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# Epidemiology and Detection of Virulent Genes of *Mycobacterium tuberculosis* in India

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**ABSTRACT**

With its capacity to remain dormant inside the host, *Mycobacterium tuberculosis* (MTB) causes the exceptional illness known as tuberculosis (TB), which has been associated with significant mortality and morbidity for decades. Pulmonary tuberculosis (PTB) is caused by the pathogen infecting the lungs' alveoli, which initiates the condition.Currently, TB ranks within the top ten global deaths. In 2019, 7 million people will be infected with TB worldwide (5,946,816) cases. Karnataka (64,346), one of the top 13 states with the highest TB burden in India, continues to have a significant problem with unfavorable conditions like PTB, which are caused by the original infection in the lungs. Furthermore, urban areas of the state, like Mysuru district (2,544), are among those with a high PTB burden. While TB primarily affects the lungs, it can manifest oral symptoms like ulcerations and swollen lymph nodes, albeit less commonly. Moreover, medications used in TB treatment can have side effects affecting oral health, such as dry mouth and oral candidiasis. Immune suppression resulting from TB can increase susceptibility to oral infections, while poor oral health can potentially exacerbate TB symptoms. Additionally, there's a theoretical risk of TB transmission through saliva if oral lesions are present, though respiratory transmission is more common **Objectives:** The PTB epidemiology in Mysuru district was the region focused in the study. **Methods:** For the detection spatial distribution of the infection using GIS and molecular analysis for detection of virulence genes. The regions with the highest incidence and hotspots were targeted. **Results:** The hotspots zones show the unpredictability of TB transmission in the Mysuru region. A global incidence-based spatial analysis from 2011 to 2019 identified possible TB transmission hotspots and their changes based on the epidemiology study. The Mysuru metropolitan region was found to have the most PTB cases and comes in first among districts with a lot of cases. The staining quantification scale was used to choose the samples, which were then decontaminated and digested. The samples were subjected to direct MTB DNA isolation & PCR amplification. With the help of specific primers and a modified PCR procedure, the dangerous genes fadD33, MmpL10, and WhiB3 were amplified. **Conclusion:** The DNA isolated from the sample's amplified virulent genes help identify the spread of virulent strains. In some regions with a high incidence of PTB, these findings could be utilized to assess the incidence, dissemination, and circulating virulent strains in society.

**Keywords:** Mycobacterium tuberculosis, Incidence, Cause of Death, Decontamination, Geographic Information Systems, Virulence, India, Tuberculosis, Pulmonary, Spatial Analysis, Digestion, Staining and Labeling, Lung, DNA, Polymerase Chain Reaction

## INTRODUCTION

*Mycobacterium tuberculosis* (MTB) and humans have had host-pathogen connection for 50,000 to 70,000 years [1]. MTB uses virulence factors to overcome host immunity as it enters the human host by an aerosol and establishes an infection in the lung. The knowledge of the pathophysiology of MTB has advanced significantly during the past few decades. The mechanisms behind MTB virulence, however, are still largely unclear. Although there is an 85% success rate for treating non-drug resistant pulmonary infections linked to active TB, the management of TB in endemic countries is challenging due to a dearth of suitable diagnostic equipment. Current techniques include smear microscopy, sputum culture, nucleic acid amplification tests (NAATs), and others. Sputum culture is the gold standard diagnostic procedure for PTB, but it can take up to two weeks to yield a definitive result. The high costs of NAATS, which have nearly similar sensitivity to sputum culture, prevent them from being used more widely in impoverished countries, where TB is most prevalent [2,3].

Approximately 33% of individuals worldwide are at risk of contracting the disease due to the current TB epidemic. Most TB cases were discovered in the most affected areas of 11 south-east Asian nations (44%), Africa (24%), and 37 western Pacific countries. Most cases of TB were found in India (27%), followed by eight other nations, including China (9%), Indonesia (8%), Philippines (6%), Pakistan (6%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) and least cases were found in 22 countries of the eastern Mediterranean (8%), America (3%) and 44 countries of Europe region (3%) [4]. Among other nations, Russia, China, and India all have high TB burdens. Despite the use of anti-TB medications, around 9 million people worldwide develop active cases

of PTB each year, consequently, it is one of the more prevalent infectious lung illnesses in people and a major worldwide health issue. [5].

