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Polymerase Chain Reaction (PCR) based Diagnosis of Extra-Pulmonary TB in Clinical Samples by Targeting IS6110 Sequence

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Abstract: ***India has the highest burden of TB. Mycobacterium tuberculosis (TB) is one of the oldest and biggest killer of the humans compare to all other infections. Tuberculosis (TBC) is an infectious disease caused by some species of genus "mycobacterium". Accurate and early detection of mycobacterium tuberculosis decrease incidence of tb. There is a several techniques available but PCR based technique is most common for diagnosis. TB is divided mainly in two parts that are Pulmonary (in side of lungs) and Extra-pulmonary (outside of lungs) .We have focused on extra-pulmonary TB. A small amount of mycobacteria inhalation can able to infect us. Mycobacterium tuberculosis is a slow growing bacterium and doubles at every 18 to 24 hours. This project was based on PCR method. First of collect the sample and DNA extraction. Then run PCR for the PCR product. Load the sample in Agarose gel electrophoresis for checked the results of sample. India has the highest burden of TB. Mycobacterium tuberculosis (TB) is one of the oldest and biggest killer of the humans compare to all other infections.Tuberculosis (TBC) is an infectious disease caused by some species of genus "mycobacterium". Accurate and early detection of mycobacterium tuberculosis decrease incidence of tb. There is a several techniques available but PCR based technique is most common for diagnosis.TB is divided mainly in two parts that are Pulmonary (in side of lungs) and Extra-pulmonary (outside of lungs) .We have focused on extra-pulmonary TB.A small amount of mycobacteria inhalation can able to infect us.Mycobacterium tuberculosis is a slow growing bacterium and doubles at every 18 to 24 hours. This research was based on PCR method. Different samples of extra-pulmonary were collected from the laboratories, which were taken for the diagnostic purpose. Those samples were checked for the absence or presence of the any mycobacterium species by amplifying the IS6110 sequence.***

Keywords: TB, MTB, TBC, *Mycobacterium tuberculosis*, PCR

1. Introduction

India is 2nd most populated and developing country. India is the country having highest burden of tuberculosis according the WHO data of 2011(Manganelli, Dubnau, Kramer, & Smith, 1999).Accurate and early detection of bacteria in clinical samples is a crucial step for decreasing the incidence of TB disease. There is several techniques available based on different principle to detect bacterial infection in sample. Smear examination, culturing, ELISA based methods and PCR – based techniques are common for diagnosis(Amin et al., 2011).*Mycobacterium* is a genus of bacteria having species *tuberculosis*, which is a causative agent of most TB cases(Soolingen et al., 1997).*Mycobacterium tuberculosis* (MTB) is first discovered by Robert Koch in 1882.Complete sequenced H37Rv strain of the *M. tuberculosis* genome consists of 4.4 x 10^6 bp and having approximately 4000 genes.

Tuberculosis (TBC) is an infectious disease caused by some species of genus "*mycobacterium".Mycobacterium tuberculosis* is the most common bacterium responsible for causing TB. Based on site of infection, TB is divided mainly in two parts that are Pulmonary (in side of lungs) and Extra-pulmonary (outside of lungs). Extra-pulmonary Tb has further many sub types that depend on the organ or part of body infected (Maher D et al. 1997).

Pulmonary TB (PTB):
A cough for 2 weeks or more is a most common sign to define a person for a suspect of TB.TB infection can be got by inhaling the air droplets from an infected person's sneeze or cough. PMT generally develops in the minority people whose immune system does not successfully eliminate the primary infection. The disease develops within a week after got infected, or infection lie dormant for many years before causing the disease.

Extra Pulmonary TB (EPTB):
The term EPTB is used to describe the isolated occurrence of tuberculosis at body site other than the lung.TB can spread to each and every organ of the body. Main organs or parts can be infected with MTB are brain, bones, abdomen, heart, lymphatic system, urinary track and lungs(Amin et al., 2011).Some types of EPTB areLymph node tuberculosis, Pleural effusion and empyema thorasis, Abdominal Tuberculosis, Neurological TB, Pericardial TB, Bone and Joint TB, Genitourinary tuberculosis, Female genital TB, Cutaneous TB, and TB of the breat(K. Sharma & A. Mohan 2004).

A small amount of mycobacteria inhalation can able to infect us. The transmission of bacteria usually occurs by cough, spit, sneeze, and talk of infectious people, thus propel the tuberculosis bacilli. At the time of primary infection, organism spreads systematically, the on later stage, it may be activated at genital site. Mycobacterium tuberculosis is a slow growing bacterium and doubles at every 18 to 24 hours.BCG is made of a live form of attenuated form of bovine tuberculosis bacillus – *Mycobacterium bovis.* The BCG vaccine is 80% effective in preventing human tuberculosis but its preventing effects are varying according

to geography (Mh, Stell, & Sa, 2008).In developing countries, specifically in India, Genital tuberculosis is responsible for the major proportion of women having infertility (Muir and Belsey 1980). A research concluded that 5% to 10% women of world having genital tuberculosis, but it may vary 1% in United States to 18% in India (Muir & Belsey, n.d.).

