Evaluation of Molecular and Ziehl-Neelsen stain for detection renal tuberculosis in urine sample

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Abstracts

Background: The aim to present study was performed to compare between the PCR method and Ziehl-Neelsen stain (Acid fast stain) for detection of renal patients with genitourinary tuberculosis.

Methods: A total of 50 renal patients with genitourinary tuberculosis were included in the study. The urine sample was collected from Nephrology and Urology word SS Hospital, IMS, BHU, Varanasi. The detection of *Mycobacterium tuberculosis* is used by Ziehl-Neelsen stain (AFB) and Polymerase chain reaction method.

Results:

The total number of disease patients was within 11 to 70 years (39.5 ± 14.4) age groups were studies. According to the results of PCR the highest percentage of tuberculosis patients in relation to the total number of disease patients was within 21 to 30 years age group (28%) followed by 31 to 40 years age group (16%), while the least percentage was detected in the age group 11 to 20 years (5%). It was also found that 33 males (66%) and 17 females (34%) with the ratio of 1.94:1 were infected with the disease. In this study Ziehl-Neelsen stain was negative in all patients but polymerase chain reaction for detection of *Mycobacterium tuberculosis* was 88% patients were positive.

Conclusion: Our study concludes that polymerase chain reaction is a superior technique detection of renal patients with genitourinary tuberculosis comparison to Ziehl-Neelsen stain.

Key words PCR, Ziehl-Neelsen stain and Renal tuberculosis

Introduction

Mycobacterium tuberculosis (MTB), the causal agent of tuberculosis (TB), is estimated to have infected one-third of the world's population and every year causes 8 million new tuberculosis cases and 2 million deaths.[1] It normally affects the lungs (pulmonary TB) but can affect other sites as well (extra pulmonary tuberculosis).[2] Genitourinary tuberculosis is the second most common extra pulmonary manifestation of tuberculosis, affecting 8–15% of the patients among pulmonary tuberculosis. [3] From the kidneys, the lungs are affected during hematogenic dissemination, with consequent involvement of the bladder and ureters through regressive infection of the collecting system. The genital organs are complicated through hematogenic (epydidimus and prostate) prostate and epydidimus) or retrograde canalicular distribution. [3,4] The diagnosis is often delayed, specifically in developing countries, due to the growth insidious with only some nonspecific symptoms, beside with a need of consciousness of physicians.[5,6] Because of its dangerous evolution and late onset of symptoms, the diagnosis and cure are infamously delayed, resulting in significant morbidity (e.g. end-stage renal failure, testicular destruction and shrunken bladder. [7,8,9] Genitourinary TB (GUTB) is generally caused by metastatic spread of organisms through the blood stream during the preparative infection. Active disease results from the reactivation of the initial infection. The diagnostic criterion for genitourinary tuberculosis is the isolation of *Mycobacterium tuberculosis* from urine. This is not simple to achieve, as the discharge of organisms into the urine is sporadic and more prominently involves few organisms.[10] Direct smears are frequently negative and do not discriminate tuberculosis from non-tuberculosis *Mycobacterium*. Culture, which is further sensitive, takes 6 to 8 weeks because of the slow growth rate of mycobacterium. [11] Although the Ziehl-Neelsen (Z.N.) stain is fast and low-cost, it lacks sensitivity. [12] Due to arrival of polymerase chain reaction (PCR) as a diagnostic tool, a new possibility has been opened for TB diagnosis. [13] PCR is a potential means to remove the sensitivity and specificity restrictions of mycobacterium traditional diagnosis methods. [14] In a clinical laboratory, it is desirable to use detection methods that have small complication and that are relatively easy and secure. The present study was performed aiming to compare between the PCR methods and other conventional method such as Ziehl-Neelsen stain (Acid fast stain) for diagnosis of renal patients with genitourinary tuberculosis.