For the most accurate diagnosis and the best course of treatment, the percentage of PTB cases that have been reported must be monitored. Adults with sputum smear positive cases of TB were found to have more than 80%, which is highly contagious. Within 10 years of diagnosis, patients with PTB that have been bacteriologically proven have a high mortality rate of 70% [6]. Patients are more likely to acquire MDR-TB (Multi drug resistant TB). If PTB is not promptly recognised and treated the infection is more likely to get adverse conditions like EPTB, MDR-TB and XDR-TB. The demanding nature of MDR patients' treatment plans makes it important to concentrate on reducing the risk of MDR strains in order to stop the spread. Therefore, adequate therapy with consistent follow-up, early detection and identification of PTB patients who have positive bacterial smear results may lower the likelihood of MDR-TB and XDR-TB spreading [7-9].

Many developing nations have been able to stop the spread of TB by combining efficient prevention techniques with social and economic growth. As a significant public health concern, TB eradication is a priority for many low- and middle-income nations. However, India has the largest burden of TB in the world with 27% of estimated incident cases. The World Health Organisation has approved the Directly Observed Therapy, Short Course (DOTS), which was first implemented in India in 1997 as a component of the National TB Elimination Programme (NTEP), formerly known as the Revised National TB Control Programme (RNTCP).These programme has assumed the initiative in national TB control. Although incidence and prevalence are the two most important components in an epidemiological survey, the majority of data on TB incidence comes from brief studies and yearly reports from the central tuberculosis division. But many

regions and nations have not yet conducted TB prevalence studies [10] which may lack in the knowledge of the region with TB spread.

With 83,094 TB cases and 54% of PTB cases, Karnataka in India's southern west is one of the regions with the highest reported rates of TB. The state holds Bangalore City at the highest level in the state's differential ranking of the top 10 TB cases recorded each year. In terms of the total number of reports, the Mysuru district ranks fifth. Different risk factors and other determinants, such as high risk populations (geriatrics and men), undernutrition, unsanitary living conditions, indoor air pollution, irregular medication intake, etc., may be the cause of the infection's spread in the area [11,12].

In the Karnataka state province, Mysuru city is one of the districts that include a variety of urban and rural settings, as well as residential, commercial, and industrial zones. According to Anderson et al. (2014), the district contains an estimated 1.6 million residents and a mild climate that enables farming, business, and information technology as the mainstays. The city gradually underwent expansion and commercialization, as well as a decline in agriculture and mountain areas that lowered the quality of the surrounding air. As a result, there is a positive correlation with regional growth and the spread of PTB. The mode of PTB spread in the regions can be known by new technology for the epidemiological aspects [13-15]. One such intervention study is the Geographical Information System (GIS), which captures, manipulates, collects, interprets, organizes and displays all types of geographic data. Based on the interpolation method, the interpolated location the TB cases are discovered with taluk wise variability.

**Detection of virulent gene and its agent**

The MTB genome consists of 4,000 genes and 4 million base pairs [16]. About 50% of these genes are still classified as unknown, uncharacterized, or having putative activities, however, per [17].

TB is still one of the main causes of death worldwide despite a variety of variables. The inability of MTB to persist in human tissue in a non-replicating state untouched by anti-mycobacterial medications and cause disease [18]. Some of the genes are crucial for MTB's virulence, which determines its capacity for host survival.

