

Role of Nucleic Acid Amplification Tests (NAATs) in Tuberculous Pleural Effusion: Where It Fits In Routine Diagnostic Workup?

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Abstract

Background: Commonest cause of pleural effusion in India is tuberculosis (TB) and poses a diagnostic difficulty because of the low sensitivity of culture technique

Methods: Prospective study conducted during Jan 2012 to Sept. 2013 with an objective to find role of Nucleic acid amplification tests (NAATs) in tuberculous pleural effusion. We also observed comparison of NAATs i.e. MTB DNA PCR with other conventional diagnostic techniques like pleural fluid biochemistry, ADA (adenosine deaminase level), cytology and culture for mycobacterium tuberculosis, included 100 cases with signs, symptoms, history and radiological features suggestive of tuberculous pleural effusion. All the cases were subjected to pleural fluid analysis, smear for AFB, ADA, cytology, AFB culture on LJ media & MTB DNA PCR. Statistical analysis was done by using t-test and chi-test.

Results: Out of total 100 cases with pleural effusion, 09% cases were sputum positive for AFB, 3% pleural fluid samples positive for AFB, 28% were culture positive, 74% were DNA PCR positive, and 85% cases had ADA >40 units/liter, 87% cases had a LN Ratio greater than 0.75. Sensitivity, specificity, PPV & NPV of PCR for MTB was observed 92.86%, 33.33%, 35.13% & 92.30% respectively ($p < 0.01$). In PCR positive cases, there was no significant association between ADA levels in pleural fluid culture for MTB positive and negative ($P > 0.4$). In PCR negative cases; there was statistically significant association between ADA levels in pleural fluid culture for MTB positive and negative results. ($P < 0.05$) Combined yield of pleural fluid culture, ADA > 40 units/liter, DNA PCR and LN ratio > 0.75 gave a positive diagnostic yield in 98% of cases, 2% with diagnostic dilemma were diagnosed by pleural biopsy.

ATT response was observed in 78% cases in 2 weeks, 98% cases in 4 weeks and 100% cases at the end of 6 weeks.

Conclusion: In cases with exudative pleural effusion with Lymphocyte in pleural fluid >50% and L/N ratio > 0.75 with ADA < 40 units, MTB DNA PCR (NAATs) will be very useful in confirming tuberculosis as a cause for pleural effusion. Results of NAATs in this situation are very useful, sensitive, less time consuming and comparable to pleural fluid culture. Hence we recommend MTB DNA PCR in these cases.

Keywords: Tuberculous pleural effusion; NAATs; ADA; MTB; ATT

Introduction

India is the highest tuberculosis burden country with World health Organization (WHO) statistics for 2010 giving an estimated incidence figure of 2.3 million cases of tuberculosis for India out of a global incidence of 9.4 million cases [1]. WHO statistics also show that India is 17th out of the 22 high burden countries in terms of tuberculosis incidence rate. The estimated tuberculosis prevalence figure for 2010 is given as 3.1 million [2]. 40% of the Indian population is infected with tuberculosis bacteria, the vast majority of whom have latent rather than active tuberculosis [2].

Tuberculosis can potentially involve any system or organ of the body. Pulmonary tuberculosis is most common presentation; extrapulmonary tuberculosis is also an important clinical problem

[3,4]. Pleural effusion is one of the common complications of primary tuberculosis or in conjunction with pulmonary infiltrate typical of post primary tuberculosis.

The obvious explanation for the development of the tuberculous pleural effusion is that the delayed hypersensitivity reaction increases the permeability of the pleural capillaries to protein, intense inflammatory reaction in the parietal pleura impedes the lymphatic drainage from the pleural space and leads to the accumulation of pleural fluid [5].

The diagnosis of tuberculous pleuritis depends on the demonstration of tubercle bacilli in the sputum, pleural fluid, or pleural biopsy specimen, or the demonstration of granulomas in the pleura. Pleural fluid smear for AFB is positive in less than 10 percent instances in most reports, while mycobacteria can be cultured from pleural fluid in 10-70 percent cases in various studies [6].

