MOLECULAR DIAGNOSIS OF TUBERCULOSIS IN KANDY AND MATALE DISTRICTS USING SPUTUM SAMPLES

R.K.B.S. Vasanthakrishnan [1], S.H. Jayasena[1], A. Siribaddana[2] and S.B.P. Athauda[1*]

[1] *Department of Biochemistry, Faculty of Medicine, University of Peradeniya*
[2] *General (Teaching) Hospital, Kandy*

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis*, is one of the most wide spread infectious diseases and the leading cause of death, due to a single infectious agent in many parts of the world including Sri Lanka. Sri Lanka too has a high incidence of TB, where a total of 8497 TB patients were registered in 2007 and 8996 TB patients in 2008 by the National Programme for TB Control and Chest Diseases. As TB is highly infectious for humans, it is of paramount importance that the disease be diagnosed as early as possible to stop the spread of the disease. Direct microscopy with Ziehl-Neelsen (Z-N) staining of clinical specimens for acid-fast bacilli although, rapid, inexpensive and easy it lacks sensitivity and specificity. The use of the Polymerase Chain Reaction (PCR) for the detection and identification of *M. tuberculosis* in clinical samples was reported to be rapid and sensitive technique. Several studies have reported on the use of the Polymerase Chain Reaction (PCR) for the detection and identification of MtbC in clinical samples (Kox *et al.*, 1994; Singh *et al.*, 2000; Piersimoni and Scarparo, 2003)

The objectives of this study were identification of the best method to extract high quality genomic DNA from mycobacterium isolated in Sri Lanka and development of molecular diagnostic technique to identify the *Mycobacterium tuberculosis* complex (MtbC), which is the major causative agent of tuberculosis in human.

Methodology

Sputum specimens were collected from the patients of chest clinics of Kandy and Matale districts, who are suspected for TB. Initially DNA was extracted from Lowenstein-Jensen (LJ) cultures by using available methods and then the purity and quantity of the genomic DNA was evaluated by measuring the absorbance at 260 and 280 nm. The three methods used were 1) CTAB (Cetyltrimethyl ammonium bromide)/NaCl method, 2) 2% SDS-10% Triton X- 100 followed by ethanol precipitation, 3) Tris-EDTA (TE) boiling method. The methods were also evaluated with sputum samples, initially the sputum samples were decontaminated by NALC-NaOH method.

DNA was extracted Using CTAB/NaCl method (Amita, 2002). The decontaminated pellet was resuspended in 10mM TE buffer and then kept at 80^0 C for 60 min followed by centrifugation at 9000rpm for 5min and then solution A (containing TE buffer and Lysozyme) was added and kept at 37^0 C for 1 hour followed by

DNA isolation using the CTAB/NaCl method and kept at -30^0 C until used for PCR amplification.

Detection of MtbC DNA by PCR

Isolated DNA was subjected to PCR. The primers for PCR were based on the repetitive sequence (IS986) of M. tuberculosis (Abe Chiyoji *et al.*, 1993). The two primers used were, PT-8 and PT-9 corresponding to bp105 to 124 (5'-GTGCGGATGGTCGCAGAGAT-3') and 626 to 645 (5'–CTCGATGCCCTCACGGTTCA-3'), respectively of the IS 986 sequence. PCR amplification gives a 541bp product which is present only when the DNA from bacteria belonging to the MtbC is present. The reaction mixture for PCR consists of 20 µl total volume. The thermo cycling parameters included an initial denaturing at 94^0 c for 5 min, 40 cycles of 94^0 C for 45 sec, 65^0 C for 1min and 72^0 C for 1.5 min and a final extension at 72^0 C for 5 min. The PCR products (20µl) were examined for bands by agarose gel electrophoresis in 1 X Tris-Borate-EDTA buffer.

Results

Among the three methods were used to extract the genomic DNA it was found that the CTAB/NaCl method was the best to obtain a significant DNA yield. Out of a total of 278 sputum samples analyzed by PCR it was found that 132 samples were positive by PCR. Results are shown in Table 1.

Discussion

Approximately 47.5% patients were found to be carrying MtbC in their sputum by PCR method. Eight of the patients who gave a negative result for Z-N smear test were identified as positive for MtbC by PCR. Out of 193 Z-N smear positive samples, 28 samples were scanty by smear and of these 19 gave a positive result for PCR. This clearly suggests that our genomic DNA extraction followed by PCR detection is a better method for molecular diagnosis. Further studies are in progress to analyze reason for giving 69 PCR negative results among 193 Z-N smear positive samples.

Table 1. Summary of the PCR results

PCR Results / AFB smear Results	Positive	Negative	Total
Negative (n = 85)	08	77	**85**
Positive (n = 193)	124	69	**193**
Total	**132**	**146**	**278**

Conclusion

The above results strongly suggest the importance of using sensitive molecular diagnostic technique for early diagnosis of TB in Sri Lanka. Further using this method it is possible to diagnose the presence of Mycobacterium in patient sputum samples within 36 hours.

References

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