IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 20, Issue 3 Ser.12 (March. 2021), PP 42-48
www.iosrjournals.org

Combined result of PCR assay by using IS6110 and TRC4 primer can more reliably diagnose pleural tuberculosis.

Sanjida Khondakar Setu, [1] Abu Naser Ibne Sattar[2], Sharmeen Ahmed [2] Md Towfique Hasan Firoz [3]

[1]*Assistant Professor, Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka 1000, Bangladesh,*
[2]*Professor, Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka 1000, Bangladesh,*
[3]*Assistant Professor, Department of Radiation Oncology,*
National Institute of cancer Research & Hospital (NICRH), Bangladesh

Abstract:
Background: *Pleural tuberculosis is the second most common form of EPTB cases in Bangladesh. The reported extrapulmonary tuberculosis (EPTB) prevalence in Bangladesh is 4.8% to 21.98% which shows an increasing trend. Conventional diagnostic techniques required more time and less sensitive due to paucibacillary nature of M. tuberculosis. Various studies showed that using a single primer in PCR assay may fail to detect all cases. This study was carried out to evaluate PCR assay by using IS6110 and TRC4 primers to detect Pleural tuberculosis.*
Material and Methods: *A total of 53 samples from clinically suspected cases of pleural tuberculosis of all age and sex group were sent to Microbiology and Immunology Department ,Bangabandhu Sheikh Mujib Medical University for laboratory investigation of M. tuberculosis, fulfilling the inclusion criteria were enrolled in the study from March 2013 to February 2014. Culture in Lowenstein Jensen media, Z-N stain microscopy, ADA assay, cytology, histopathology and other biochemical test was performed. Conventional PCR was performed by using two pairs of primes IS6110 and TRC4. Amplified products for IS6110 and/or TRC4 were indicative of M. tuberculosis.*
Results: *Out of 53 suspected Pleural tuberculosis cases, 31 were confirmed as pleural TB and rest 22 were diagnosed as non-TB cases according to diagnostic criteria. Among 31 Pleural tuberculosis cases 7(22.58%) were positive by microscopy and culture .PCR test was found positive in 27 (87.09%) cases. Regarding culture as the gold standard, TRC4 primers had a higher sensitivity (66.66%). But when combined assay of the two primers were considered, the sensitivity increased to 70% in detecting pleural TB.*
Conclusion: *The use of TRC4 can be helpful for improved detection of M .tuberculosis. This study showed that, combined assay of PCR using TRC4 and IS6110 primers can increase the sensitivity of PCR test. It was also observed that a clinicopathological correlation with microbiological and molecular test result can play a significant role in diagnose the pleural TB cases.*
Key Words*: pleural Tuberculosis, IS6110 and TRC4, PCR assay.*

Date of Submission: 16-03-2021 Date of Acceptance: 31-03-2021

I. Introduction:

Tuberculosis (TB) still remains the most important bacterial infectious disease worldwide as approximately 10.0 million new cases and 1.2 million TB deaths were estimated globally in 2019[1]. Geographically, the burden of TB is highest in South-East Asia and Africa. Bangladesh has 3.6% of the world's TB cases[1].

In Bangladesh, the Extra pulmonary tuberculosis (EPTB) prevalence was reported to be 21.98% to 25.5%[2]. One of the most common sites of extrapulmonary tuberculosis is tuberculous pleural effusion (20%)[3]. In our country, tubercular pleural effusion is the second most common form of presentation followed by among all EPTB cases [2].

The diagnostic criteria have limitations in extra-pulmonary TB due to the paucibacillary nature of extra-pulmonary specimens and non-specific signs and symptoms of the disease [4].

Studies have shown that positive Mantoux test, elevated ESR, cytology, elevated adenosine deaminase level in pleural fluids may be important components in the diagnosis of pleural tuberculosis[5,6] . But all the above mentioned methods has not established as confirmatory. These inadequacies necessitate the development of an improved, sensitive and specific test for early detection of mycobacterium in clinical specimen.

PCR is a highly sensitive assay that amplifies target nucleic acid region and uniquely identify *Mycobacterium tuberculosis* which may even detect 1-10 organisms within 3-6 hours in different clinical specimens[7,8]. Negi *et al* (2005) showed that mycobacterial culture negative clinical specimens obtained from cases who were being treated and responded afterwards with anti-tubercular treatment (ATT), were positive by PCR test and thereby suggesting that the DNA amplification method could detect even nonviable mycobacteria[9].

