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ISOLATION AND CHARACTERIZATION OF *MYCOBACTERIUM TUBERCLE* ISOLATES FROM PULMONARY TUBERCULOSIS PATIENTS USING CONVENTIONAL AND MOLECULAR TECHNIQUES

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ABSTRACT
Tuberculosis is a major public health issue that affects all age groups of peoples all over the world which is caused by Mycobacterium tubercle bacilli. Tuberculosis remains one of the most serious infectious disease worldwide**.** Dissemination of tuberculosis infection in the population occurs mainly by inhalation of contaminated aerosols from patients with active pulmonary disease. Rapid isolation and molecular characterization of Mycobacterium tubercle isolates are essential for accurate diagnosis, epidemiological surveillance and detection of drug resistance strains. The present study was carried out to isolate and characterize Mycobacterium tubercle isolates obtained from 150 pulmonary tuberculosis patients using conventional Microbiological, Biochemical and Molecular techniques. Sputum samples were processed by Ziehl – Neelsen staining and cultured on Lowenstein – Jensen (LJ) Media. Molecular confirmation was performed using Polymerase chain reaction, targeting the IS6110 Sequence. Further characterization of isolates was carried out using Spoligotyping and MIRU- VNTR typing methods. Out of 150 sputum samples, 118 (78.6%) were smear positive, while 110 (73.3 %)yielded positive cultures. PCR confirmed 106 isolates as Mycobacterium tuberculosis. Molecular analysis demonstrated considerable genetic diversity among isolates. Multidrug- resistant strains were also identified through detection of mutations in resistance – associated genes. This study clearly demonstrates that molecular techniques are highly sensitive, rapid and reliable tools for the characterization of Mycobacterium tubercle isolates and could be significantly contribute to tuberculosis control and management programs.

KEYWORDS: *Mycobacterium tuberculosis*, Molecular epidemiology, PCR, Spoligotyping, MIRU-VNTR, Pulmonary tuberculosis, Public health.

INTRODUCTION

Tuberculosis is a chronic infectious disease worldwide caused by *Mycobacterium tubercle bacilli*. Despite advances in diagnosis and chemotherapy, TB remains a major global public health challenges especially in developing and under developed countries (WHO, 2025, Saskia et al, 2025). Pulmonary tuberculosis is the most common form and act as the principal source of transmission (Hemeg et. Al, 2023, You et al 2024, WHO 2020, 2025). According to recent global reports, 10 million of new tuberculosis cases occurs annually with increasing concerns regarding Multidrug- resistant tuberculosis (Ranganathan et al, 2024, Cryptic 2024, Kishori et al 2025). Molecular epidemiology plays a crucial role in understanding transmission dynamics, strain diversity, evolution of *Mycobacterium tuberculosis* strains and emergence of Multidrug resistant tuberculosis (MDR-TB) (Jagielski et al 2025, WHO 2025, Kamerbeek et al 1997, Soni et al 2001). Many effective conventional antimicrobial drugs have been available, however, emergence of multidrug–resistant tuberculosis(MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) has overshadowed the effectiveness of the current first and second line drugs. (Shakya et.al. 2012). Conventional diagnostic methods such as sputum smear microscopy and culture methods are time consuming and lack sensitivity . (Bartolomeu et.al 2024, Saskia et.al 2025, Patterson et.al 2024).Molecular techniques Polymerase chain reaction (PCR), Spoligotyping and Mycobacterial interspread repetitive unit variable number tandem repeat (MIRU – VNTR) typing have significantly improved the speed and accuracy of Mycobacterium tuberculosis detection and characterization. (Soni et.al 2001, Mousavi et.al 2024, Jagielski et.al 2025, Jesudason 2026, Zhang et.al. 2023, Sharma et.al 2026). Spoligotyping detects polymorphisms in the direct repeat region of Mycobacterium tuberculosis genome where as MIRU-VNTR typing analyzes tandem repeat loci to differentiate strains. (Kamer beek et.al. 1997, Van Embdenet.al. 1993, Sharma et.al. 1996, Jagieski et.al. 2025 , Jesudason 2026, Telenti et.al. 1993, Zamani et.al. 2016, Gauther et.al. 2015, Streiti et.al. 2015, Hemeg et.al. 2023, You et.al 2024). Recovery of mycobacterium avium paratuberculosis from patients with inflammatory bowel disease, Crohns disease and chronic gastrointestinal disease indicates a close association of mycobacterium with a number of other diseases

