Original Article

TB Diagnostic Techniques

Comparison of Conventional TB Diagnostic Techniques with PCR IS6110 in Tertiary Care Chest Hospital Lahore Pakistan

1. Somayya Tariq 2. Sabeen Akhtar 3. Atiqa Ambreen 4. Saba Riaz

1,2. PG Students of M.Phil, Deptt. of Microbiology and Molecular Genetics, University of the Punjab 3. Consultant of Chest Disease, Gulab Devi Chest Hospital, Lahore 4. Asstt. Prof. of Microbiology and Molecular Genetics, University of the Punjab.

ABSTRACT

Objective: To compare the efficiency of conventional diagnostic techniques and Insertion Sequence (IS)6110 based PCR assay for M. tuberculosis in pulmonary and extra-pulmonary specimens from tertiary care chest hospital.

Study Design: Observational study.

Place and Duration of Study: This study was conducted at Gulab Devi Chest Hospital, Lahore from August to January 2013.

Materials and Methods: A total of 1599 (1417 pulmonary and 182 extra-pulmonary) non-duplicate clinical specimens, obtained over a period of six months, were tested by conventional techniques such as Ziehl-Neelsen staining (ZN), Lowenstein Jensen (LJ) medium and Fluorescent staining. MTB was extracted through DNAzol method. Insertion Sequence (IS) 6110 based PCR assay was used for M. tuberculosis from pulmonary and extra-pulmonary specimens. Of the 1599 specimens, 781 were suspect cases while 818 were MDR (follow up) cases. Mean age of TB patient was ±33 years. 18% of follow-ups and 20% of suspects were <20 year in age, 52% follow-ups and 36% suspects were about 20-40 years, and 30% follow-ups and 33% suspects were >40 years of age.

Results: It was seen that, among MDR cases (follow-ups) 68% were males and 32% were females. Similarly, among TB-suspects, 58% were males and 42% were females. Of total 168 suspected pulmonary samples ZN (48.2.7%), fluorescent microscopy (79.7%), LJ culture (52.9%) and PCR (91.6%) were positive for M. tuberculosis. In total 143 suspected extra-pulmonary samples, ZN (34.95%), fluorescent microscopy (45.5%), LJ culture (39.8%) and PCR (87.4%) were positive.

Conclusion: In contrast to conventional methods of TB diagnosis, PCR is more quick, sensitive, reliable and cost effective technique.

Key Words: MDRs, Bacteriocins, Lactobacilli, Antibiotic Resistance, MAR, Antibacterial activity

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INTRODUCTION

Tuberculosis (TB) a common and life threatening infectious disease and it is a burning issue for several years in health care setting. Pakistan stood on fourth position among TB burden countries.[1] It is globally prevalent chronic disease, caused by *Mycobacterium tuberculosis*, and is present devastatingly in the developing countries like Pakistan. But if accurately diagnosed and properly treated, this disease is quite curable. Accurate laboratory findings with accessibility is the need in the war against TB.[2] In 1993 global emergency was declared on tuberculosis by the World Health Organization. According to the latest estimates included in the "Global Tuberculosis Report" (2013) are that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths, showing subtle decline in overall ratio.[3] TB is slowly declining but MDR and XDR TB is becoming a serious challenge in the attempt to eliminate TB. In mycobacteriology, smear microscopy is still the most rapid easiest and cheapest procedure with specificity of over 99% (*Mycobacterium*spp). However, the sensitivity of microscopy is not up to the requirement since 25% to 50% smear microscopy of a respiratory specimen provides false-negative results.[4] Thus, where a positive smear test of a respiratory specimen helps in making a presumptive diagnosis of tuberculosis, a negative test does not rule out the disease.[5] However, the good laboratory practice requires the confirmation of any fluorescence microscopy smear-positive results by a ZN stain. Thus, the advantage of the rapid detection of acid-fast bacteria (AFB) by fluorescence microscopy is reduced. The recent re-emergence of tuberculosis has brought the shortcomings of the laboratory diagnosis of tuberculosis to light. In the last few years of the 20th century, the introduction of broth-based cultivation (BACTEC) and molecular biological methods(NAAT