The role of nucleic acid amplification tests i.e. MTB DNA PCR in the diagnosis of tubercular pleural effusion has been evaluated extensively as an alternative diagnostic tool and has yielded variable results, with sensitivities ranging between 42 and 100% and specificities ranging between 85 and 100% using various PCR targets such as IS6110, 65kDa, TRC4, GCRS, etc [7]. Low sensitivity values can be explained by the low bacillary load and the presence of substances that inhibit amplification in pleural fluid. There is possibility of a false positive PCR finding due to the presence of old healed tuberculosis infection in a patient having non tubercular effusion due to other diseases [8].

In present study we evaluated role of pleural fluid DNA PCR in tuberculous pleural effusion in high burden settings where case load is remarkable and compared its usefulness with parameters like pleural fluid biochemistry, microbiology, ADA and pleural fluid culture.

Materials and Methods

This is a prospective study, conducted in department of Pulmonary Medicine, MIMSR Medical College, Latur, India during Jan 2012 to Sept. 2013 with an objective to find role of Nucleic acid amplification tests (NAATs) in tuberculous pleural effusion. We also observed comparison of NAATs i.e. MTB DNA PCR with other conventional diagnostic techniques like pleural fluid biochemistry, ADA (adenosine deaminase level), cytology and culture for mycobacterium tuberculosis.

All patients of age more than 12 years, either admitted or attending outdoor clinic of department of pulmonary medicine at our hospital, who presented with signs, symptoms and history and radiological picture suggestive of tuberculous pleural effusion and willing to participate in the study were enrolled after proper counseling. The protocol was explained to patient/ care provider before enrolment and informed consent was taken from each patient. Detailed clinical history was taken and physical examination was carried out in all patients. Sputum smear for acid fast bacilli was examined at our designated microscopy centre.

Inclusion criteria

1. Patients of both sex and age of more than 12 years. 2. Medical history compatible with tuberculous pleural effusion 3. Pleural effusion by clinical examination, chest X ray and ultrasonography (Figure 1) 4. Exudative pleural effusion by Light's criteria

Exclusion criteria

1. Age less than 12 years. 2. Patient's refusal for pleural fluid aspiration. 3. Cases with empyema thoracis. 4. Transudative pleural effusion. 5. Patients with well documented chronic history of heart failure, nephritic syndrome, liver cirrhosis. 6. Hemothorax/empyema following trauma to the chest wall. 7. Contraindication to thoracentesis like patient on mechanical ventilation, uncooperative patients, bleeding diathesis, patient on anticoagulation therapy, local cutaneous condition such as pyoderma or herpes zoster infection. 8. Patients on antituberculous chemotherapy. 9. Patients with Diabetes mellitus and HIV infection.

All the patients were subjected to following

Full history taking with special emphasis on family history and personal history of Tuberculosis. Thorough clinical examination

including general physical examination and local examination was performed. Radiological examinations including plain chest xray posteroanterior (PA) and lateral view performed whenever necessary. Other investigations like complete blood counts, sputum for AFB (acid fast bacilli), tuberculin testing. Written informed consent for thoracentesis was taken as protocol. Pleural aspiration/ Thoracentesis was done in 6th intercostals space in midaxillary or posterior axillary line after clinical localization of pleural effusion on respective side of thoracic cavity. We have USG facility in our department; in selected cases we used USG guidance for thoracentesis especially in loculated and minimal pleural effusion. Pleural fluid analysis including gross and microscopic examination was performed. Pleural fluid for nucleic acid amplification tests DNA-PCR (deoxyribonucleic acid polymerase chain reaction) were performed for in all the study samples. Other pleural fluid tests like microbiology (ZN and Gram's stain), biochemistry, ADA, cytology & pleural fluid AFB culture were performed.

