacterium* TB isolates tested, and as can be shown in Figures 3, 16(80%) of them tested positive for the *katG* gene.

Fourteen (70%) of 20 *Mycobacterium* TB isolates tested positive for the presence of the *rpoB* gene. Gel electrophoresis was utilized to find the (280 bp) amplicon, and the findings were compared to an allelic ladder [Figure 4].

In this study, the determination of TNF-α, TNF-β, IL-12, and IL-17 in 20 *M. tuberculosis* isolates was investigated, and the results showed that, the highest mean of TNF-α level was found among patients with RTI infected with *M. tuberculosis* (620.53 ± 18.455 ng/mL) comparing with the control group (280.69 ± 108.80 ng/mL) with a significant difference between the two groups ($P < 0.0001$), TNF-β level was found among patients with RTI infected with *M. tuberculosis* (630.32 ± 25.150 ng/mL) compared with the control group (230.13 ± 20.818 ng/mL) with a significant difference between the two groups ($P < 0.0001$), IL-12 level was found among patients with RTI infected with *M. tuberculosis* (690.82 ± 34.669 ng/mL) compared with the control group (249.22 ± 6.2316 ng/mL) with a significant difference between the two groups ($P < 0.0001$) and IL-17 level was found among patients with RTI infected with *M. tuberculosis* (650.13 ± 34.460 ng/mL) compared with the control group (220.24 ± 12.078 ng/mL) with a significant difference between the two groups ($P < 0.0001$), all results are shown in Table 2.

Discussion

In 37.6% of cases, *M. tuberculosis* was isolated. These results are consistent with research by[18], in which *M. tuberculosis* was isolated from 38% of patients with RTIs. Understanding the pathophysiology of tuberculosis requires the identification of *M. tuberculosis* virulence determinants that play a role in human disease.[19] One of the virulent *M. tuberculosis* genes may have a role in the bacteria's ability to survive inside human macrophages, according to recent research.[20] The disease's onset is caused by the inhalation of aerosol particles containing *M. tuberculosis*.[21] To comprehend tuberculosis pathogenesis, it is essential to characterize virulence determinants of *M. tuberculosis* that is applicable to human diseases. A virulent *M. tuberculosis* gene may have a role in the bacteria's ability to survive inside human macrophages, according to a recent study.[20] For mycobacteria, the ability to cause disease, or virulence, is contingent on their capacity to invade host cells and thwart the microbicidal activities of macrophages. Due to the close relationship between the *Mycobacterium* genre and humans, the mycobacterial genome appears to encode bacterial factors that reflect a highly evolved and coordinated program of immune evasion strategies that interfere with both innate and adaptive immunity, causing disease even in fully immunocompetent host.

Several genes related to virulence and genes involved in the virulent lifestyle of *M. tuberculosis*.[9] The inflammatory response and the final result of mycobacterial infections are heavily influenced by the cytokine network.[22] Protective immunity and pathogenesis against tuberculosis are both greatly aided by TNF-α. To confine a mycobacterial infection, granuloma development is crucial, and it works synergistically with gamma interferon to boost nitric oxide metabolite synthesis and aid in mycobacterial death. The levels of cytokines in bronchoalveolar lavage fluid specimens from individuals with pulmonary tuberculosis were higher than those with less severe illness.[23] Cytokine analysis of blood has been recommended in another study as a means of differentiating between people with active tuberculosis and healthy controls. The increased reactivation of tuberculosis (including miliary and extrapulmonary disease) in patients with Crohn's disease and rheumatoid arthritis following therapy with monoclonal anti-TNF-α antibodies demonstrates the importance of TNF-α in regulating bacilli in the latent stage.[24] Both IL-12 and IL-17 activities were observed. The presence of IL-17 causes an increase in IL-12 production, which may be an immunological mechanism for dampening Th-1 reactions. To cause their typically severe form of sickness, they may block an intrinsic mechanism.[25,26]

Conclusion

Compared to conventional biochemical tests and the automated Vitek 2 system, the identification of *M. tuberculosis* by employing a particular primer gene was more specific. There were identified virulence genes that were found to be crucial to pathogenicity.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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