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Molecular detection of Multi-Drug Resistant Mycobacterium species in a Tertiary Care centre

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ABSTRACT

Tuberculosis (TB) - one of the leading causes of adult death in the Asia-Pacific Region, is an infectious disease caused by Mycobacterium tuberculosis (MTB). TB remains a major public health issue especially in developing nations due to the lack of adequate rapid diagnostic testing facilities. Drug resistance in tuberculosis was observed nearly fifty years ago. The risk is now due to the emergence of new strains which are most resistant to the potent anti-tuberculosis drugs. The resistance to drugs in Mycobacterium tuberculosis is conferred by mutations with the genes encoding drug targets or drug converting enzymes. In suspected extra pulmonary tuberculosis cases also, fast and accurate laboratory diagnosis is of primary importance, since the techniques which are followed from past years for detecting acid-fast bacilli have many limitations. This study may also help in standardizing the technique for rapid identification of MDR TB in extra pulmonary TB patients in this Tertiary care Hospital. The current study describes the importance and use of Polymerase Chain Reaction (PCR) for the detection of Multidrug-resistant Mycobacterium species. The target genes encoding resistance to Isoniazid and Rifampicin were detected by Polymerase Cain reaction and Agarose gel electrophoresis. Among the 359 samples, 2% were resistant to both Isoniazid and Rifampicin and the prevalence of drug resistance was found to be more in adult age groups.

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INTRODUCTION

Tuberculosis (TB) is a deleterious infectious disease characterized by the presence of nodules in the tissues, especially the lungs. TB is an airborne disease which is caused by *Mycobacterium tuberculosis* (MTB) (Sukeshrao, 2009). It usually affects the lungs, which lead to fever, severe coughing, and chest pain. Many researches in the past few years have provided valuable insight to the mode of TB transmission, its diagnosis, and treatment. The disease being second to HIV/AIDS in causing high mortality rates is now an overwhelming burden on public health. India has new TB patients annually contributing to 27% of the worlds TB burden (Catejan, 2017). About 2.79 million new TB patients are been reported annually. Drug resistance was observed nearly 50 years ago even in the early days of chemotherapy. Multi Drug-Resistant Tuberculosis (MDR-TB) which is defined as resistance to both Isoniazid (INH) and Rifampicin (RIF), is a worldwide public health problem particularly in areas where poor settings are only available for adequate diagnosis and treatment (Anupurb *et al.*, 2014).

Resistance to drugs in *Mycobacterium tuberculo-*

sis (*M. tuberculosis*) is mostly conferred by mutations within the genes encoding drug targets or drug converting enzymes. Genetic studies have demonstrated that up to 98% of the resistance is conferred to a single point mutation in the RNA polymerase gene rpoB which comprises a defined region of 81 base pairs which is commonly referred as Rifampicin Resistance Determining Region (RRDR) (codons 507-533) (Riska *et al.*, 2000). On the other hand, Isoniazid resistance (INH) is due to mutations that occur in one or more genes. But among them, kat G S315T mutation is the most common mutation, accounting for 50-95% of clinical resistance to INH (Zhang and Yew, 2009). DNA amplification with the aid of PCR has allowed great progress in the rapid and accurate diagnosis of infections due to non-cultivable organisms that require complex media or cell cultures and prolonged incubation times. Amplification techniques for the diagnosis of tuberculosis are of considerable interest since it shortens the time required for detecting and identifying *Mycobacterium tuberculosis* in different specimens. Several methods such as DNA sequencing, Single Strand Confirmation Polymorphism (SSCP)-PCR analysis, Restriction Fragment Length Polymorphism- PCR, Multiplex Allele-Specific (MAS)- PCR, genotype MTBDR plus and many more are designed to observe specific mutations which are responsible for *M. Tuberculosis* drug resistance (Al-Mutairi *et al.*, 2011). Among these techniques, DNA sequencing of PCR products has been considered as a gold standard.

MATERIALS AND METHODS

Sample processing

359 pulmonary samples (sputum, broncho alveolar lavage, endotracheal aspirate, pleural fluid) were collected from In-patient and Out-patient department of Saveet□a Medical College and Hospital. All the sputum samples were stained by Auramine staining and Zeihl Neelsen staining and was subjected to Modified Petroff's method for concentration. The concentrated sputum samples were again stained by Zeihl Neelsen staining and Auramine staining method for the statistical analysis of sensitivity and specificity of the methods before and after the concentration of sputum specimen.

Zeihl Neelsen staining

The sputum smears were spreaded with Carbol fuchsin reagent, heat-fixed, kept for 5 minutes. Then the smears were washed under running tape water. 25% Sulphuric acid were flooded over the smears and kept for 3 minutes and again, the smears were washed again and flooded with methylene blue and kept for 1 minute. Washed under running tape water, the smears were air-dried and observed under oil immersion field objective. The positive smears were confirmed by RNTCP grading protocol.