Materials and Methods

Patients

Fifty patients clinically suggested to have renal tuberculosis were chosen from the Nephrology and Urology Department, Sir Sundar Lal Hospital, Banaras Hindu University, Varanasi during September 2012 to December 2016. They were suffering with dysuria, hematuria, flank pain and pyuria. The study was approved by Institute of Medical Sciences, Human Research Ethics Committee, Banaras Hindu University, Varanasi, India. All participants were thoroughly informed about the objective of the study, as well as the risk and precaution.

Written consent was taken from each patient. For each patient, early morning urine samples were collected on three consecutive days. The specimens were collected, pooled each patient's samples and centrifuged at 3000g for 20 min. Supernatant was removed and the pellet was divided into two parts. First part was used for AFB by the Ziehl-Neelsen method and other part was used for Polymerase chain reaction analysis.

Ziehl-Neelsen stain (AFB)

Purpose: Applied in the manifestation of acid-fast bacteria belonging to the genus 'mycobacterium', which contain the causative agent for tuberculosis. [15]

Procedure

1. The neutralized material was smeared over a clean and sterile glass slide. 2. The smear was made dry, fixed and covered with carbol fuschin and was gently heated steaming for 10 minutes. Precaution was taken not to make the strain dry.3. The slide was washed with running tap water. 4. Then the running stain was decolorized by adding 20 % sulphuric acid drop by drop till no more stain come off. 5. Then the smear was counter stained with methylene blue (0.5 gm in 100 ml DW). The solution was kept for one minute and then washed with running tap water. 6. Then the slide was examined by light microscope under oil immersion objective to see any bright rod like structure again blue background.

DNA extraction and Quantification

Bacterial DNA was extracted from urine sample *by Mycobacterium tuberculosis* commercial available Kit *(*Shanghai ZJ Bio-Tech Co., Ltd.) and Quantification of DNA was done by Qubit 2.0 Fluorometer (Invitrogen by life technology made in Austria).

PCR for the detection of *M. Tuberculosis*

Primer sequences *IS6110*

Two oligo nucleotide primers within the IS6110 sequence (24), designated primer 41 (20 bp; 5'-CCTGCGAGCGTAGGCGTCGG-3'; located at nucleotides 935 to 954) and primer 43 (21 bp; 5'-TCAGCC GCGTCCACGCCGCCA-3'; located at nucleotides 638 to 658) were synthesized at the Centers for Disease Control (Atlanta, Ga.). A total of 85 µM of reaction mixture was overlaid with 50 µl of mineral oil prior to the addition of 25 µl sample DNA. The reaction mixtures contained 1.5 mM magnesium chloride, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 50 mM potassium chloride, 200 µM (each) nucleotide (dATP, dCTP, dGTP, and dTTP), 0.5 µM (each) primer, and 2.5 U of Taq polymerase (Promega Corp., Madison, Wis.) was overlaid with 50 µl of small oil before to the adding of 25 µl of sample DNA Extracts from serial dilutions of M. tuberculosis were amplified at 30, 35, and 40 cycles to determine the optimum number of cycles.[16] The amplification procedure was performed as follows. Each cycle consisted of 94°C for 2.2 min for denaturation and 68°C for 3.25 min for annealing and primer extension. On the basis of these optimization studies, extracts from clinical samples were subjected to 35 cycles of amplification. Negative and positive controls were included in each amplification experiment.

Amplification products were analyzed by electrophoresis in an ethidium bromide stained 1.5% agarose gel. [17] The amplified product of the expected 317 bp fragment was considered positive for the presence of *M. tuberculosis.*