affecting human health (Singh et.al.2016). Information on genomic diversity, prevalence and various risk factors associated with tuberculosis in different sub regions could be very much useful to locate the hot spots that required targeted interventions for the control and transmission of disease. Therefore, the present study has been undertaken to isolate and characterize Mycobacterium tubercle isolates from different sputum samples of pulmonary tuberculosis patients using different conventional and molecular methods and to confirm Mycobacterium tuberculosis isolates using PCR amplification , characterization of M.TB Strains using Spoligotyping and MIRU -VNTR typing, identify genetic diversity among MTB isolates and to detect drug resistance–associated Mutations in differential clinical samples.

Objectives of the study: The present study aims:-

- To isolate Mycobacterium tuberculosis from sputum samples collected from different pulmonary tuberculosis patients.
- To confirm MTB isolates using PCR amplification.
- To characterize MTB strains using Spoligotyping and MIRU – VNTR typing.
- To identify genetic diversity among MTB isolates and to detect drug resistance – associated mutations.

Materials and Methods

A total of 150 clinically suspected pulmonary tuberculosis patients were enrolled in the study and sputum samples were collected from these patients visited Mangalayatan University Hospital, Aligarh (UP), National JALMA Institute of Leprosy and Other Mycobacterial Diseases (ICMR) Agra and Department of Tuberculosis and Chest Diseases, S.N. Medical College &
Hospital, Agra (UP) for treatment and confirmatory diagnosis of Tuberculosis. A cross sectional hospital based study was conducted over the period of 18 months from January 2025 to June 2026. After appropriate counseling and written consent, the patients were included in the study. Their detailed present clinical history and previous history of Tuberculosis treatment, Chest and radiological findings, weight loss and any other associated illness and physical characteristics of the study participants were recorded. Patients above 20 years of age presenting with persistent cough, fever, weight loss and haemoptysis were included in the study, after obtaining their written consents. Early morning sputum samples were collected in a sterile leak proof sputum collecting vials, following latest bio safety guide lines of WHO. The Clinical samples collected were transported to department of Microbiology for further processing on the same day. The sputum samples were stored at 4°C till further processing.

All the sputum samples were processed for smear microscopy and isolation of M. tuberculosis on Lowenstein – Jensen (LJ) medium. All the microbiological tests were conducted in Bio safety level-3 (BSL- 3) Laboratory of Department of Microbiology of Mangalayatan University Hospital, Aligarh and National JALMA Institute of Leprosy and Other Mycobacterial Diseases (ICMR) Agra.

Processing of Sputum Samples for Smear Microscopy and Culture: All the sputum samples were subjected to decontamination by modified Petroffs method as reported by Kent and Kubica 1985.All the sputum samples were processed for smear microscopy, isolation and detection of M. tuberculosis using Ziehl – Neelsen (ZN) acid fast staining and cultured on Lowenstein–Jensen (LJ) medium. Briefly 3-5 ml of sputum samples were homogenized in a shaker for 15 minutes with an equal amount of 4 percent NaOH. After centrifugation at 3,000 rpm for 15 minutes, the pellet was neutralized with 20 ml of sterilized distilled water. The tubes were centrifuged at 3000 rpm for 15 minutes. After that LJ medium was inoculated from the sediment using inoculation loop wire. The culture tubes inoculated were kept in incubator at 37°C for 6 to 8 weeks and regularly examined every week for the presence of Mycobacterial growth. Any suspected growth on LJ medium was confirmed by ZN staining for acid fast bacilli and the positive growths were sub-cultured on fresh LJ media for further identification and genotyping, using Spoligotyping, PCR, and MIRO-VNTR typing.

Identification of isolates: Identification of the growth recovered on LJ medium was carried out using standard biochemical tests viz Nitrate reduction, Catalase activity at 68°C and Niacin tests as per the method described by Vestal 1977.