Auramine staining

The sputum smears were made, heat-fixed and flooded with Auramine O solution for 20 minutes. After washing the slides, 0.1% acid alcohol was added as a decolouriser. The slides were again washed off and added with 0.5% Potassium permanganate for 1 minute. Washing off and air-dried, the smears were observed under a fluorescent microscope. The samples which showed smear positivity was further processed. The smear grading was done following the RNTCP grading system.

Modified Petroff's method

All the sputum samples were liquified and were decontaminated with an equal volume of 4% Sodium hydroxide and mixed thoroughly by pulse vortexing. The mixture was subjected to centrifugation at 3000 rpm for 15 minutes. The supernatant, which is at the top, was discarded, and the settled pellet was again treated with an equal volume of sterile distilled water. Then the mixture was again centrifuged at 1500 rpm for 15 minutes and the supernatant was discarded. The pellet was again washed in an equal amount of distilled water, centrifuged at 1500 rpm for 10 minutes. Then collected in sterile collection tubes and stored (Anupurb *et al.*, 2014). Zeihl Neelsen and Auramine staining was done. The concentrated specimens were subjected to the extraction of Mycobacterial DNA Table 1 and Table 2.

Molecular detection of IS6110, rpoB and katG gene

In this study, 51 samples found to be positive in Auramine staining after concentration were taken for DNA extraction and amplification. i. Sekiguchi *et al.* (2007) developed a DNA sequencing method to detect mutation in eight genome regions, including rpoB for Rifampicin, katG and mabA for Isoniazid, embB for ethambutol, pncA for pyrazinamide, rpsL and rrs for Streptomycin and gyrA for Levofloxacin. In the present study, mutations in 3 genome regions, including IS6110 for identifying Mycobacterium species, rpoB for Rifampicin and katG gene for Isoniazid, were detected. The samples were subjected to genotypic detection of IS6110(identification of Mycobacterium species), rpoB gene (Rifampicin resistance) and katG gene(Isoniazid resistance).

Table 1: List of primers

Target	Forward	Reverse	Amplicon
IS 6110	5'-GGATCCTGCGAGCGTAGGCGTCGG-3'	5'-CCTGTCCGGGACCACCCGCGGCAA-3'	200
rpoB	5'-CGAGGTGCCGGTGGAAAC-3'	5'-GTCGTCGTGCTCCAGGAAGG-3'	721
katG	5'-GAGCCCGATGAGGTCTATTG-3'	5'-GTCCTTGGCGGTGTATTGC-3'	498

Table 2: Acid-fast bacilli positivity in Auramine and Zeihl Neelsen staining before and after concentration of sputum samples

Samp	Zeihl Neelsen staining				Auramine staining			
	Before concentration		After concentration		Before concentration		After concentration	
Sputum	+	–	+	–	+	–	+	–
	49	278	51	276	51	276	51	276

RESULTS AND DISCUSSION

The agewise and sex-wise distribution of 51 positive samples were depicted in Figure 1. All the 51 samples were found to be positive for the presence of Mycobacterium species (IS6110). Prevalence of Mycobacterium in this Tertiary care Centre is explained in Figure 2. 2% showed resistance to both Rifampicin(rpoB gene) and Isoniazid(katG gene) and 98% samples were negative to both Rifampicin(rpoB gene) and isoniazid(katG gene). The prevalence of MDR TB is represented inFigure 1.

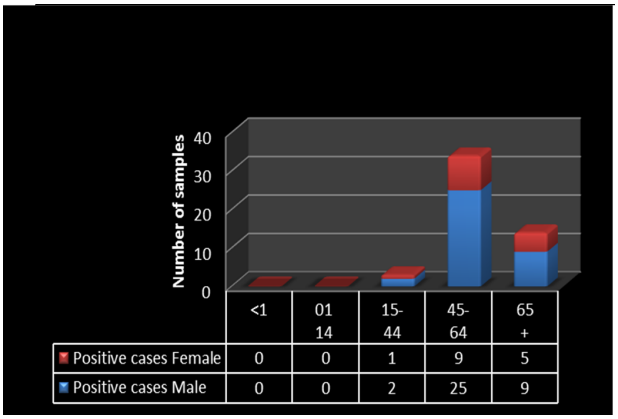

Figure 1: Sex wise and age-wise distribution of positive samples

Shenai *et al.* (2009) speciated many clinically important Mycobacteria, which are resistant to Rifampicin, Isoniazid and Streptomycin in *Mycobacterium tuberculosis* complex. Later on, a study conducted in Japan in 2014 by (Ueyama *et al.*, 2014) *Mycobacterium species* were speciated using multi-