Studies revealed that the sensitivity of PCR ranges from 42% to 93% depending on the clinical specimens targeting various parts of the genome such as IS6110, 65kDa, *devR*, TRC4, GCRS, MPB64 etc for the detection of *Mycobacterium tuberculosis*[10,11,12]. Among them, most of the studies have generally targeted the multi-copy IS6110 sequence of the genome with a sensitivity of 80.5%[13,14]. However, the absence or the presence of only a few copies of IS6110 sequence has been reported in some strains, particularly those from Southeast Asia[13]. A large number of clinical isolates of *M. tuberculosis* from South India had either a single copy (40%) or no copy (4%) of IS6110 resulting false negative results[15].

TRC4, a repetitive element with three open reading frames (ORFs) has been demonstated to be highly sensitive and specific targets as an ideal target for PCR assays to identify M. *tuberculosis* with a sensitivity of 91%; even in strains carrying no copies of IS6110 intuberculous patients[15,16,17]. Studies also have demonstrated that an increased diagnostic yield can be achieved by PCR assays using multiple primers than that by using single primer to elude misdiagnosi[18,19,20].

Considering the increasing number of pleural TB cases in Bangladesh, there is an urgent need for rapid and accurate diagnostic modalities to detect pleural TB. The present study was undertaken to evaluate the combined result of PCR assay in the detection of *Mycobacterium tuberculosis* using IS6110 and TRC4 primer from tubercular pleural effusion.

II. Materials and Methods

Study population: A total of 53 samples of tubercular pleural effusion collected from clinically suspected cases of pleural tuberculosis of all age and sex group fulfilling the inclusion criteria were enrolled in the present study. All samples were subjected to ADA testing and cytological, biochemical and microbiological examination. Other supporting investigations were collected from patient case files

Study Design: Cross sectional type of comparative study.

Study Location: Department of Microbiology and Immunology, Bangabandhu Sheik Mujib Medical University (BSMMU) Dhaka, Bangladesh.

Study Duration: March 2013 to February 2014.

Based on the diagnostic criteria out of the 53 patients, 31 were diagnosed as pleural tuberculosis and rest 22 were non-tuberculous group. Treatment response with anti tuberculour drugs were followed up in all patients in the TB group and showed marked improvement. A positive to treatment response was defined as the improvement of clinical and / or imaging findings of two months after two months of treatment.

Inclusion criteria: [9, 21, 22].

Pleural TB cases were diagnosed by clinical menifestation and/or imaging evidence suggesting TB with one or more of the three criteria:

1. Positive tuberculin test and, significant level of ADA value (≥ 40 U/L) and lymphocytic exudative body fluid (protein level ≥0.45g/l and /or glucose ≤ 2.5 mmol) **,**
2. Detection of AFB by microscopy or isolation of mycobacteria by culture from clinical samples and
3. Clinical features compatible with TB, having positive tuberculin test and / or high ESR and clear response to anti-TB drugs.

Exclusion criteria:

- Patients who have taken anti tubercular drugs and refused to be a part of the study were excluded

Sample collection and processing:

At least 40 ml fluid was aseptically collected in a sterile tube containing heparin or EDTA with a sterile syringe by attending Physician using aspiration technique.Five ml was sent for routine bio chemical (glucose, protein) test and 15 ml for cytological analysis. Gram stain, routine bacteriological culture,L-J culture, ADA testing,AFB smear for Z-N staining and PCR assay were performed by remaining 20 ml[23]. These specimens were processed within four hours of collection.Collected samples were centrifuged at 3000 x g for 30 minutes. After centrifugation of pleural fluid, supernatant was taken for ADA assay.ADA level was determined by colorimetric method. After that, deposit was inoculated immediately onto L-J culture medium for mycobacterial culture.Remaining deposits were used for AFB smear for Z-N stain and PCR assay or stored at -20^{o} C in a freezer until tested[24]. All the steps including sample processing, smear preparation and culture were done in a biosafety level-2 cabinet. Other investigations such as Hb per cent, total count (TC), differential count (DC), ESR, tuberculin test, chest X-ray were included as supportive indicator in this study.

