

“Evaluation of PCR – Based Techniques for the Detection of Mycobacterium Tuberculosis Complex in Sputum Samples”

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Abstract: Tuberculosis has been one of the major causes of death globally. Due to the high risk of contact transmission. This has led to the increase in the morbidity and mortality rates and several countries have been endemic, especially in the developing countries. Ranked as the second leading cause of morbidity from communicable diseases after HIV/AIDS. Study evaluated the Mycobacterium T. complex from sputum samples and perform PCR using IS6110 gene sequence to detect MTB complex. Cross-sectional study design with sample of 100 collected from the OPD and IPD conveniently at the department of micro-biology. Study period was from Aug., 2012 to Aug., 2015. Sputum samples for smear culture was collected as per the RNTCP guidelines. About 70% of the males and 30% of females were susceptible. Of which at 72 weeks reported 86% with cough. Those with severe fever were about 73%, chest pain 60%, breathlessness 70% and loss of appetite 78%. Among the 100 sample in the ZN staining, 14% was positive and 86% negative. In the PCR sputum sample, 67% was negative and 33% positive. On comparing ZN smear positive and PCR, the sensitivity of PCR test was 100% in smear positive samples. While smear negative were also positive in PCR and sensitivity of PCR was 20.9%. In conclusion, isolation of 100 samples using microscopy and PCR indicated that there were discordance in 19% cases. Great care should be taken in designing primer pairs for the insertion sequence IS6110 to avoid false negative or false positive results. Pulmonary T. patients are more likely to be present with the specific symptoms. The yield of sputum smear positive Pulmonary T. cases can be improved if patients with more than 2 weeks history of cough are screened to diagnose such cases. Therefore there is urgent need for modern techniques especially in developing countries to facilitate better outcome of diagnosis and public health control measures.

Keywords: Mycobacterium Tuberculosis, Mortality, ZN staining, Sputum positive

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I. Introduction

The world health organization (WHO), South –Eastern Asia Region has reported Tuberculosis (TB) to be a major public health concern. India alone accounts to about 26% out of the 36% global burden of diseases. An estimate of about 3.4 million cases continue to occur year after year and especially 450,000 that died in 2012. Majorly from these five countries: Bangladesh, Thailand, India, Indonesia and Myanmar. Out of these patients, the percentage with HIV status was 39% and about 6% were reported positive. HIV- TB patients on co-trimoxazole preventive therapy was 89% and 69% on antiretroviral therapy (ART). More also, multi-resistant drugs level are low in the region at 2.2%. Allowing this to translate into about 0.0009 million multi drug resistant (MDR-TB) in 2012 among the TB cases reported [1]. The genus Mycobacterium bacterial, non-motile-sporulated rod like shaped, grouped into the rank of actinomycetes an estimated amount of 60-71% guanine plus cytosine [G+C] in the genomic deoxyribonucleic acid (DNA), in the wall with high lipid content and might be the highest among all bacteria [2]. In the ethology of TB within the M. tuberculosis complex called the tubercle bacilli, have different host like the zoonotic pattern and the reservoir. The M. Africanus, M. canetti, are subtypes of M. tuberculosis that are usually in humans. While M. bovis and M. microti are TB causative agents in animals but can be transmitted to humans [1].

Microscopic morphology of the habitat

In smear stained with carbon fuchsin or auramine, the tubercle bacilli, examined under the microscope, appears straight or slightly curved rod. When under the microscope does not allow mainly M. tuberculosis from the other mycobacterium. There are factors necessary for the growth and condition of the bacilli. Age is one of such factors of the culture, with the bacilli varying in size, and in shape, from short to long

rod. The dimensions of the bacilli have been reported to be 1-10 µm in length (usually 3-5 µm), and 0.2-0.6 µm in width. Therefore, the length of the microorganism is comparable to the diameter of the nucleus of a lymphocyte. Unlike some fast growing mycobacterium and other actinomycetales, M tuberculosis is rarely pleomorphic; it does not elongate into filaments, and does not branch in chains when observed in clinical specimens or culture. In the experimental macrophage infection, intracellular bacilli were described as being significantly elongated compared to broth-grown bacilli and, remarkably, to display bud-like structures [3]. Once the disease has been controlled, dying bacilli become sparser, often faintly and unevenly colored, due to partial loss of the internal contents. Of course, irregular staining may also be the consequence of technical defectiveness of dyes or staining procedures [4].