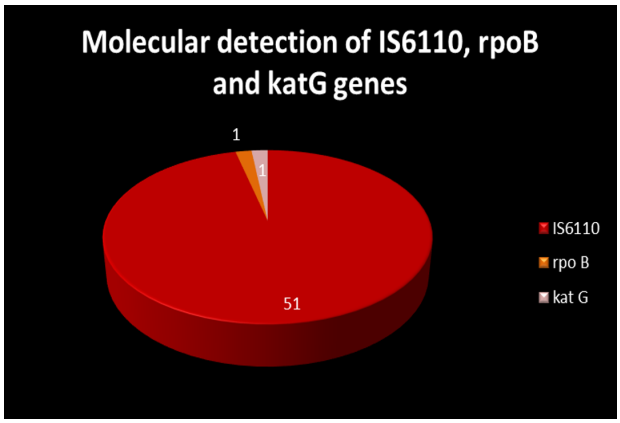

Figure 2: Prevelance of MDR TB

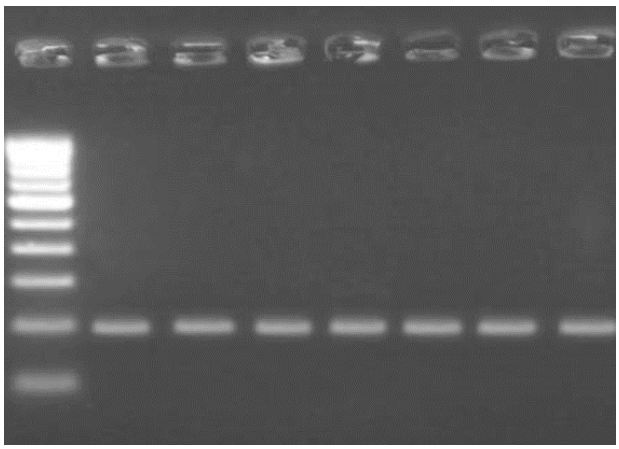

Figure 3: Genotypic detection of IS6110 sequence. [Lane 1 DNA ladder Lane 2,3,4,5,6,7,8 Samples positive for IS6110(200bp)]

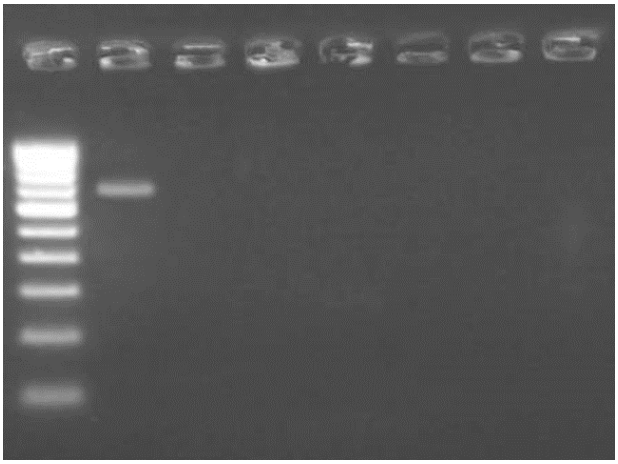

Figure 4: Genotypic detection of rpoB gene
Lane1 DNA ladder
Lane 2 Samples positive for rpoB (721bp)
Lane 3,4,5,6,7,8 Samples negative for rpoB (721bp)

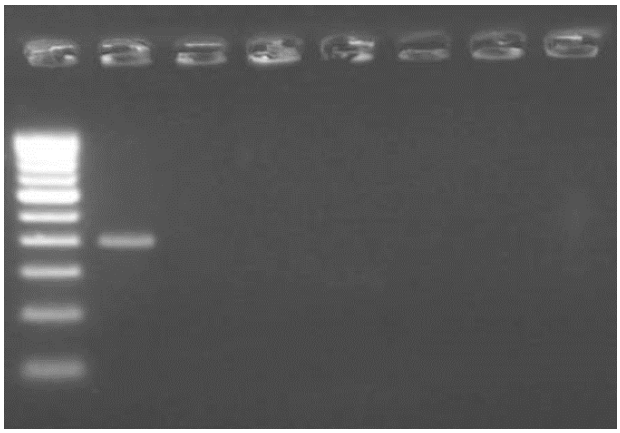

Figure 5: Genotypic detection of katG gene Lane 1 DNA ladder
Lane 2 Sample positive forkatG (498bp)
Lane 3,4,5,6,7,8 Samples negative for katG (498bp)

plex PCR that detected cfp32, RD9 and RD12 genes. In this study, the identification of Mycobacterium species was only done. Figures 3 and 4and Figure 5 were the electrophoretrogram images of IS6110, rpoB and katG, respectively.

The 2%, which showed resistance to both rpoB gene and katG gene, were from the adult age group (45-64) and were from male patients. (Saeed *et al.*, 2018) and Sharma *et al.* (2010) reported that the prevalence of MDR-TB was more in male compared to females. The highest drug resistance ratio was found in the adult age group.

CONCLUSION

From this study, it is clear that Auramine staining stands efficient on comparison and can be used as an alternative to Zeihl Neelsen staining since it detects the bacilli even before concentration with high sensitivity. Nearly 2%of *Mycobacterium species* in this study are found to be resistant to the first-line group of drugs, highlighting the need to apply rapid diagnostic tests for the identification and detection of drug-resistant tuberculosis for effective treatment and there by preventing the spread of tuberculosis in the community. Routine surveillance of resistance to anti TB drugs will enhance the timely detection of MDR TB cases and helps to prevent further transmission of the disease. The information obtained will provide baseline data that can be used to design further research for the prevention of MDR TB in our set up.

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