Amplification of Mycobacterial DNA

DNA was extracted from specimens per the manufacturer's instructions using the QIAGEN QIAamp DNA mini kit (QIAGEN, Valencia, CA)**.** Identification of M.tuberculosis done by using two pairs of primers designed to amplify specific sites of Mycobacterial genome are: IS6*110*a(5'-CCT GCG AGC GTA GGC GTC GG- 3') and IS6*110* b (5'- CTC GTC CAG CGC CGC TTC GG-3') and TRC_4primer 1(5' – GAC AAC GAC GTG CGC CTA CT – 3') and TRC_4primer 2(5' – GAC CGA ATT AGC GTA GCT CC – 3'). Primer IS6*110*and primerTRC_4amplify fragment with a length of 123 bp and 173 bp respectively [16,20]. Primers were obtained from Tuberculosis Research Centre (TRC-ICMR) Chennai, India.

PCR assay:

A 20 µl PCR mixture and 5 µl template DNA was mixed to prepare 25 µl reaction mixture of PCR. PCR mixture was prepared according to the number of sample tested at a time in a microcentrifuge tube with master mix 5µL, primer forward 1µl,primer reverse 1µl and volume made up to 20 µl with dH2O. 20 µl PCR mix for each sample is then transferred to 0.2 ml PCR tubes. Then 5 µl of specimen DNA added to each tube making total volume of reaction mixture 25 µl/tube. Amplification was carried out in a thermal cycler (MJ Rsearch, PTC-200 Peltier thennal cycler) involving the following steps, which were pre-programmed in the machine.

Table no I: Programmed steps of PCR in the thermal cycler [16, 20].

Steps	**IS6110(35 cycle)**	**TRC4(25 cycle)**
l. lnitial denaturation	94℃ for 5 min	95℃ for 5 min
2. Denaturation	94℃ for 2min	94℃ for 30 sec
3. Annealing	68℃ for 2min	58℃ for 30 sec
4. Extension	72℃ for 1 min	72℃ for 30 sec
5. Looping(step 2-4)	31 times	31 times
6. Final extension	72℃ for 7 min	72℃ for 5 min
7. Hold A	4℃ for 24 hrs	4℃ for 24 hrs
8. End	End	End

Each run of the PCR assay included one positive control and one negative control to compare the obtained results. DNA of H37RV strain was used as positive control and PCR grade water as negative control. The PCR products were electrophoresed on a 1.5% agarose gel in 1× TBE buffer containing Ethedium bromide at 10 mg/ml concentration; 3 µl of 100bp ladder marker was also loaded. Electrophoresis done at 5 mAmp/ cm' for 1.5 to 2 hours. After completion of electrophoresis, illumination of the gel done under U-V light (254-366 nm) and at the same time image recorded using a gel documentation (gel doc) system (BIORAD, Italy). The bp size was determined by using software included in gel doc system.

Statistical analysis:

After collection all data were checked and edited then analyzed by using computer based SPSS (Statistical Package of Social Science) software version: 17.00with a 95% confidence interval (95% CI).The results of individual tests were compared byChi square (χ^2) test and ANOVA test. p value < 0.05 was taken as minimum level of significance. Significance was calculated by Z-test of proportion.

III. Result:

A total of 53 clinically suspected patients of having pleural tuberculosis were enrolled in this study. The patients were divided in two groups –tuberculous(58.49%) and non-tuberculous22 (41.50%) group Table no 2. The mean age of the study subjects was 34.17 ± 1.42 years with age ranging from 1- 75 years.

Table no 2: Distribution of study population as per diagnostic criteria (n=53)

Study population	**No. of cases (%)**
Extrapulmonary tuberculosis(EPTB) cases	31(58.49)
*Nontuberculous (non- TB) cases	22(41.50)

*The samples collected from the 22 non-TB pleural fluids cases included from the patients diagnosed as malignancy(15), parapneumonia(03) and congestive cardiac failure (04)

Out of 22 non-Tb pleural fluid samples 1(4.54%) was positive in PCR by using IS6110 primer. None were positive by mycobacterial culture and TRC4 primer. The major presenting clinical features included fever, malaise, chest pain, cough, weight loss and anorexia. Out of 31 pleural tuberculosis cases 25(80.64%)) were tuberculin positive and mean ESR was 87.46 ± 2.34. All of the 31 pleural fluid samples from patients in the

study had ADA ≥ 40 U/l with mean of 69.17±3.78 and lymphocytic exudative effusions with mean lymphocyte differential count of 84.28 ± 7.28. Of all cases, 45.71% cases had positive imaging evidence suggestive for tuberculosis. All the thirty one pleural tuberculosis patients responded to anti-tubercular treatment.