Genital Tuberculosis (Type of EPTB)

Primary genital tuberculosis is very rare, where secondary genital TB is more common. It is always secondary to infection of lungs, kidneys, gastrointestinal tract, bones and joints.Fallopian tubes are involved 100% in cases of secondary infection, 50% in endometrium, 20% in ovary, 5% in cervix, <1% in vagina and vulva (Dawn C. S 1998 and Arora V. K et al. 1994). Partner with genital TB disease or infection can direct inoculate tubercle bacilli at vulva orvagina by sexual intercourse (Maurya, Kant, Nag, Kushwaha, & Dhole, 2012).The genital TB is occur in 10% cases of pulmonary TB and account for 5% in all female with pelvic infection (Dawn C. S 1998). Genital TB shows the Infertility, pelvic pain, menstrual disorders, and amenorrhea likes symptoms. Among them, infertility accounts for 60% of cases, where some patients are also asymptomatic (Dawn C. S 1998). Infertility due to Genital TB is occur because of pathology in endometrium and fallopian tube which makes block and stops the ovum transport (Schaefer G 1972 and Kumar A et al. 1997). Genital tuberculosis is a chronic disease and it show symptoms very rare with few specific complaints. Infertility was reported in 44% of total genital tuberculosis, where pelvic pain was present in 25% and abnormal vaginal bleeding in 18% noted. Vaginal discharge and amenorrhea were present in about 5% of cases, while bleeding in menopause period were detected in 2% of genital TB patients (Mh et al., 2008).

PCR assay is most reliable, highly sensitive, specific and rapid tool available to date for the diagnosis of M. tuberculosis in all samples with pulmonary and extra pulmonary TB (Amin et al., 2011).GeneXpert/RIF is less susceptible compare to routine PCR – based techniques because in PCR based methods, generated amplicon contamination can give false positive results (Blakemore et al., 2010).

2. Material and Method

Total 25 extra pulmonary samples were transported to our institute from the different laboratories. Samples were collected with the aim of diagnosis, so the ethical approval is not required for this study. We didn't disclose any identity or information related to identity. Samples were stored at 4^0C till the DNA extraction. Tissue samples were processed with QiAamp DNA blood mini kit as described in manufacturer's protocol. Fluid samples were processed with manual protocol. Samples were pellet down with high speed centrifugation. Pellet was given two to three wash with phosphate buffer. Then pellet was dissolved in 200µl of triton X – 100. Mixture was incubated at 100^0C for 15 min. Then after the mixture was centrifuged at high speed and supernatant was used directly for PCR reaction preparation. We checked DNA quality and quantity with Nanodrop Lite (Thermo scientific) and Agarose Gel.

Quality and quantity of tissue samples were satisfactory; where same of fluid samples were not satisfactory,so fluid samples were excluded from the study from this step.

PCR reaction was made with the total volume of 25µl.two primers (Forward - 5'GGATCCTGCGAGCGTAGGCGTCGG3' and Reverse - 5'CCTGTCCGGGACCACCCGCGGCAA3') were used with final concentration of 20pM. Other components were 12.5 µl Mastermix (Takara Emeraldamp), DMSO 1µl (100%) and water. After making the reactions, tubes were put in thermal cycler machine. The cycling conditions were 95^0C for 15min, 40 cycles of 95^0C for 30sec, 62^0C for 30sec and 72^0C for 30 sec. final extension was done with 72^0C for 5 min.

After the PCR cycles over, the tubes were stored in freezer at -4^0C. PCR product was checked on 1.5 % agarose gel for presence or absence of 200bp product fragment.

3. Result& Discussion

- **DNA Quantification Data**

Table 1: Data of DNA ration of purity and concentration.

Sample code	Ratio A260/A280	Concentration (ng/µL)
DK01T	1.85	157.0
DK02T	1.76	122.9
DK03T	1.88	41.2
DK04T	1.95	45.5
DK05T	1.82	139.1
DK06T	1.72	20.0
DK07T	1.78	27.5
DK08T	1.79	27.3
DK09T	2.59	1788.5
DK10T	0.65	26.4
DK11T	0.63	40.1
DK12T	0.81	34.3
DK13T	1.83	21.4
DK14T	1.55	11.9
DK15T	1.87	39.3
DK16T	1.43	10.8
DK17T	1.69	9.4
DK18T	1.67	23.6
DK19T	1.74	14.0
DK20T	0.89	48.3
DK21T	0.69	24.4
DK22T	1.66	19.4
DK23T	1.79	72.7
DK24T	1.91	87.8
DK25T	1.85	49.6