Structure of cell wall

As the most distinctive anatomical feature of the bacillus, the cell envelope has been the main object of research. Progressive chemical, molecular and ultra-structural research has produced robust knowledge on the synthetic pathways and structure of the mycobacterial cell envelope [5]. The envelope, which has been profusely represented by schematic models, is composed of the plasma membrane, a cell wall, and an outer capsule like layer. Surrounded, as in almost all bacteria, by a cell wall that protects the cell contents, provides mechanical support and is responsible for the characteristic shape of the bacterium. The mycobacterial cell wall, however, is constituted by an inner peptidoglycan layer, which seems to be responsible for the shape-forming property and the structural integrity of the bacterium. Indeed, the degree of peptidoglycan unusual high number of cross-links in the cell wall of M. Tuberculosis is 70-80 % whereas that in E. coli is 20-30 % [6].

Acid Fastness

Unlike Gram-negative bacteria, mycobacteria do not have an additional membrane in the outer layers of the cell wall. They are structurally more closely related to Gram-positive bacteria. However, mycobacteria do not fit into the Gram-positive category as the molecules attached to the cell wall are distinctively lipids rather than proteins or polysaccharides. Frequently, they do not retain the crystal violet and appear as “ghosts” after Gram staining. The waxy cell wall of mycobacteria is impermeable to aniline and other commonly used dyes unless these are combined with phenol [7]. To discover the causative agent of TB, Robert Koch had to develop a specific staining process using alkaline dyes. Soon after, Ehrlich discovered the acid fastness of the tubercle bacillus, which has been the prominent characteristic of mycobacteria up until now. The expression “acid-fastness” describes the resistance of certain microorganisms to decolorization with acid-alcohol solutions after staining with aryl methane dyes such as carbolfuchsin. This feature is of utmost practical importance in identifying the tubercle bacillus, particularly in pathological specimens [8].

Incipient Tuberculosis and Symptoms

In socio-epidemiological studies of a poor rural population in India in the early 1960s, it was found that 95% of patients who were positive by direct smear microscopy were aware of one or more symptoms suggesting tuberculosis. About 70% complained of cough as the leading symptom, while the rest gave greater importance to other complaints [9]. About two-thirds had symptoms of only 1–3 months’ duration [10].

In another prospective study on case detection in a population of about 6 million, some 1600 smear-positive patients were interviewed about symptoms [11]. 73% of patients complained of cough, ranking it first or second in importance as a symptom. The remaining 20% complained of fever or influenza like illness, and only 7% denied having any subjective symptoms. The duration of symptoms was also similar, with 62% having had symptoms for less than 3 months and 83% for up to 6 months [12]. It thus seems that symptoms are present in more than 90% of patients with sputum positive by direct smear microscopy, and that these symptoms are apparent in the early phase of the disease. Since more than 90% of infectious patients develop perceptible symptoms within a few weeks of the onset of tuberculosis, early detection is possible – not by traditional mass radiography, but also by sputum examination of symptomatic persons [13]. The WHO Expert Committee on Tuberculosis emphasized the importance of case detection among patients with symptoms. Patients with cough of several weeks’ duration should have their sputum examined by microscopy as the first priority for case detection. If found to be sputum-positive, these patients are the first priority for treatment [14].

Clinical Features in pulmonary tuberculosis are generally similar in HIV-infected and HIV-negative patients. However, there are less frequently reported cough cases with HIV patients may be as a result of less cavitation, inflammation, and endobronchial irritation due to the reduction in cell-mediated immunity [15].