Results of AFB smear and Mycobacterial culture of EPTB samples are shown in Table no 3. Out of 31 pleural fluid samples, 5(16.13%) were positive in mycobacterial culture alone and 2(6.45%) were positive by both AFB smear and culture.

Table no 3: Results of AFB smear and Mycobacterial culture in pleural fluid samples among pleural TB cases (n=31)

	Number	%
Only AFB smear positive	0	0
Only mycobacterial culture positive	5	16.13
Both AFB smear & culture positive	2	6.45
Total Bacteriological test positive	7	22.58

Comparison between different combination of two primer with AFB smear and culture result among PCR positive pleural tuberculosis cases has been demonstrated in Table no 4. Among 7 smear and culture positive cases, both IS6110 and TRC4 showed 74.19% PCR positivity. By using PCR only IS6110 and only TRC4 showed 1428% and 28% positivity respectively. Among 24 smear and culture negative cases, 79.16% were PCR positive by using both IS6110 and TRC4 primer simultaneously and only TRC4 showed 4.16% positivity.

Table no 4: Comparison between different combinations of primers with AFB smear and culture result among PCR positive pleural tuberculosis cases (n=31)

Test	**Number of sample**	**PCR positive by n=27**		
		Both IS6110 & TRC4 n (%)	Only IS6110 n (%)	Only TRC4 n (%)
AFB smear &/or culture positive	7	4(57.14%)	1(14.28%)	02(28.57%)
Both AFB smear & culture negative	24	19(79.16%)	0(0%)	01(4.16%)
Total	**31**	**23(74.19%)**	**1(3.22%)**	**03(9.67%)**

Note: Two different primers were used. Samples positive by any of the primer considered PCR positive

The primer evaluation considering culture as a gold standard in diagnosis of pleural tuberculosis cases was revealed in Table no 5. The sensitivity and specificity of IS6110 were 62.5% and 95.45% respectively. The sensitivity and specificity of TRC4 were 66.66% and 100% respectively. The combined results of PCR assays using both primers showed the highest sensitivity characteristics compared to the individual IS6110 and TRC4 assay. The sensitivity and specificity of combined PCR assay were 70% and 95.45% respectively.

Table no 5: Primer evaluation in diagnosis of EPTB among the study population.

	IS6110 (%)	**TRC4 (%)**	**Combined PCR assay (%)**
Sensitivity	62.50	66.66	70.00
Specificity	95.45	100	95.45
PPV	83.33	100	87.50
NPV	91.21	95.45	91.21

Note: Culture was considered as gold standard.

IV. Discussion

The diagnosis of pleural tuberculosis poses a special challenge as it is often missed or misdiagnosed due to its atypical presentations. The poor performance of conventional microbiological techniques, due to its non-uniform distribution of microorganisms; the paucibacillary nature of the specimens, the diagnosis is largely based on clinical suspicion and non-microbiological parameters in pleural specimens. Polymerase chain reaction (PCR) is the best known Nucleic acid amplification (NAA) test which amplifies target nucleic acid regions that uniquely identifies the *Mycobacterium tuberculosis* complex. Kumar *et al* (2013) has summarized from various studies that the sensitivity of PCR testing has been varied ranging from 42% to 93% mainly due to different target nucleic acid sequences and the clinical specimens, but no definitive study among Bangladeshi population has been carried out before comparing the use between combined assay of dual primer with the single primer assay[25,26].

In this study, diagnostic efficacy of PCR was evaluated in relation to the conventional techniques. A total of 53 suspected pleural tuberculosis cases were included in this study. Among them 31 patients were finally diagnosed as pleural tuberculosis and 22 were non-TB cases. Pleural fluid samples collected from this suspected pleural tuberculosis cases were subjected to AFB smear, AFB culture by L-J media and PCR. To assess a better target for PCR amplification, two sets of Mycobacterium tuberculosis complex specific primers, namely IS6110 and TRC4 were used. Then the combined assay of these two primers was compared with the single assay.