Poly glycolipid PGL production is possible in some MTB species [16]. PGLs, lipids, and related PDIMs are formed by an enzyme that is comparable to polyketide synthase (pks). The metabolic process carried out by the pks 1-15 genes results in the production or transportation of pthiocerol dimycocerosate (PDIM). The methylmalonyl coenzyme A-containing pks-like enzyme extends to this compound, which is crucial, particularly for pathogenicity. According to a number of recent studies, FadD26 had been thought to be an acyl coenzyme A (acyl-CoA) Synthetase involved in the degradation of fatty acids. Phthiocerol must be biosynthesized using a polyketide synthase. One of the MTB genome's genes, fadD33 (also known as Rv 1345) involved in lipid synthesis, is comparable to the fadD gene in E. coli [19]. The initial stage of fatty acid breakdown is the conversion of free fatty acids to acyl-CoA thioesters by the acyl-CoA synthase enzyme in E. coli [20,21]. An acyl-CoA synthase is also encoded by the fadD33 gene.

Additionally, the complex molecule synthesize, a crucial but least studied gene in the MmpL a transport protein, as well as one of the mutant phenotypes and its inability to transport PMID all belong to a large community [22,23]. There are 13 MmpL-coding genes in the MTB genome. Genes for polyketide biosynthesis (pks gene) co-localize with large mycobacterial membrane proteins [19]. These proteins take part in the transport of complex lipids because they contain lipid breakdown genes (papA, fadD) in MTB [24,25]. MmpL is a part of the PDIM transport process [25, 23], which, by providing a molecule precursor, is also necessary for the production of sulfolipid [27,28] and the MmpL proteins also mediate in MTB drug [29].

The persistence or latent stage of MTB is believed to be comparable to bacterial sporulation according to the WhiB family of MTB transcription regulators, which interacts with the mycobacterial sigma factor and is assumed to be necessary for the transcription of most mycobacterial housekeeping genes. Members of the WhiB family identify and respond to the pathogenicity-related host genes NO and $O_2$ using their iron-sulfur (Fe-S) clusters. Redox homeostasis in MTB, the existence of Whi homologues in the dsDNA siphoviridae bacteriophage family, and horizontal gene transfer from this family, which also contains Whi homologues, all contribute to the explanation of the mechanism of action of Whi members.

Due to the irregular spread of strains with virulent genes, there is a risk of significant spread among PTB patients in the region. Based on the idea that MTB strains altered their genomes as necessary to adapt to their changing settings, leading to the pathogen's genetic diversity with VFs, the molecular study was conducted. The Mysuru district underwent an epidemiological examination of PTB transmission by MTB strains with virulent genes. The identification of virulent genes in mycobacterial stains can be useful for both the discovery of rearrangements in medication combinations against pathogenicity and the molecular elucidation of disease processes.

**MATERIALS AND METHODS**

*Study area:* Karnataka's southernmost district, Mysuru, is situated at coordinates 12.2958° N and 76.6394° E. The city has residential, commercial, and industrial zones as well as a variety of urban and rural environments. In 2019, the population of the Mysuru district was estimated at 10 lakh.

Because of the region's temperate temperature, which ranges from 17 to 37°C, the local economy is based primarily on farming, manufacturing, and data technology [12].

*Epidemiology study:* The number of TB cases in the Mysore district that have been recorded were counted using gis analysis [30]. With a view to discovering the PTB gene transfer pathogenicity in the Mysuru district. The sputum samples were collected between 2017 and 2020 at Princess Krishna Jammanni Sanatorium (PKTB), Mysuru. Based on an epidemiological survey, cases of TB patients from RNTCP in Mysuru were studied using GIS mapping. For the study, demographic information and the percentage of patients with both beneficial and detrimental instances were looked at. Additional data, such as location, gender, and age, were obtained and processed.

*Sample collection:* About 120 sputum positive samples were collected and processed. One of the ideal methods for the decontamination and digestion of sputum samples is NaOH/NALC (sodium hydroxide/Sodium citrate) method. The samples were treated with equal volume of 3% NaOH and 2.9% Na Citrate followed by fluorescent staining on the standard recording of AFBs according to Tuberculosis Laboratory Biosafety manual, WHO and the Mycobacteriology manual [31]. DNA isolation was processed by the CTAB/NaCl method with minor changes. MTB detection in the quantified samples: The samples were subjected for the PCR amplification with the primers F-5'-CAAGGCTTCAATTCCGGTGATGCC-3', R5'-TGGTCCGGTTCATACTCGGGCTGG-3'. The virulent genes viz., fadD33, MmpL10 & WhiB3 were targeted in the selected samples.