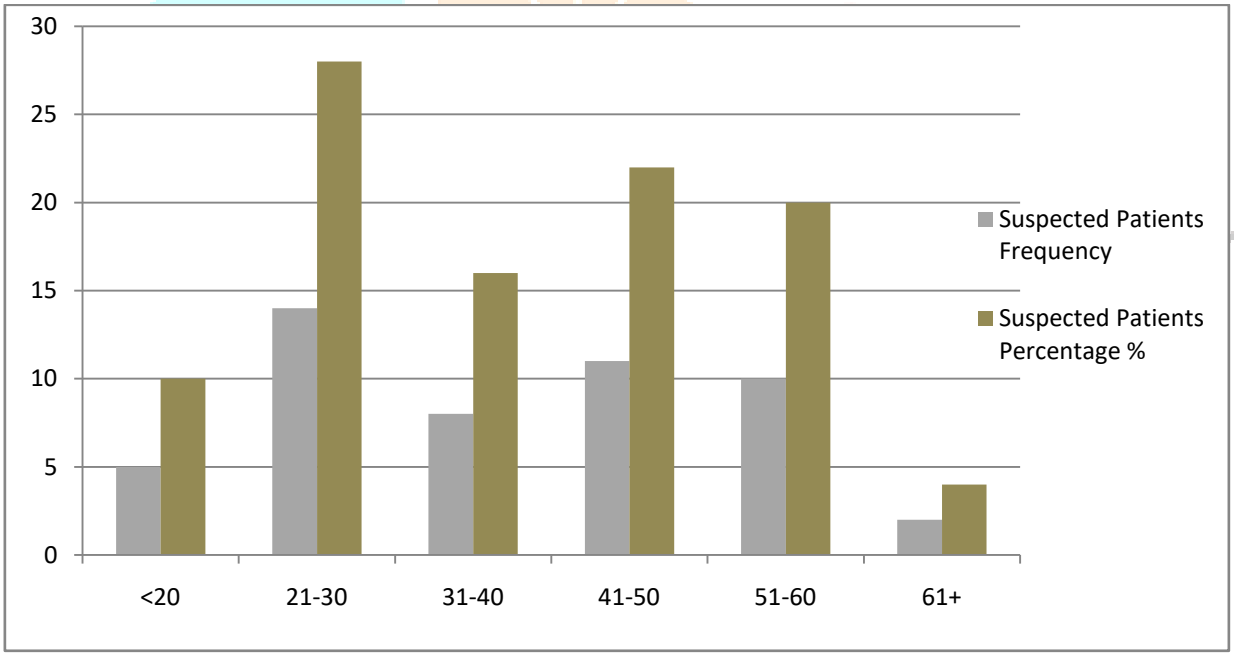
Statistical analysis

Data were analyzed using SPSS, version 16 (SPSS Inc., Chicago, IL) for windows, which were included statistical tables (frequencies, percentages and Pearson correlation)

Result

According to the results of PCR shown in Fig. 1, the highest percentage of tuberculosis patients in relation to the total number of disease patients was within 21 to 30 years age group (28%) followed by 31 to 40 years age group (16%), while the least percentage was detected in the age group 11 to 20 years (5%). It was also found that 33 males (66%) and 17 females (34%) were infected with the disease.

Fig. 1 Distribution of tuberculosis patients among different age groups



The first method by using Ziehl-Neelsen stain for detection of *Mycobacterium tuberculosis*. In this study Ziehl-Neelsen stain was negative in all urine specimens. Second method by using PCR for detection of MTB was 88% patients were positive (table 1)

Table.1 Comparison between polymerase chain reaction and Ziehl-Neelsen stain according to suspected TB patients

Methods		**Frequency of positive cases**	**Percentage %**	**Significance**
Ziehl-Neelsen stain (AFB)	50	0	0	41.667 (p<0.001)
Polymerase chain reaction	50	44	88	

Figure 2. Gel electrophoresis for PCR product of 317bp (1.5%) agarose for 15 minutes at 100 volt.