On comparing the detection rate of pleural tuberculosis cases between bacteriological examination and PCR assay, PCR test was found much more superior than AFB smear and L-J medium culture in this study ($p < 0.005$). Among 31 TB cases PCR showed positivity in 27(87.09%) cases. Trajman*et al.* (2007) also showed PCR positivity of 82.4%in pleural fluid [27]. Only culture was positive in 5(16.13%) cases and both AFB and culture positive in 2(6.45%).(Table-IV). This higher positivity rate of PCR in this study than that of the AFB smear and culture is comparable with the study of Negi *et al*(2005) who showed sensitivity of PCR ,AFB smear by Z-N stain and L-J medium culture 74.4%,33.79% and 48.9% respectively[9].

Only seven cases were found positive by bacteriological test (AFB smear & or culture by L-J medium). This low yield of AFB smear and culture may be due to the paucibacillary nature of pleural fluid samples. The lower positivity of AFB smear and culture in the present study is comparable with that of many other studies where the positivity of AFB smear and culture was respectively <l0% and 12-70% in pleural fluid[11, 28].

Of 22 non-TB samples, a case of malignant pleural effusion was false positive by PCR assay using IS6110 primer. It can be explained by that, in country like Bangladesh where the prevalence of tuberculosis is very high, the immunosuppressive nature of malignant lesion is capable of reactivating a latent tubercular infection; hence, malignancy and tuberculosis may coexist [11]. Study also showed that the central sequence (IS) of IS6110 may be found to be homologous with other atypical mycobacteria such as *M.avium-M.intracellulare* complex which may cause false positive result[29]. This could be also due to cross contamination during tube to tube transfer[26].

Out of seven bacteriological test positive pleural TB cases, unexpectedly 1 (14.28%) case was found negative by PCR. This false negativity of PCR test can be explained either by sampling errors, inefficient extraction of DNA, or the presence of PCR inhibitors[30].

All the PCR positive with bacteriological negative cases were responded to antitubercular treatment with significant clinical improvement. Cheng *et al* (2004) stated that, in patients particularly with clinical and radiological features suggestive of active tuberculosis, a negative AFB culture cannot exclude a clinical diagnosis of tuberculosisas demonstrated in our patients[31]. There can be a variety of reasons for a positive TB PCR but negative AFB culture and AFB smear. For example, the *M. tuberculosis* count may be low at the tissue level or the organisms may not be viable but the presence of mycobacterial DNA in tissues could still be easily detected by PCR at the early stage[31,32].

Among the PCR positive cases, 87.09% cases were positive simultaneously by two primers.TRC4 gene-based PCR showed highest 80.64% positivity and IS6110 showed 77.41%positivitybut the difference was not significant ($p > 0.05$). Three (9.67%) cases were found to be negative by IS6110 that were positive by TRC4 primer. These are not false negatives because among these three cases, two cases were positive by bacteriological test and one case was responded to antitubercular treatment subsequently. Other studies in India and in other geographical regions of the world have also reported similar results [9,16,20]. Reason of this may be due to absence or low copy number of IS6110 in genome of some isolates of Mycobacterium tuberculosis mostly reported from south-east India, Tanzania, Malaysia, Oman and Denmark[15,33].

The combined results of two assays showed the better positivity compared to the individual IS6110 and TRC4 assays. Considering culture as a gold standard the sensitivity of IS6110 was 62.50% and TRC4 was 66.66 %. But when combined assay was considered, it was increased to 70%. Using one or more sets of primers especially from a repetitive element like TRC4 could be advantageous in increasing the sensitivity of PCR. This study confirms the utility of combined assay to detect M. tuberculosis from pleural fluid samples[16, 20].

Despite the higher sensitivity of PCR, AFB and culture remain an important technique for diagnosing of pleural TB. In this study, if the PCR positive result had been accepted as the only diagnostic criteria, it would have missed one bacteriologically confirmed case. So, a combination of conventional techniques and PCR in conjunction with clinical parameter must be applied for the diagnosis of paucibacillary nature in pleural tuberculosis to achieve maximum sensitivity.