Sputum Smear Microscopy remains the cornerstone of tuberculosis diagnosis, even in areas of high HIV prevalence. Systematic studies in sub-Saharan Africa have shown that most HIV-infected pulmonary tuberculosis patients are sputum smear-positive, although the proportion of patients with smear-negative, suspected pulmonary tuberculosis is greater in HIV-infected than in HIV-negative tuberculosis patients. HIV-infected, smear-positive patients also tend to excrete significantly fewer organisms in sputum than HIV-negative patients, which can lead to acid-fast bacilli being missed if insufficient high-power fields are examined by microscopy [16, 17]

Laboratory Diagnosis

Smear examination has several operational advantages over culture: the results are available sooner, correlate with infectiousness, and identify both patients at high risk of death from tuberculosis if untreated and patients who require more drugs in the initial treatment regimen because of greater bacterial load. The timing of the diagnostic procedures will depend on the prevalence of tuberculosis in the community. In areas with a high prevalence of tuberculosis, smear examination should be the initial test. For diagnosis of pulmonary disease in areas with a lower prevalence of tuberculosis, smears and chest radiography may be performed simultaneously, a short course of antibiotics nonspecific for tuberculosis may be given, or a chest radiograph may be used as an auxiliary diagnostic procedure before smears and culture [18]. Screening is a public health activity intended only to detect and cure sources of infection, and is additional to diagnostic activities in persons consulting spontaneously. Because the objective is primarily to benefit the community, the procedure must be simple, convenient for the individual, and free of charge, and should not detract from the patient's original purpose in attending the clinic [19].

Sputum smear microscopy has a fundamental role in monitoring the response to treatment of infectious cases of pulmonary tuberculosis. Smear examination should be performed at the end of the initial phase of treatment; if smears are still positive, the intensive phase should be extended for an additional month. Smears should be examined during and at the end of the continuation phase to confirm cure. The conversion rate at 2–3 months (defined as the proportion of initially smear-positive patients with negative smears out of the total who started treatment) is a good operational indicator. It shows the capacity of the programme to maintain patients on treatment, obtain smear samples, and eliminate sources of infection, and it is an early surrogate of the treatment outcome indicator [20]. In order to assess the qualitative performance of sputum examination in rural health institutions, several studies were carried out by the National Tuberculosis Institute, Bangalore, India [21]. The authors concluded that non-specialized staff of general health institutions is capable of carrying out satisfactory smear microscopy. Taking into consideration the short period of training usually received, it may be expected that, with continuous supervision and corrective retraining, the performance of such microscopists could be maintained at a satisfactory level [22].

The following high risk groups, designated by the center for Disease Control and Prevention (CDC) advisory council for the elimination of tuberculosis, should be screened for tuberculosis: Close contacts (those sharing the same household or other enclosed environment) of persons known or suspected to have tuberculosis, Persons with HIV, Persons who inject illicit drugs or other locally identified high risk substance users (such as crack-cocaine users), Persons who have medical risk factors known to increase the chance for disease if infection occurs, Resident and employees of high risk congregate settings (such as correctional institutions, nursing homes mental institutions, other long term residential facilities, and shelters for the homeless, Healthcare workers who serve high risk clients, Foreign born persons, including children, recently arrived (within 5 years) from countries that have a high incidence or prevalence of tuberculosis, Some medically underserved low income population, High risk racial or ethnic minority populations as defined locally, Infants, children, and adolescents exposed to adults in high-risk categories.

Objectives of the study is to evaluate mycobacterium tuberculosis complex from sputum samples and to perform PCR using IS6110 gene sequence to detect MTB complex

II. Material And Methods

Place of Study: Department of micro-biology Santhosh Medical College, Ghaziabad Uttar Pradesh 201009, India. Study sample: A cross sectional study design and study duration was from August, 2012 to August, 2015.

Sample size: 100 sputum samples were collected from the patients at the OPD and IPD in the microbiology department conveniently. Inclusion criteria: Symptoms of tuberculosis: Patients with chronic cough ≥ 2 weeks. Patients with fever ≥ 2 weeks and patients having cough with or without sputum. Exclusion criteria: Patients who refuse to give consent form and patients not willing to participate.

Analysis: This study compared the slides between the ZN slide method culture, PCR technique including their sensitivity and specificity.

Procedural for Sample Collection

Written informed consent was collected from microbiology department, Santhosh Medical College before the commencement of the study. Well-designed questionnaire was used to gather the demographic information regarding the patients. Sputum sample for smear and culture were collected as per the RNTCP guideline. [23, 24].