*Optimization of the PCR:* The optimal extraction method, quantities of primer pairs, deoxynucleotide triphosphates (dNTPs), and MgCl2 were considered for optimizing the process variables. A heavy suspension of MTB in phosphate buffered saline (PBS) was extracted and 10µl aliquots were subjected to 30 cycles of amplification with various concentrations of primers, in a 10x PCR buffer with 1.25 UTaq polymerase (Qiagen). A master mixture of 10x reaction buffer,

primers, dNTPs, MgCl2, and water was created when the ideal concentrations were identified. It was then divided into 25 l aliquots and refrigerated at -20°C until use. A cycle consisted of denaturation at 95°C for 5 min, primer annealing at 95°C for 45s, re annealing for 60 °C for 45s, 72°C for 45s and 72°C for 45s and final extension at 72°C for 8min. The products were gel electrophoresis on a 2% agarose with 123-bp DNA ladder (Thermo Fisher Scientific) marker.

## RESULT AND DISCUSSION

***Epidemiology of PTB in Mysuru*:** To determine the current state of the disease in the Mysuru district, the epidemiological features of PTB, including incidence, dissemination, and hotspots, were researched. If the most affected region is targeted, it may be possible to prevent the region with a history of TB from maintaining its position among the top 5 districts. Mysuru is one of the districts that is most impacted from 2011-16 based on the map. In the Mysuru district, 13,943 PTB cases in all (2011–18) were reported. With 5,172 (41%) PTB cases reported from 2011-18, the metropolitan Mysuru region was also determined to have the highest rate. The Mysuru district's regions with the highest reported PTB patients were chosen for the collection of sputum samples.

***Sample staining*:** the sputum samples from the hotspot regions were collected and subjected for further processing. The microscopic evaluation of the samples with Auramine rhodamine (AO) or fluorescent microscopy were conducted.

The samples were discovered to fall within the range of scales based on the standard scale (1+, 2+, & 3+). About 120 sputum positive samples were obtained based on the quantity and calibre of the material. We chose to stain only the samples that had mucoid and purulent characteristics. Under a microscope, the stained samples had the appearance of long, slender, slightly curved bacilli. The samples were also submitted to decontamination and digestion using the (N-acetyL-cysteine)

NALC/NaOH procedure, as well as molecular research, which was started by the isolation of MTB genomic DNA from sputum samples.

***Quantification of MTB DNA*:** One of the best techniques for the samples that were gathered. With certain adjustments, the decontaminated samples were submitted to genomic separation. Using a UV spectrophotometer, DNA concentration was estimated at 260 nm. Using electrophoresis on an 8% agarose gel, the purity of fragments of the obtained genomic DNA was evaluated. Utilizing electrophoresis and the mass ruler low range 1000k, the complete amount of DNA of the PCR result was estimated. The amplification settings were defined during the primer design process using the standard Tm condition with a 20ng DNA quantity.

The standard strain H37Rv strain was amplified as a positive control, and a single band of 285 bp DNA was found in the samples. The major virulence genes, WhiB3 (Redox and pH responsive transcriptional regulator subclass of Whi gene), FadD33 (fatty acid CoA ligase), and mmpL10 (Mycobacterial membrane protein large), were also found in the DNA samples isolated (Figure 1-3). The PCR master mix, primer, and targeted genes were used to complete 35 cycles at varying temperatures according to the selected gene (10ng).

Determining the amplification's sensitivity using genomic DNA extracted from sputum samples. The legend of Figure 1 provides a description of the PCR conditions.Using the reference strain H37Rv and a 1000k ladder, genomic DNA from three or more sputum samples is amplified in lanes A and B.

The initial batch of samples was used for standardization and amplification in order to find the pathogenic gene WhiB3 in MTB strains. For the Whi B3 gene, all samples had bands at the 100-bp marker and in the 246-bp region (Figure 1). The distribution of the pathogenic gene was discovered to be age-independent; the first set of samples consisted of patients aged 5-50 or older.