- **Agarose Gel – Electrophoresis of PCR Products**

Table2: Sample type, result and well code for locating the lane of fig. 4.1 & 4.2

Well code	Sample code	Sample Type	Result
1	DK01T	Endometrium Tissue	**Positive**
2	DK02T	Endometrium Tissue	Negative
3	DK03T	Endometrium Tissue	**Positive**
4	DK04T	Endometrium Tissue	**Positive**
5	DK05T	Lung Biopsy	**Positive**
6	DK06T	Lymph Node	Negative
7	DK07T	Lymph Node	Negative
8	DK08T	Tissue Sample	Negative
9	DK09T	CSF	Negative
13	DK13T	Tissue Sample	Negative
14	DK14T	Tissue Sample	Negative
15	DK15T	Tissue Sample	Negative
16	DK16T	Tissue Sample	**Positive**
17	DK17T	Abdominal Tissue	Negative
18	DK18T	Pleural Tissue	**Positive**
19	DK19T	Lymph Node	Negative
22	DK22T	Endometrium Tissue	Negative
23	DK23T	Endometrium Tissue	Negative
24	DK24T	Endometrium Tissue	**Positive**
25	DK25T	Endometrium Tissue	Negative

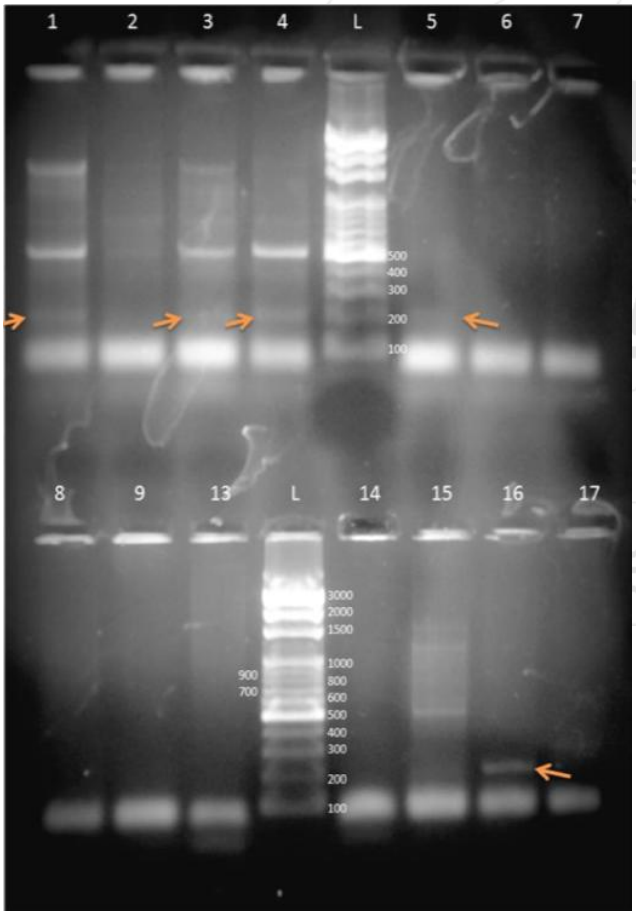

Figure 1: Agarose gel electrophoresis image – 1(For sample # DK01T, DK02T, DKO3T, DK04T, DK05T, DK06T, DKO7T, DK08T, DK09T, DK13T, DK14T, DK15T, DK16T, DK17T

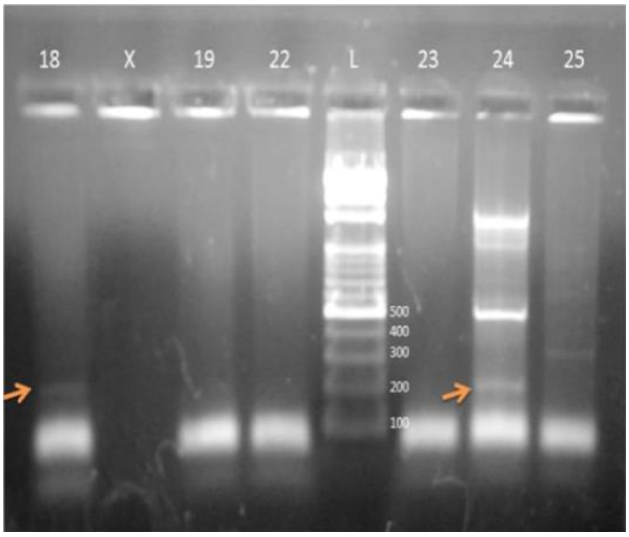

Figure 2: Agarose gel electrophoresis image–1(For sample DK18T, DK19T, DK22T, DK23T, DK24T, DK25T)

In present study we have taken total 25 different extra pulmonary samples and extracted their DNA. We didn't get satisfactory DNA purity of fluid samples with sample number DK10T, DK11T, DK12T, DK20T and DK21T (Table No. 1). So those 5 samples were excluded from the further study. Total 20 samples were successfully studied for the presence or absence of the mycobacterium tuberculosis complex by targeting insertion sequence (IS6110).