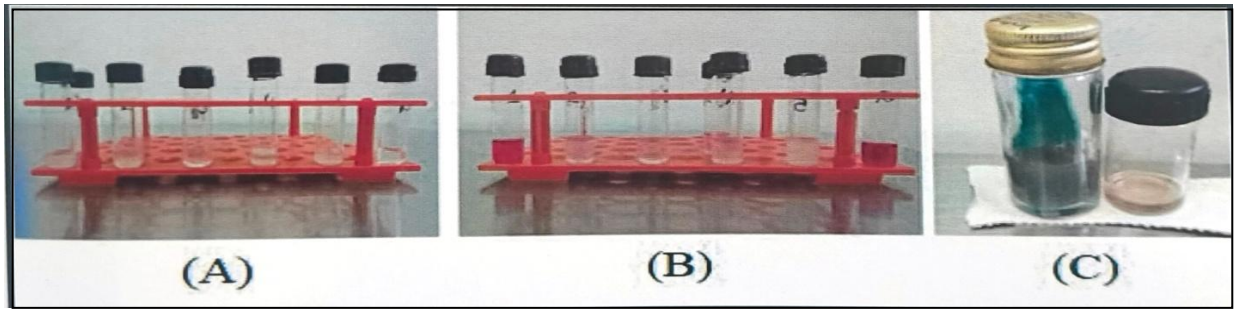

Figure.1. Biochemical identification of Mycobacterium tuberculosis isolates using A: Catalase Test B: Nitrate reduction Test and C: Niacin Test

All the Mycobacterium tuberculosis positive isolates were processed to genomic DNA extraction using Physical, Chemical and Enzymatic method (Singh et.al. 2021). The extracted genomic DNA of M. tuberculosis isolates were subjected to Spoligotyping using the method of Sharma et.al. 2008. The direct repeats region and interspread known space region in the extracted genomic DNA of M. tuberculosis isolates was amplified using DRa and DRb primers. The amplified PCR products were hybridized on a membrane pre-coated with 43 covalently bound spacer oligonucleotide derived from known spacer sequence of M. tuberculosis complex. The presence of hybridization signals were visualized using X-ray films after incubation with streptavidin-peroxidase and ECL detection. The genomic DNA of M. tuberculosis (H37 RV) and M.bovis (BCG) were used as positive control and distilled water was used as negative control. The hybridization signals of spacer oligonucleotides were recorded in the form of binary code that was converted into octal code and analyzed with the international database SITIVIT WEB_2. Mycobacterial –interspersed- repetitive units (MIRU) and Variable number of tandem- repeats (VNTR) typing is an important genotyping method for Mycobacterium tuberculosis.

Mycobacterium tubercle isolates were genotypically differentiated using 15-locus MIRU-VNTR typing (Gauthier et al., 2015). According to Gauthier et al. (2015), the DNA was extracted from the Mycobacterium TB isolates using the boiling procedure and 15 primers for MIRU-VNTR and PCR. By using 1.5% agarose gel electrophoresis to detect the PCR result, the copy number of each repeating allele was ascertained by comparing it to the PCR product of the standard strain, H37 RV. The MIRU-VNTR method's positive and negative controls were the H37 strain and distilled water, respectively (Zamani et al. 2016). Using MIRU-VNTR with aline software, the similarity of the MIRU-VNTR panel for each patient's sample was assessed.

PCR amplification of tandem repeats loci was performed and fragment sizes were analyzed to determine the repeat numbers.

For epidemiological research of Mycobacterium TB, MIRU-VNTR typing procedures were found to have a higher discriminatory power than Spoligotyping alone as recorded during the investigation. These typing methods have been found extremely useful for differentiation between mycobaterical tubercle isolates.

RESULTS

In present study, genomic diversity of M. tubercles isolates from pulmonary tuberculosis patients was studied. Out of 150 sputum samples, 118 (78.6%) were found smear positive and 32 (21.4%) were found smear negative samples. The results of present study were found consistent with many other studies carried out in hospitals and TB control programs in North India.

The gold standard method for diagnosing tuberculosis is the culture method (WHO 2025). Out of the 150 sputum samples used in this investigation, 110 (73.3%) were found to be culture positive for the presence of Mycobacterium tubercle bacilli, whereas 40 (26%) were found to be culture negative

Figure.2.Isolates recovered on LJ medium with positive and negative control used.

A. Mycobacterium. tubercle isolates recovered from pulmonary tuberculosis patients on LJ medium. B.Non tuberculous mycobacterial isolates recovered from suspected pulmonary tuberculosis patients on LJ media.