The first batch of isolates was tested for the pathogenic gene Mmpl in Mtb strains using amplification and standardization. For the Mmpl gene, all samples had bands at the 100-bp marker and in the 423-bp region (Figure 2). The distribution of the pathogenic gene was shown to be independent of patient age; the first set of samples included patients in the 5–50 age range.

The bands identified on the gel indicated that the DNA ladder from the samples was a positive virulent strain. In contrast to mmpL10 bands, which were only seen in runs 1 through 8 of samples, and FadD33 bands, which were only seen in run 5, WhiB3 bands were seen in all runs of samples (Figure.3). The majority of samples show that the virulent strain was spread throughout the PTB patients' localities. The control of the virulent gene in the cell metabolism makes the reference strain H37Rv virulent. One of the MTB complexes, a significant pathogen in PTB patients, was discovered to be MTB. This demonstrates that areas with PTB patients may be at risk in threat of EPTB, MDR-TB, and XDR-TB among these strains. The virulent genes and their families play a crucial function in lipid metabolism and are necessary for the survival of the viruses as trans-membrane proteins and regulatory proteins.

Virulent amplified genes in the isolates: To find out if the virulent genes were present, the samples were amplified. The reverse primer was amplified at 28°C to 30°C for 63 minutes at a temperature ranging from 56°C to 77°C for the forward primer. The initial batch of DNA samples included the MTB strain banding patterns. The pathogenic gene family was represented by the banding patterns of DNA isolates labeled 2–7 with negative control.

The initial batch of samples was used for standardization and amplification in order to find the pathogenic gene WhiB3 in MTB strains. All samples displayed bands for the WhiB3 gene at the 100-bp marker, as well as bands for the mmpL10 gene at 426-bp and the fadD33 gene at 412-bp

(Figure 1-3). The distribution of the pathogenic gene was discovered to be age-independent; the first set of samples consisted of patients aged 5-50 or older.

The chosen gene was found to be in the MTB strain, which is related to the H37Rv, however the virulent genes in each sample were unable to recognise the pathogenic strain in the samples that were acquired. Patients who were 15 years of age or older and had these band arrangements showed success. In the samples that were taken, older patients were seen to be affected by these virulent strain. The findings suggest that the selectively virulent genes may contain virulent elements that are crucial to the organism's survival and infection.

**Discussion**

We presented a generalized strategy for the PTB epidemiology in the research area of Mysuru district, focusing on the spread of virulent genes. This strategy was developed so that the assay would provide the most hotspot discovered regions and particular virulent genes transmission regions with PTB cases, which might ultimately be helpful in a clinical diagnosis and understanding the spread that helped in TB eradication measures. Targeting the types of TB spread settings may result from focusing on the regions with the most PTB cases at the regional and local levels.

The nations with the highest TB burden were noted in a related study by Charchyard and Sweindells in 2019 [32]. According to a study by Pareek et al. 2016 [33], Africa and Asia have high burden settings with 58- 28% cases. According to Crampin et al 2008 [34] study, the majority of PTB cases were found to be recorded in urban areas and the most populous regions of India. The incidence of PTB with culture findings and swab results was around 605 per 100,000 and 323 per 100,000, respectively, according to a survey conducted in India. Similar research was done in Karnataka, where it was stated that 61 million people were at risk and that TB prevalence was

100,000 people. According to a study by Deepak et al., 2012 [35] 80% of the PTB cases in the study are TB cases in the Udupi region. Mysuru district, the study region, was in the top 5 most reported TB regions. In addition, it was discovered that out of the seven other talukas, the Mysuru city region had the most recorded PTB incidences, with 41% cases from 2011-19. Similarly Ranganath et al., 2013 [36], Meundi et al., 2015 [37] and Kumar et al., 2011[38] on the PTB prevalence in rural areas, detention center inmates, and MDR-TB trends in PTB. A prevalence study of TB in Mysore city by Chadha et al., 2017 [39], studied awareness of the risk factors in the disease transmission Rashmi et al., 2016 [40].