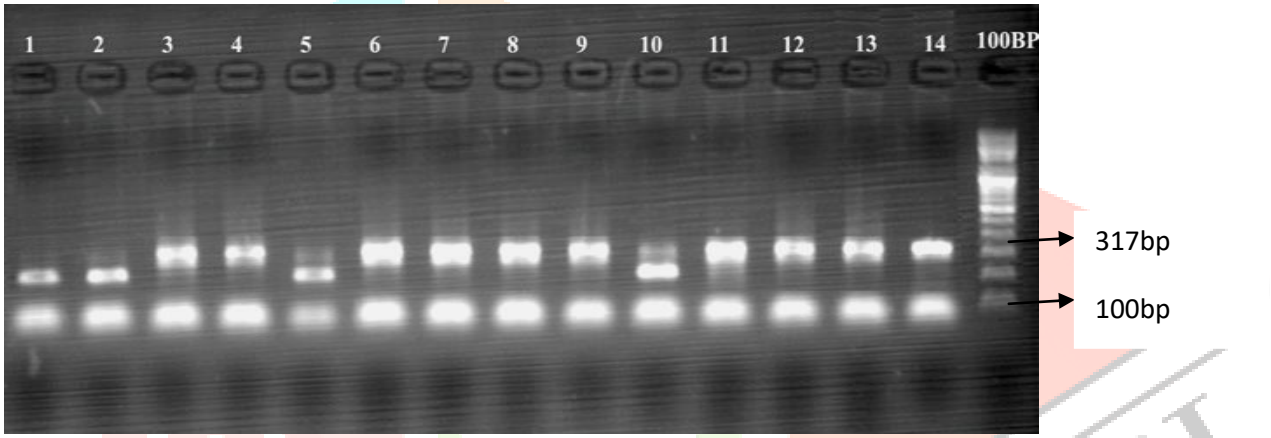


Figure 2 explained the agarose gel picture of PCR products of renal patients with tuberculosis patients. Lanes 1 to 14 contain PCR products of patients and Lane 15 contains DNA marker in 100bp. Internal control band was formed at 100bp and positive band at approximately 317bp. Lane 1 exhibited negative control while Lane 14 positive control. This figure showed that Lanes 3, 4, 6, 7, 8, 9, 11, 12 and 13 were positive and 2, 5 and 10 were negative.

Discussion

Renal tuberculosis is the most general site of extra pulmonary tuberculosis. This disease can result in cessation and destruction of renal mass and remedial can lead to obstruction, strictures and infection causes' renal functional loss and failure. [18]. Renal tuberculosis is still one of the major health problems in the many countries. One of the standard reasons for the malfunction of tuberculosis control programs is incapability to detect infectious cases early sufficient. Mycobacteria are typically present in relatively tiny number in the majority clinical samples including urine. [19] The first method by using Ziehl-Neelsen stain for detection of *Mycobacterium tuberculosis*. In this study Ziehl-Neelsen stain was negative in all urine specimens (table 1).

Although the Ziehl-Neelsen stain is rapid and easy on the pocket, but it lacks sensitivity. The detection of tuberculosis is based on conventional methods i.e. examination of Z N staining smear and culture on Lowenstein Jensen (L J) medium, while the detection criterion for genitourinary tuberculosis has based on the isolation of *Mycobacterium tuberculosis* from urine. This is not easy to obtain, as the discharge of organisms into the urine is sporadic and, more importantly, involves few organisms. Consequently, single specimen was probable to be false negative and at least 3 first morning specimens should be collected to provide the highest yield. [20] Another highly developed method such as polymerase chain reaction (PCR) has been useful in this study. Moreover, the results of this study agreed with [21] who reported that a significant variation has been noticed between PCR and Ziehl-Neelsen stain for detection tuberculosis infection. Furthermore has found that a significant difference with PCR and Ziehl-Neelsen stain for investigating renal tuberculosis and the percentage of positivity were lower when using Ziehl-Neelsen method. [22] PCR was supported that incidence of renal tuberculosis in males is more than females due to their excessive revelation to the sources of tuberculosis infections.[23]

Conclusion

PCR is a technique used to amplify very small quantity of a specific genomic sequence fast. The occurrence of a very small number of bacteria may be detected within few hours, the elevated sensitivity of PCR is mainly useful in paucibacillary situations such as extra pulmonary tuberculosis (renal tuberculosis). Also establish that PCR was not only rapid but also more correct than conventional methods for the detection of *M. tuberculosis, then*, PCR is a rapid and sensitive instrument to detect Mycobacterium tuberculosis in urine samples for Urinary tuberculosis patients.