Out of 20 samples we got 7 samples positive for infection of TBC. According to the results we got, 35% is the frequency of tuberculosis in total number of suspected patients. Here we didn't take any normal samples beside suspected, so our data can't give the idea of TB prevalence in general population. Out of 20 samples, there were 12 samples of endometrium tissue, 1 sample of lung biopsy, 4 sample of Lymph node tissue, 1 sample of abdominal tissue and 1 sample of pleural tissue. Out of 12 endometrium tissue samples, we got 5 samples positive, 1 positive sample from Lung biopsy, and 1 positive sample from plural tissue. As these results were noted, we got 41.66% endometrium TB in suspected patients, where it is accounts for 25% from overall suspected patients of extra pulmonary TB. Same as 100% frequency in pleural and lymph tissue were noted in suspected patients, where their overall frequency in extra pulmonary TB is 5%. These all samples are of location nearby Mahesana city, so we can consider the total prevalence is 35% in suspected TB Patients in Mahesana territory.

Mean age of females have been suspected for endometrial TB is 38 years ranging from 30 years to 45 years. Where mean age of the patient confirmed positive for endometrium TB is 40.66 ~ 41%, which shows that more prevalence in female at the age of or near to menopause period. This data of our study is completely opposite to the data of Marcus et. al. which was stated that prevalence of genital TB is more (62%) in female with post menopause in developed country and less (28%) in developing countries. It may because of less sample size or geographical difference. They stated that the prevalence of genital TB is directly proportional to pulmonary TB in particular area. A researcher showed that a very high incidence of pulmonary TB were noted in the

Spanish civil war and World War II, which decline very quickly after 1961, then after a year later quick decline in genital TB were noted too (Markus SF et. al 1994 and Nogales-Ortiz F et al. 1979). The mean age of our all samples is 41.47 ~ 41% ranged from 20 years to 75 years. We recorded 35 % prevalence of TB in suspected patients which is very less compare to Amin et. al., they noted that 94% suspected extra pulmonary TB and 6 % pulmonary TB (Amin et al., 2011).

PCR is a method of choice for TB diagnosis because it produces results very rapid with high accuracy and specificity compare to other screening methods. Greco et. al. find the TB DNA in 46.5 smear negative TB samples by PCR amplification method (Greco et al., 2009).Nested PCR (nPCR) is more sensitive and more specifically detects the bacterium compare to PCR. Therese et. al. concluded that 95.8% cases were noted positive out of 100% culture positive clinical samples by nPCR. They targeted IS6110 and MPB64 genes for nPCR. They stated that if only one PCR target gene (IS6110 & MPB64) is used, then there will be a 4.2% chances of missing positive cases(Therese KL, Gayathri R, Dhanurekha L, Sridhar R, Meenakshi N, 2013).GeneXpert is approved and recommended by WHO and FDA for detection of MTB. Xpert MTB/RIF kit has a 95% Sensitivity and 100% specificity where in CobasTaqMan MTB Kit shows 78% sensitivity and 98% specificity reported by Causse et al. (Causse, Ruiz, Gutie, & Casal, 2011).

Genital tuberculosis is associated with infertility in females. Hassoun et al. reported that 1.8% cases shows genito-urinary site involvement in all other tuberculosis cases (Ali Hassoun et al., 2005). In our study, patients suspected with endometrium TB have the infertility problems so this data is relates our results in this manner. One more study in South Africa found an incidence of 6% of culture positive tuberculosis in an infertile population (Margolls, Wranz, Kruger, Joubert, & Odendaal, 1992).

That is reported by Soussis et al. that they got 28.6 % success rate with IVF in 13 patients with histologically proven genital TB (Soussis, Trew, Matalliotakis, Margara, & Winston, 1998). Thus IVF is the only possible treatment of tubual and endometrial tubercular infertility (Neelam, Mohanlal, & Namita, 2005).

4. Conclusion

PCR is rapid and highly sensitive method to detect the mycobacterium tuberculosis complex. It is a superior to staining and microscopic methods, where it is little less specific than GeneXpert method. Conventional PCR is little tough in the detection of TBC because there are many target genes are available. These all target genes have their own merits and demerits. So the study should be continuing on the way to achieve a more specific and more sensitive technique for the TBC.

Attention towards the extra pulmonary TB must be given same as the pulmonary TB, because it is asymptomatic in most cases and less symptomatic in some cases, so it can show sever disease whenever it cause. Infertility is also a major concern.

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