The results showed that Mysuru district had the highest number of reported PTB cases, and further genetic analysis sample collection was started. A significant number of cases were found to be centered in Mysuru's urban regions; large cases were detected in 15 patients who were over the age of 65, and the majority of PTB cases were found to be in men, who made up 80% of those infected throughout all talukas in the Mysuru district. As a result, the PTB cases were reduced, and sputum samples were taken in the districts of the city's hotspots. After sputum positivity was confirmed, the samples were further subjected to decontamination and digestion. Only a few studies used a similar decontamination technique by Sharma et al. 2012 [41] and Chatterjee et al 2013 [42]. The slides show the appearance of one field bacilli in grades 1+, 2+, and 3+ according to WHO recommendations. Similar procedures and grade standards were used by Bonnet et al.2011 [43] for MTB staining. All of the samples fell within the acceptable ranges on these scales.

The main virulent genes were modified in the current investigation, and their presence in the isolated MTB DNA from the hotspot sites was examined. It performed well in comparison to other sample preparation techniques examined by Sritharan & Barker in 1991 [44]. The MTB, which included almost 4,000 genes in all, contained virulent genes. Out of these, 91 genes, or 23% of the MTB genome overall, were linked to virulence, detoxification, and adaptability [45]. The majority

of these pathogenic genes encode for regulators, signal transduction enzymes, cell surface proteins, and lipid metabolism enzymes [46]. Many of these virulent genes are crucial for the generation of virulent proteins. It is yet unclear what criteria and circumstances define these harmful genes.

Perez et al. (2016) [47] analysed the pathogenic genes in M.colomiense, the second-most medically complex pathogen after the MTB complex, and found that they have enormous possibilities for the development of particular methods for diagnosis. This pathogen is hopeful, rare, and has a high level of medical complexity. The prospective MTB therapeutic targets database analysis identified 93 virulence-related proteins that are important for MTB pathogenicity. Similar to Causee et al. (2011) [48], Gous et al. (2012) [49], Yang et al. (2011) [50], Lee et al. (2013) [51], Bustin et al., 2009 [52], 2010 [53] studies targeted pathogenic gene amplification by qPCR with rapid and sensitive technique for detecting MTB in human samples. Correspondingly, the pathogenic genes FadD33, MmpL10, and WhiB3 were searched for using PCR analysis on DNA samples.

The detailed reporting of cases in the Mysuru regions led to the identification of the pathogenic genes in PTB patients. The genes that enable the bacilli to induce infection as well as these virulence factors (VFs), which are necessary for the pathogen's ongoing survival inside the host, continue to be an issue for worldwide public health. Finding new medication or vaccine targets against pathogenicity is made easier with the identification of virulence factors. There are still few computational approaches available for evaluating, detecting, and characterising virulence factors even though the amount of knowledge about VFs is growing quickly. Because numerous proteins in genomes of bacteria are currently categorised as unreliable, have limited biological characterization, or contain contradicting information, comparative genomics research is difficult. The severe PTB transmission could lead to outbreaks of diseases including EPTB, MDR-TB, and XDR-TB that are more challenging to treat.

## CONCLUSION

In the current research, the random PTB cases distribution is in the aged 0-15 and above aged group in Mysuru urban region. The lowest populated cites like Periyapatna and KR nagar and the populated regions like Mysuru and Nanjangudu with most reported cases. The city has also gradually urbanized, industrialized, and lost some of its mountainous and agricultural districts. As a result, there is a considerable correlation between regional development and the distribution of PTB. Regarding, the discovery of specific virulent genes in strains associated with significant TB outbreaks would offer fresh insights into the evolution of MTB, enhancing epidemiological observations and maybe favoring the development of efficient control methods.Additionally, there's a theoretical risk of TB transmission through saliva if oral lesions are present, though respiratory transmission is more common. Socioeconomic factors, including poverty and limited healthcare access, are shared risk factors for TB and poor oral health. Furthermore, the co-occurrence of TB and HIV/AIDS underscores the importance of addressing oral health within comprehensive care frameworks. Integrating oral health education and dental check-ups into TB care programs can improve treatment outcomes and overall well-being.

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