Reference

1- Corbett EL Watt, Walker CJ, Maher N, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Int Med. 2003; 163: 1009-1021.

2- WHO Global Tuberculosis Report. 2017.

3- Wise GJ, Marella VK. Genitourinary manifestations of tuberculosis. Urol Clin N Am 2003; 30(1):111–21.

4- Leite OHM. Tuberculosis. Prob Gen Surg 2001; 18(4):69–7.

5- Ferrie BG, Rundle JSH. Genito-urinary tuberculosis in Glasgow a review of 230 patients. A review of 230 patients. Scott Med J. 1985; 30:30–4.

6- Schubert GE, Haltaufderheide T, Golz R. Frequency of urogenital tuberculosis in an unselected autopsy series from 1928 to 1949 89. EurUro1992; l 21:216–23.

7- Wise GJ, Marella VK. Genitourinary manifestations of tuberculosis. Urol Clin N Am 2003; 30(1):111–21.

8- Wise GJ, Shteynshlyuger A. An update on lower urinary tract tuberculosis. Curr Urol. 2008; 9:305-313.

9- Zarrabi AD, Heyns CF. Clinical features of confirmed versus suspected urogenital tuberculosis in region with extremely high prevalence of pulmonary tuberculosis. Urology. 2009; 74:41-45.

10- Menzies D, Joshi R, Pai M. Risk of tuberculosis infection and disease associated with work in health care settings. Int J Tuberc Lung Dis. 2007; 11:593-605.

11- Verhagen, Hof LM, Deutekom S, et al. *Mycobacterial* factors relevant for transmission of tuberculosis. J Infect Dis. 2011; 9 :1249-1255

12- Leung, CC Yam, WC Yew, et al. T-Spot TB outperforms tuberculin skin test in predicting tuberculosis disease. Am J Respir Crit Care Med. 2010; 6:834-40.

13- Figueiredo AA, Lucon, Junior AM, et al. Urogenital tuberculosis in immune compromised patients. Int. Urol. Nephrol. 2009; 41: 327-333.

14- Dochviri TZ, Katsitadze VA, Khosiashvili, GZ. et al..Detection of Mycobacterium tuberculosis in patients with Urogenital tuberculosis by PCR method. Georgian Med News. 2005; 9: 14-17.

15- Brown M, Varia H, Bassett P, et al. Prospective study of sputum induction, gastric washing, and bronchoalveolar lavage for the diagnosis of pulmonary tuberculosis in patients who are unable to expectorate. Clin Infect Dis. 2007; 11:1415 1420.

16- Rabhi M, Shawar, Found AK, et al. Detection of *Mycobactenium tuberculosis* in Clinical Samples by Two-Step Polymerase Chain Reaction and Nonisotopic Hybridization Methods' Journal of Clinical Microbiology, 1993; 1: 61-65.

17- Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,Cold Spring Harbor. 1989.

18- Brown M, Varia H, Bassett P, et al. Prospective study of sputum induction, gastric washing, and broncho alveolar lavage for the diagnosis of pulmonary tuberculosis in patients who are unable to expectorate. Clin Infect Dis. 2007; 11: 1415 1420.

19- Moussa OM, Eraky I, Osman HG, et al. Rapid diagnosis of genitourinary tuberculosis by polymerase chain reaction and nonradioactive DNA. J. Urol. 2000; 164: 584-588.

20- Chain K,. Clinical microscopy. Manual of Clinical Microbiology,. 1995;33: 51

21- Kamyshan IS, Stepanov PI, Ziablitsev SV.. Role of polymerase chain reaction in diagnosing tuberculosis of the bladder and male sex organs. Urol., 2003; 3: 36 39.

22- Heyns P, Whitaker PM, Van Helden P, .Polymerase chain reaction in the diagnosis of urinary tract tuberculosis. Urol Res. 1996; 24:107-110.

23- Aslan G, Doruk E, Emekda G, et al. Isolation and identification of *Mycobacterium tuberculosis* from the urine samples by conventional and molecular methods. Mikrobivol. Bull. 2007; 41:185-192.