C. Positive control(H37 Ra). D. Negative control (Slant inoculated with sterilized distilled water).

The samples were determined solely by sputum smear microscopy and the L-J culture method, respectively. All the isolates were further subjected to Biochemical tests for the rapid differentiation from the members of Mycobacterium tuberculosis complex

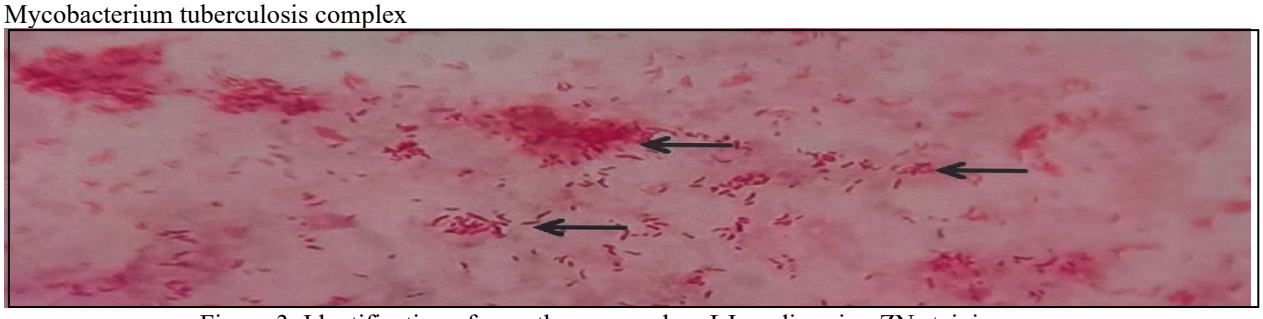

Figure.3. Identification of growth recovered on LJ media using ZN staining.

The figure shows the presence of acid fast bacilli in growth recovered on LJ medium using Microscopic examination (100X).

The acid fast bacilli (Pink Colour)as shown by black arrow are tubercle bacilli.

Molecular identification of Mycobacterium tubercle isolates

All the biochemically characterized Mycobacterium tubercle isolates were subjected to molecular identification using IS6110 PCR. In the present study, 106 (70.6%) isolates were found positive for the presence of IS 6110 sequence and 44 (29.4%) were recorded as negative. PCR targeting IS6110 demonstrated high sensitivity and specificity for confirmation of Mycobacterium tubercle isolates**.**

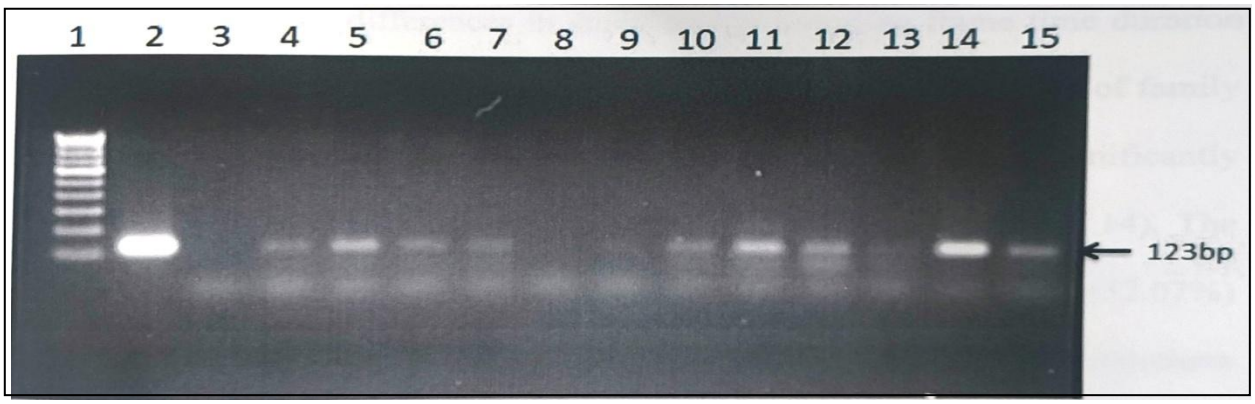

Figure. 4. Molecular identifaction of Mycobacterium tuberculosis isolates using IS6110 PCR.

(Band size : 123 bp):Lane 1 100bpmarker,Lane 2 Positive control, Lane 3 Negative control, Lane 4-15 , Clinical samples.