Secondly is direct microscopy using sputum smear preparation: Sputum smear were prepared nearer to the flame (spirit lamp). Labeled a new clean, unscratched slide. Mucopurulent portion was used for smear preparation, transferred an appropriate portion of the specimen to the slide by using a broom – stick or chrome wire loop of 5mm dm (27SWG). Smear the specimen over an area of approximately 2 by 3cm. Allowed smear

to air dry for 15 minutes. Fixed the smear to the slide by passing it over the flame 3 to 5 times for 3 to 4 seconds each [24].

Preparation of staining solution (Z.N. staining) [25]: (a) carbol- fuchsin with basic fuchsin (Hi media of 10 gm, absolute alcohol 100 ml, phenol 50 gm, distilled water 900 ml. Procedure: Weigh 10 gm of basic fuchsin dye in a balance & transfer it to 250 ml Erlenmeyer flask. Add 100 ml of absolute alcohol & dissolve the dye by placing it in a water bath at 60 degrees centigrade. Measure 50 ml of phenol and add to the basic fuchsin solution and mix gently. Transfer the contents into a 1000 ml measuring cylinder. Add distilled water to make up the final volume to 1000ml. Pour the solution through filter paper (Watman No. 1) and store filtered solution in a glass bottle. Label the bottle as 1% carbol- fuchsin. (b) sulphuric Acid (H₂SO₄) 25 % using conc. H₂SO₄ 250 ml, distilled water 750 ml. Take 750 ml distilled water in a flask. Carefully add concentrated sulphuric acid to the water. Mix gently and store it in amber colored bottle and label it as 25% sulphuric acid. (c) Methylene Blue 0.1%: Methylene blue (BDH) 0.5g. Distilled water 500 ml. Procedure:- Weigh 0.5 gm of methylene blue and transfer to a 1 liter flask. Add 500 ml of distilled water. Shake well & dissolve. And store in a glass bottle.

B. Ziehl - Neelsen Staining:- Place the slide on a staining rack with the smeared slide facing up. Flood entire slide with filtered 1% carbol – fuchsin. Heat each slide slowly until it is steaming. Maintain steaming for five minutes by using intermittent heat. Rinse each slide individually in a gentle stream of running water until all free stain is washed away. Flood the slide with 25 % sulfuric acid (H₂SO₄.) solution for 2 – 3 minutes. Rinse the slide thoroughly with water. Drain off excess water from the slide. Flood the slide with 0.1% methylene blue for 30 seconds. Rinse the slide thoroughly with water. Drain excess water from the slide. Allow smear to air dry or slide dryer [26].

C. Observation: - Stained smear were scanned under oil immersion. Apply one drop of liquid paraffin oil (heavy) immersion oil to the left edge of the stained smear. Stained smear were scanned systematically from left to right side. Grading the smear done according to WHO guidelines [27].

Polymerase Chain Reaction (PCR)

Processing of samples:

The deposit of the sputum samples obtained after processing with modified petroff’s methods were used for DNA extraction which were then used for amplification of gene sequence by PCR.

DNA was extracted using commercially available QIAmp DNA mini kit, QIAGEN, Germany with one initial additional step. The preliminary processed materials were kept at 80oC for 10 min for inactivation of possible mycobacterium. The material was then further processed as per the guidelines of the manufacturers of the kit to obtain the DNA. DNA sequences were amplified in the PCR thermal cycles model 2700 (Applied Bio systems).

Amplification

In each independent PCR assay, test results were compared with the results for one positive and one negative control. The positive control includes the DNA of H37Rv and negative control includes the PCR grade water. The 123 bp sequence of IS6110 gene was amplified using primers.

5’- CCT GCG AGC GTA GGC GTC GG – 3 and 5’- CTC GTC CAG CGC CGC TTC GG - 3’.

PCR protocol was observed for 50 µl reaction: 2x Master Mix 25.0 µl, forwards primer 0.5 µl (10pmole), reverse primer 0.5µl (10pmole), template 0.5 µl, water 19 µl. PCR condition was cycles 30 cycles: pre-denaturation 94°C, 5 min, denaturation 94°C, 5 min, annealing 65 °C, 45 sec, extension 72°C, 45sec, final extensions 72°C, 10min The amplification product was separated on 1.5% agrose gel. The sample showing the presence of 123 bp band under ultraviolet transillumination were considered positive.