V. Conclusion and Limitations:

Our study demonstrated that considering combined assay by using two primers (IS6110 & TRC4) will increase accuracy of *M. tuberculosis* detection in pleural TB. Also it may be very useful in cases those are highly suspected for pleural tuberculosis but negative for bacteriological test .This may improve clinician's ability to better diagnosis in pleural tuberculosis. In addition, rapidity of the test allows quick implementation of treatment regimen. There were some limitations in this study. PCR inhibitors could not be identified and after DNA extraction DNA concentration could not be measured.

References:

[1]. Global tuberculosis report 2020. Geneva: World Health Organization, 2020. Available online: https://www.who.int/publications/i/item/9789240013131. accessed 18 March 2020

[2]. Shahriar MD, Kamal MS, Ahasan HAM *et al.* (2010) Isolation and Identification of Mycobacterium from Extrapulmonary Specimen at NTRL, NIDCH, *J Med;* 11: 128-30.

[3]. Sharma SK and Mohan A. (2004)Extrapulmonary tuberculosis. *Indian J Med Res;* 120: 316-353.

[4]. Jain A.(2011) Extrapulmonary Tuberculosis : A Diagnostic Dilemma. *Ind J Clin Biochem*; 26(3):269–273.

[5]. Zhai K, Lu Y and Shi Z H. Tuberculous pleural effusion. J Thorac Dis. 2016 ; 8(7): 486–94;10.21037/2016.05.87

[6]. Zaric B, Kuruc V, Milovancev A et al. Differential diagnosis of tuberculous and malignant pleural effusions: what is the role of ADA? Lung. 2008*;* 186: 233-40.

[7]. Verma A, Dasgupta N, Agarwal AN *et al.*(1995)Utility of a Mycobacterium tuberculosis GC-rich repetitive sequence in the diagnosis of tuberculous pleural effusion by PCR. *Indian J. Biochem Bio-phys*; 32: 429–436.

[8]. Chowdhury MR,Bari SMN, Islam KS *et al.*(2012) Evaluation of Real Time PCR for the diagnosis of Extrapulmonary Tuberculosis and comparison with AFB Microscopy among Bangladeshi population. *Int J Nat Sci*; 2(1):26-32.

[9]. Negi SS, Khan SFB, Gupta S *et al*. (2005) Comparison of the conventional diagnostic modalities,BACTEC culture & polymerase chain reaction test for diagnosis of tuberculosis. *Indian J Med Microbiol*; 23: 29-33.

[10]. Nagesh BS, Sehgal S, Jindal SK, and Arora SK. (2001) Evaluation of polymerase chain reaction for the detection ofMycobacterium tuberculosisin pleural fluid. *Chest*; 119:1737–1741.

[11]. Chakravorty S and Tyagi JS.(2005) Novel multipurpose methodology for detection of Mycobacteria in pulmonary and extrapulmonary specimens by smear microscopy, culture and PCR. *J Clin Microbiol;* 43, 2697-2702.

[12]. Kusum S, Manish M, Kapil G *et al.*(2012) Evaluation of PCR Using MPB64 Primers for Rapid Diagnosis of Tuberculosis Meningitis. Scientific reports. Available online: https://www.omicsonline.org/scientific-reports/2161-0703-SR204.pdf. accessed 18 March 2020

[13]. Kerry HL, Benjamin WH, Kimerling ME *et al.* (2002) Molecular differentiation of *Mycobacterium tuberculosis* strains without IS6110 insertions. *Emerg Infect Dis*; 8: 1303-1305.

[14]. Kesarwani RC, Pandey A, Misra A, Singh A. (2004) Polymerase chain reaction (PCR): Its comparison with conventional techniques for diagnosis of extra-pulmonary tubercular diseases. *Indian J Surg* 66: 84-88.

[15]. Das S, Paramasivan CN, Lewis DB, Prabhakar R and Narayanan PR. (1995) IS6110 restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, south India. *Tubercle Lung Dis* 76: 550-554.

[16]. Narayanan S, Parandaman V, Narayanan PR *et al*. (2001) Evaluation of PCR using TRC4 and IS6110 Primers in detection of tuberculous meningitis. *J Clin Microbiol*; 19: 2006-8.