Genomic diversity in Mycobacterium tubercle isolates from pulmonary tuberculosis patients was also studied using Spoligotyping method for the investigation of molecular diversity and transmission of Mycobacterium tuberculosis in different communities (Lobie et.al.2020, Devi et.al. 2021, Yadav. A. et.al. 2022, WHO 2024, Saskia et.al. 2025). Spoligotyping of the M. tuberculosis strains revealed high genetic diversity among M. tuberculosis in north India. The MTB lineages identified are :- EAI . 38%, CAS 29% and Beiging lineage 14% . Predominance of EAI and CAS lineages has also been reported in molecular epidemiological studies from south Asia.

MIRU- VNTR analysis was carried out during the study. The MIRU-VNTR analysis showed considerable genetic diversity among isolated isolates and differentiated clustered strains from unique strains. The 15– locus MIRU-VNTR method demonstrated high discriminatory efficiency and improved epidemiological resolution.

Drug Resistance Analysis

Selected Mycobacterial isolates were screened for mutation in rPOB gene (Rifampicin resistance and KatgG gene isoniazed resistance). Among 106 confirmed isolates, 13 isolates were identified as multidrug resistant. It is observed that Rifampicin resistance associated mutations were recorded predominant. Molecular detection of resistance associated mutation provides rapid identification of multidrug resistance strain and support timely treatment decisions (Islam et.al. 2024 ,WHO 2025).

DISCUSSION

Advances in diagnostics molecular biology and recombinant DNA technology have provided newer techniques and tools for diagnosing several infectious diseases with considerably higher sensitivity and specificity than the conventional methods (Sharma et.al. 2026) These includes specific gene probes, PCR. Spoligotyping ,MIRU-VNTR typing analysis as well as genetically engineered antigens for immunodiagnosis. The need for such techniques is particularly felt for Mycobacterial infections and several researchers have made efforts to investigate the molecularbiology of lepra and Tubercle bacilli. (Sharma et.al. 1996, Sharma et.al. 2026 , Katoch et.al. 1993,94). These studies have led to the development several gene probes and gene amplification techniques for identification and detection of Mycobacterium Leprae and Mycobacterium tuberculosis (Sharma et.al. 2026, Katoch 1994) In practice rRNA targeting probes and PCR have been found to be more sensitive for Mycobacterium tuberculosis (Sharma et.al. 1996, 2026). The present study demonstrated the utility of molecular methods in rapid detection and characterization of Mycobacterium tuberculosis. Although culture remains the gold standard for isolation. Molecular techniques considerably reduce diagnostic delays. PCR amplification targeting IS 6110 proved highly sensitive for confirmation of Mycobacterium tubercle isolates(Mousavi et.al 2024,Bartolomeu et.al. 2024 , Hemeg et.al 2023) . Spoligotyping enabled lineage identification and showed predominance of CAS families, which is consistent with earlier studies from Tuberculosis endemic regions. MIRU-VNTR typing provided enhanced discriminatory power and effictively differentiated genetically related strains. The combined use of Spoligotyping and MIRU- VNTR typing has been shown to improve understanding of transmission patterns and epidemiological relationship among TB strains (Jagielski et.al 2025, Zhang et.al. 2023, Saskia et.al.2025, Patterson et.al. 2024) . This study also identified Multidrug resistance isolates through molecular analysis of resistance – associated genes which clearly highlighting the importance of molecular

diagnostics in Tuberculosis control programs (Diana et.al. 2024, Bhnushali et.al. 2024, Islam et.al 2024, WHO 2025, Jagielski et.al 2025, Patterson et.al.2025 , Kishori et.al. 2025,

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CONCLUSION

By utilizing a combination of conventional methodologies and advanced molecular techniques specifically PCR, Spoligotyping, and MIRU-VNTR typing, this study successfully isolated and characterized *Mycobacterium tubercule* isolates from pulmonary tuberculosis patients. These molecular epidemiologic tools demonstrated high sensitivity, speed, and reliability in achieving precise identification and strain differentiation. Consequently, such advanced approaches prove indispensable for robust community surveillance, diagnosis, outbreak investigations, and the expedited detection of multidrug-resistant tuberculosis (MDR-TB). Ultimately, integrating these molecular assays into public health frameworks significantly enhances diagnostic accuracy and epidemiological tracking, offering novel baseline data to optimize global tuberculosis control strategies.

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