III. Result

Table: 1. Clinical profile of patients (n=100)

Demographic/ Clinical features	Total No of suspects	
Sex	Male patients	70 (70%)
	Female patients	30 (30%)
Mean age	Male patients	38.15 years.
	Female patients	39.77 years.
Cough	>2 weeks	86 (86%)
	<2 weeks	14 (14%)
Fever	>2 weeks	73 (73%)
	<2 weeks	27 (27%)
Chest pain	60 (60%)	
Breathlessness	70 (70%)	
Hemoptysis	14 (14%)	
History of contact	45 (45%)	
Loss of appetite	78 (78%)	

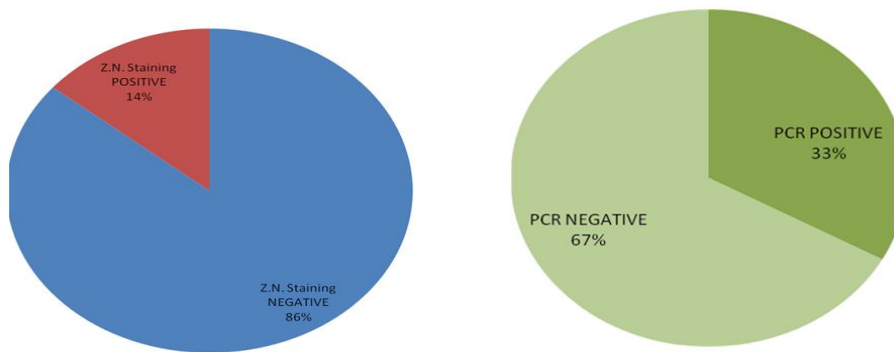


Table: 2. Comparison of Z.N. Smear Positivity & PCR.

	PCR		Sensitivity of PCR Test
	Positive	Negative	
Z.N. Smear Positive	14	0	100%
Z.N. Smear Negative	19	67	20.9%

In table 1, of the total number of patients (100), the numbers of male patients were 70 (70%) and female patients were 30 (30%). This data also shows that the average mean age of the male patients were 38.15 yrs and the 39.77 years for female’s patients. 60% of the total patients had chest pain and 45% had infection by contact of infected person followed by haemoptysis (14%). The patients also showed loss of appetite (78%). Out of the total (100) samples, 14 (14%) samples were smear positive for acid fast bacilli (AFB) and 86 were smear negative. The isolation rate of *M. tuberculosis* was 14(14%) in sputum samples in Z.N. staining. In fig 2 is Agarose gel electrophoresis of PCR technique for the confirmation of IS6110 sequence. 1, 123bp ladder. 2-13 positive samples, and positive control & negative control at the end. Out of 100 samples in table 2, 14 (14%) samples were Z.N. Smear positive for acid fast bacilli (AFB). Out of 86 Sputum samples which were smear Negative, 19 (19%) were positive in PCR. The sensitivity of PCR test was 100% in smear positive samples, While which were smear negative were also positive in PCR and sensitivity of PCR was 20.9%.

IV. Discussion

In the present study the clinical history of the patients showed that number of male patients 70(70%) was higher as compared to that of female patients 30(30%). The global data on tuberculosis prevalence has shown that the prevalence of *M. tuberculosis* is similar in males and females until adolescence; but after that it appears higher in males [28]. Several studies have explored reasons behind the gender bias in tuberculosis susceptibility and found that fear and stigma associated with TB makes greater impact on women than on men [29]. The analysis of epidemiological data by Thorson et.al [30, 31] also reflects gender to have an impact on the disease and its control. Such differences may be related to culture beliefs, traditional customs and practices, labour division within the house hold and seasonal work preventing woman from being diagnosed in time and treats properly [32]. The social and economic impact of Tuberculosis (TB) which claims lives of more than 400,000 people every year is devastating, especially as it affects the economically most productive age group [33, 34].