Correspondence: Dr. Saba Riaz,
Assistant Professor, Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore
Contact No.: 03214530648
E-mail: saba.mmg@pu.edu.pk

or PCR) gave birth to significant changes and improvements in the laboratory methods used.[6] However, the authenticity and accuracy of any new method can be assessed only by comparison with conventional techniques currently available in laboratories.[7]

The present study was conducted to compare the efficiency of conventional diagnostic techniques and Insertion Sequence (IS) 6110 based PCR assay for *M. tuberculosis* in pulmonary and extra-pulmonary specimens from tertiary care chest hospital.

MATERIALS AND METHODS

Gulab Devi Chest Hospital, Lahore is 1500 beds hospital giving tertiary health care to patients with all sorts of cardiac and pulmonary diseases, especially tuberculosis. One thousand five hundred and ninety nine samples were collected (based on clinical and radiological findings) during August to January 2013. Significant demographic data were analyzed. All important diagnostic techniques were performed to see the recent trends in TB diagnostics in collaboration with Citilab and Research Centre, Lahore. Sodium hydroxide (modified Petroff) method was practiced to process and decontaminate specimens for culture. Equal volume of 4% NaOH was added to the specimen (at least 2 ml, not more than 5 ml) contained in the centrifuge tube, vortexed and allowed to stand for 15 minutes at room temperature before filling the tube with phosphate buffer (0.067 Mol/liter, pH 6.8; Na_2 PHO_4 9.47g; KH2PO_4 9.07g). The specimens were then centrifuged at 3000g for 15 minutes and the supernatant discarded and deposits were resuspended in approximately 0.2 ml phosphate buffer. Ziehl–Neelsen (ZN) and fluorescence staining technique was performed (Auramine Fluro AFB Colour Medilines). Löwenstein–Jensen medium(Salt Solution (2.4g KH_2PO_4, 0.24g $MgSO_4$ $7H_2O$, 0.6g Magnesium citrate, 3.6g L-Asparagine, Sodium glutamate, Distilled water(600ml) Glycerol (ml) or pyruvate(g) 12 ml or 7.2 g);Egg homogenate1000 ml Malachite green (2%) 20 ml; pH about 6.8) labelled with the ID number and incubated at 37°C for 4 to 8 weeks for the isolation of Mycobacterium tuberculosis.

Extraction of the MTB DNA was done by using liquid samples directly. 130µl of sample was added in each labelled eppondorf followed by 300µl of DNAzol reagent. The mixture was mixed with pipette and waited for 5-10 minutes at room temperature. 200µl of chilled isopropanol in each mixture was added and vortexed for 1 minute. The mixtures were then centrifuged for 5 minutes and each sample was decanted on tissue paper. Then again 300µl dnazol in each sample was added, centrifuged for 5 minutes and decanted on tissue paper. 900µl 70% ethanol was added and centrifuge for five minutes. The pellets were dried at room temperature for 30-40 minutes. After that suspended themin 20µl of de-ionized water.

50µl reaction mixture was prepared which contained a buffer consisting of 50 mM KCL; 2mM MgCL2; 10 mMTris HCL (pH8.3); 200 µM dNTPs, 0.5 µM of each primer, 1.5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CO), and 5 µl of extracted DNA from the pulmonary samples. The oligonucleotide primers (Loftstrand Laboratories, Gaithersburg, MD) used were TB1 (5'-GTG CGG ATG GTC GCA GAG AT -3'and TB2 (5'-CTC GAT GCC CTC ACG GTT CA -3'). These primers amplified a target fragment of 538 bp from the insertion-like M. tuberculosis sequence elementIS6110.27. In a thermocycler amplification was done with an initial cycle of denaturation (95ºC for 5 min), 40 cycles of amplification (95ºC for 1 min, 60ºC for 1 min, and 72ºC for1 min), and finally an extension cycle for 5 min. 5µl of the PCR product was loaded in 1.5% agarose gel in 1x Tris-Borate EDTA (TE) buffer and allowed for electrophoresis in a mini gel box for 30 minutes at 120 volts.