[17]. Parandaman V, Narayanan S, Narayanan PR *et al.*(2000) Utility of polymerase chain reaction using two probes for rapid diagnosis of tubercular pleuritis in comparison to conventional methods. *Indian J Med Res*;112: 47-51.

[18]. Sekar B, Selvaraj L, Alexis A *et al.* (2008) The utility of IS6110 sequence based polymerase chain reaction in comparison to conventional methods in the diagnosis of extra-pulmonary tuberculosis. *Indian J Med Microbiol*; 26: 352-535.

[19]. Kulkarni S, Singh P, Memon A *et al.* (2012) An in-house multiplex PCR test for the detection of *Mycobacterium tuberculosis*, its validation & comparison with a single target TB-PCR kit. *Indian J Med Res*; 135: 788-794.

[20]. Barani R, Sarangan G, Antony T *et al*.(2012) Improved detection of *Mycobacterium tuberculosi*s using two independent PCR targets in a tertiary care centre in South India. *J Infect Dev Ctries*; 6(1):46-52.

[21]. Fatima J, Karoli R, Chandra A *et al*. (2012) Polymerase chain reaction and adenosine deaminase activity in tuberculous ascites. Indian Journal of Medical Specialities; 3 (2):170-173.

[22]. Kelam MA, Shah BA, Ganie FA *et al.* (2013) The diagnostic efficacy of polymerase chain reaction and adenosine deaminase in tubercular effusion. *Sky. J. Med. Med. Sci*; 1(1):7 - 15

[23]. Porcel JM. (2013) Handling Pleural Fluid Samples For Routine analyses. Available online: <http://www.toraks.org.tr/uploadFiles/book/file/3720131048192 HANDLING.pdf. accessed 18 March 2020

[24]. Kantor IND, Kim SJ, Frieden T *et al.* (1998) Laboratory service in tuberculosis control, Geneva, World Health Organization. (WHO/TB/98.258). pp: 55 (Part-III).

[25]. Kumar M, Kumar R, Srivastava AK *et al*.(2013) Sensitivity of PCR *IS6110* in relation to culture and staining in Pott's disease. *Indian J Neurosurg*; 2:46-51.

[26]. Rosso F, Michelon CT, Sperhacke RD *et al.* (2011)Evaluation of real-time PCR of patient pleural effusion for diagnosis of tuberculosis. *BMC Research Notes*; 4:279. Available online at: http://www.biomedcentral.com/1756-0500/4/279. accessed 18 March 2020.

[27]. Trajman A, Kaisermann C, Luiz RR *et al*.(2007) Pleural fluid ADA, IgA- ELISA and PCR sensitivities for the diagnosis of pleural tuberculosis. *Scand J Clin Lab Invest.*; 67(8):877-84.
[28]. Aggarwal, A. N., D. Gupta, and S. K. Jindal. . Diagnosis of tubercular pleural effusion. Indian J. Chest Dis. Allied Sci. 1999; 41:89-100.
[29]. Kent L, McHugh TD, Billington O *et al.* (1995) Demonstration of homology between IS6110 of *Mycobacterium tuberculosis* and DNAs of other Mycobacteriumspp. *J Clin Microbiol*; 33:2290-3.
[30]. Singh KK, Muralidhar M, Kumar A *et al*.(2000) Comparison of in house polymerase chain reaction with conventional techniques forthe detection of *Mycobacterium tuberculosis* DNA in granulomatous lymphadenopathy. *J Clin Pathology*; 53:355-61.
[31]. Cheng VCC, Yam WC, Hung IFN *et al*.(2004) Clinical evaluation of the polymerase chain reaction for the rapid diagnosis of tuberculosis. *J Clin Pathol*;57: 281–285.
[32]. Chawla K, Gupta S, Mukhopadhyay C *et al.* PCR for *M. tuberculosis* in tissue samples. *J Infect Developing Countries.* 2008; 3(2)83-87.
[33]. Fumokong NG, Tang TH, Al-Maamry S *et al*. Insertion sequence typing of *M tuberculosis*: Characterization of a widespread sub-type with single copy of IS6110. *Tuberc Lung Dis.* 1994; 75: 435-440.

Sanjida Khondakar Setu, et. al. " Combined result of PCR assay by using IS6110 and TRC4 primer can more reliably diagnose pleural tuberculosis." *IIOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*, 20(03), 2021, pp. 42-48.