Our result showed that 86% of patient had coughed more than two weeks. Earlier study done in 2002 in a different setting showed 47% increase in sputum positive cases among chest symptomatic with >2 weeks cough [35]. Baily et al in 1967 reported an increased yield of 16% smear positive cases when the screening criterion used was cough of >2 weeks (44 of 622 with >2 weeks vs. 37 of 275 with > 3 weeks) [36]. Also most of the patients showed chest pain and loss of appetite followed by haemoptysis and history of contact. In the present study the smear positive rate was approximately higher for men than for women suggesting that the sex differences reflects biological phenomena rather than lower access to TB diagnosis for women. These may include not only true differences in TB incidence, but also differences in the bacillary load of sputum specimen and thereby in the sensitivity of smear examination [37]. This result correlates with Mashreket.al. [38], but not with that of Oliver and Harvey from Australia, who reported the majority in older ages (after 70 yrs.).

The sensitivity of smear microscopy can be affect by many factors, counting sputum quality, smearing grounding, staining procedures, assessment time and the amount of training received in correct smear examination. The sensitivity of microscopy increases due to concentration of sputum specimens, e.g. by centrifugation. The result obtained in the study of 100 isolates by microscopy and PCR are reported in Table 2. A total of 14(14%) specimens were positive by ZN microscopy versus 33(33%) PCR positive. However, discordance was present in 19(19%) cases. 19 specimens were positive in PCR and 19 specimens were negative in ZN microscopy. Hence, there is an urgent need of the new techniques; for safe, rapid and correct diagnosis of

TB Molecular techniques has becomes trends in rapid diagnosis of tuberculosis. Some of the studies reported lower efficiency of PCR in detection of MTB by amplification 16S r-DNA and hsp65 genes and IS6110. Therefore the marker sequence like “IS6110” is the best choice. This exists only in MTB-DNA amplification. The insertion sequence “IS6110” can also be used for distinguishing of the members of MTB complex from other mycobacteria in routine tests. Early diagnosis of tuberculosis is essential for clinical management and public health control measures. In a study carried out in India by Negi et al. that compared different PCR protocols targeting different *M. tuberculosis* gene sequences, PCR targeting IS6110 was found to have better positivity (77%) than other molecular targets like 65 kDa (75%), 38 kDa (72%), and the 85B protein (73%). Among the samples with negative results provided by conventional tests, IS6110-PCR showed greater positivity (26%) than PCR carried out for other targets. The PCR targeting of IS6110 has been widely reported by various reference centres for TB diagnosis that reported that it was simple and reproducible [39].

In the present study, we compared the results of PCR with the ZN microscopy. Fast DNA preparation method was used for PCR for rapid detection of *M. tuberculosis*. A total number of 100 isolates were studied. All the isolates were tested by ZN microscopy and by PCR. In smear negative specimens the bacterial load was low, often challenging for the rapid diagnostic methods such as PCR. This PCR protocol involves less manual handling procedures so that minimum risk of contamination. The 90% specificity of the carefully designed primers perfectly distinguishes MTB from other mycobacterial species in microscopy. PCR based method could reduce the false negative result in routine service. PCR would be used to supplement conventional diagnostic methods.

V. Conclusion

Z.N. Staining microscopy of sputum smears is simple and inexpensive, quickly detecting infectious cases of pulmonary TB but giving sometimes false negative result. To implement proper public health control measures, ZN microscopy is also essential for community. We proposed (recommended) that great care should be taken in designing primer pairs for the insertion sequence IS6110 to avoid false negative or false positive results. For diagnostic PCRs, multiple targeting regions such as IS6110 could be fine policy to enhance correctness of *M. tuberculosis* detection in clinical specimens. Men are more vulnerable to get TB as compared to women. This vulnerability is because of their social contacts, exposure to dust, smoking and consumption of alcohol. Pulmonary tuberculosis patients are more likely to present with specific symptoms. The yield of sputum smear positive pulmonary tuberculosis cases can be improved if patient with >2 weeks history of cough is screened to diagnose such cases. The data suggests that in TB epidemic areas, most of the cases of TB can be diagnoses appropriately by simple and cheap methods which are generally available at district hospital level. Although acid fast bacilli (AFB) microscopy, and PCR remain the cornerstone of the diagnosis of TB.

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