RESULTS

A total of 1599 TB cases were processed from August to January (2013) in tertiary care chest Hopital. Out of which 818 were the follow-up and 781 were the TB-suspect cases. On average 163 patients every month visit the laboratory for MDR-TB evaluation and 156 individuals come as TB suspects (Fig. 1). The six month record reveals the mean age of TB patient was 33 years. Three categories of the age of the patients were formed. 18% of follow-ups and 20% of suspects were <20 year in age, 52% follow-ups and 36% suspects were about 20-40 years, and 30% follow-ups and 33% suspects were >40 years of age. 11% of TB-suspects were not aware of their age. It is seen that, among MDR cases (follow-ups) 68% were males and 32% were females. Similarly, among TB-suspects, 58% were males and 42% were females (Table 1).

Table No.1: Tuberculosis prevalence in gender and age

TB Cases (1599)	Gender		Age (years)			
	Male	Female	<20	20-40	>40	Unknown
Follow-up (818)	32	68	18	52	30	-
Suspects (781)	42	58	20	36	33	11

Three hundred suspected cases (168 pulmonary and 143 extra-pulmonary) were processed for ZN, fluorescent microscopy, LJ-culture and PCR. Out of 168 pulmonary samples 147 were sputum and 21 were BAL. On the other hand extra-pulmonary samples include: lymph node 33, Pus 30, plueral fluid 48, urine 12, tissue 17, CSF 2, Pericardial fluid 1. PCR IS 6110wereperformed and positive samples showed a band on 538 bp. PCR results were more positive (91.6%) in pulmonary samples as compared to extra-

pulmonary samples (87.4%). Similarly, all other techniques showed high percentage of positivity in pulmonary suspected cases than exta-pulmonary suspected cases. Among the suspected pulmonary TB group of 168: ZN was positive in 97 (48.2%), Fluorescent microscopy in 129 (79.7%), LJ culture in 89 (52.9%) and PCR in 154 (91.6%). Whereas, in the 143 suspected extra-pulmonary TB group: ZN was positive in 39 (34.95%), Fluorescent microscopy in 50 (45.5%), LJ culture in 57 (39.8%) and PCR in 115(87.4%) [Table 2].

Table No.2: Comparison of ZN staining, Fluorescent Microscopy, LJ culture and IS 6110 PCR

Specimens of suspected tuberculosis		n=311	ZN staining		Fluorescent Microscopy		LJ Culture		PCR	
			Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Pulmonary	Sputum	147	85	62	112	35	78	69	135	12
	BAL	21	12	9	17	4	11	10	19	2
	Positive (%)	168	97 (48.27%)		129 (79.7%)		89 (52.9%)		154 (91.6%)	
Extra-Pulmonary	Lymph Node	33	11	22	15	18	9	24	28	5
	Pus	30	11	19	14	16	9	21	21	9
	Pleural Fluid	48	16	32	20	28	22	26	41	7
	Urine	12	3	9	4	8	6	6	10	2
	Tissue	17	9	8	12	5	9	8	13	4
	CSF	2	-	2	-	2	1	1	1	1
	Pericardial Fluid	1	-	1	-	1	1		1	-
Total	Positive(%)	143	39 (34.95%)		50 (45.5%)		57 (39.8%)		115 (87.4%)	

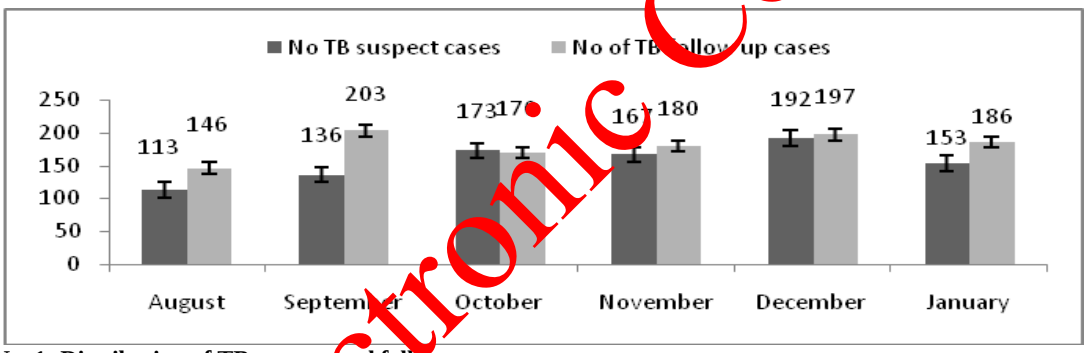

Figure No. 1: Distribution of TB suspect and follow-up cases

DISCUSSION

Many of the TB cases may be treated successfully with the appropriate chemotherapy, but diagnosis remains a huge hurdle to TB elimination. It is well-known fact that conventional methods to diagnose tuberculosis carry several limitations, which have encouraged the use of molecular techniques for AFB diagnosis.[8] Pulmonary and extra pulmonary TB types are different on the basis of the location of the infection which could be inside or outside lungs, respectively.[9] It is great success with PCR in diagnosis of MTB from gastric aspiration.[10] Pulmonary disease is believed to be more common, but extra-pulmonary remains quite severe because of unapparent and nonspecific outcome.[11] Auramine-rhodamine stains (FM) showed better sensitivity for extra-pumonary samples.

Three of the extra pulmonary samples: lymph node, urine and pleural fluid showed positive results for FM staining and so that for the culture, but they were negative for the ZN staining. Here, we found more smear-positive by FM staining. Additionally, in most of the cases ZN staining require two samples for reporting the results one at fasting condition and second after the breakfast or meal but FM is independent of this fact and yield even better results with only one sample. On the sensitive basis FM was observed to be a more cost-effective technique than ZN and applying FM on the sputum specimen.[12] By applying FM we can put on a larger number of patients on regimen which are positive for tuberculosis. This will lead to the better improvement outcomes in the treatment of the tuberculosis. Moreover with FM more patients can be diagnosing in lesser time and more importantly in an efficient way.[13] In case of MDR TB diagnosis, FM also has added advantage as in the progressing months of the treatment the sputum samples yield fewer bacilli which sometimes not detectable by the ZN staining. In general, tuberculosis are diagnosed by conventional methods which include sputum smear microscopy, chest radiographic findings and culture studies.[14] These methodologies prove unproductive due to low

Mycobacterium levels in the specimen or time consuming procedures.[15] Another important technique is nucleic acid amplification based assays are the most appropriate choice for the identification of MTB.[9] These molecular techniques have eliminated diagnostic limitations with better detection rates in smear negative samples with a high degree of sensitivity and specificity in both pulmonary and extra-pulmonary cases.[16] Direct detection of the *M. tuberculosis* from the sample using NAATs (Nucleic Acids Amplification Techniques) is one of the most promising and accurate way to evaluate the presence of the tubercle bacilli.[17] When compare with the culture, these techniques take an advantage of being rapid and free of labor.

CONCLUSION

Fluorescent microscopy is more sensitive than conventional microscopy when compared to culture as the gold standard. In contrast to conventional methods PCR is the most rapid, sensitive and reliable technique for pulmonary and extra-pulmonary tuberculsosis. Furthermore, MTB is still one of the worrisome issue being highly prevalent in this region.

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Conflict of Interest: The study has no conflict of interest to declare by any author.

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