Laboratory distribution of pleural fluid obtained with diagnostic thoracentesis

- 1) Biochemistry- 5 ml – Glucose, Protein, pH, ADA levels
- 2) Hematology- 5 ml – White Blood cell count
- 3) Bacteriology- 10 ml for M. tuberculosis Culture
- 4) Pathology – 5 ml for M. tuberculosis DNA PCR

DNA extraction and PCR protocols (Nucleic acid amplification test)

DNA extraction was performed in an identical manner for all patients' samples using High Pure PCR Template preparation kit (provided from Roche Co.). The kit is designed to purify nucleic acids from different requested specimens for PCR test. It contains a primary step as a pre-lysis for some specific specimens such as tissue or even embedded tissue. The main steps are started of applying proteinase K and binding buffer on samples, then use of inhibitor removal, washing and elution buffers respectively. At each step reagents were added to the filter tube. Supernatant was passed through collection tube after centrifugation. This procedure will highly reduce the contaminations and increase the efficiency of the recovery rate of the nucleic acids as much as possible. PCR carried out on the prepared purified nucleic acid use of *M.tuberculosis* PCR kit. It contained specific primers to target transposable element (IS6110) for amplification 330 base pair of template. 5 µL of template, 10 µL PCR buffer, 10 µL mixture (containing specific primers and d NTP, 2.5U taqpolymerase) were mixed and amplified with the recommended program. The applied PCR kit was constructed in a format of competitive PCR with internal control. Provided specific primers could also amplify a product from fragment encoding 900 base pair as internal control to ensure of proper extraction and removal of any expected inhibitors. This fragment was added before commencing extraction procedure.

The kit also contained specific labeled probes for specific and internal products to enable us for detection the amplified products by the fluorescence detector, called Fluorescent Amplification-based Specific. The PCR depends on the ability to alternately denature (melt) double-stranded DNA molecules and renature (anneal) complementary single strands in a controlled fashion. As in the membrane-hybridization assay described earlier, the presence of non complementary strands in a mixture has little effect on the base

pairing of complementary single DNA strands or complementary regions of strands. The second requirement for PCR is the ability to synthesize oligonucleotides at least 18–20 nucleotides long with a defined sequence. Such synthetic nucleotides can be readily produced with automated instruments based on the standard reaction scheme.

Pleural fluid ADA

In our study, we considered ADA level of 40 units/liter as a cut off for interpretation, value above 40 units/liter suggestive of tuberculous pleural effusion.

Pleural fluid ZN (Ziehl-Neelsen) staining

The pleural fluid which was collected in sterile container was centrifuged, the supernatant was discarded, 0.5 ml of the sediment deposit was used to prepare slides. The slides were then covered with strong carbolfuchsin, heated to steaming and left to stand for 5 minutes. The cycle was repeated three times before the excess stain was washed off with running tap water. The slides were then decolorized by flooding with 20% sulphuric acid for 3 to 5 minutes and then washed with water before they were counter stained with 0.3% methylene blue for 2 minutes. The slides were again washed. The slides were examined under the 100X oil immersion objective and 10X eye piece. A minimum of 100 fields were examined per slide before declaring it positive or negative. A slide was considered positive if it had at least one bacillus, which appeared as red, beaded rods.

Sputum for AFB

Same technique for processing, staining and interpretation of sputum samples used as in for pleural fluid.

Pleural fluid Culture of mycobacterium tuberculosis

The pleural fluid which was collected in McCartney Bottle was centrifuged, the supernatant was discarded, the deposit was mixed with double the volume of 4% NaOH, which was kept in a shaker for 20 minutes and was subjected for centrifugation, after centrifugation at 3,000xG for 15 minutes, 0.5ml of the sediment was inoculated into 2 LJ tubes. For the culture of *M. tuberculosis* in pleural fluid Lowenstein Jensen (LJ) media was used (Figure 2). Lowenstein-Jensen culture media was inspected weekly for mycobacterial growth for 8 weeks (Figures 3 and 4). Bacterial growths in LJ was identified by biochemical tests specifically niacin production and nitrate tests to detect *M. tuberculosis*. On LJ media, they produce characteristic non-pigmented colonies, with a general rough and dry appearance simulating breadcrumb.

Statistical analysis

Statistical analysis was done mainly by student t test to compare two variables and Chi square test when comparing many variables. P value was considered significant if it was below 0.05 and highly significant in case <0.001. A significant value of t was seen from table for degree of freedom. In our study, p value < 0.05 was considered as significant with either negative or positive correlation on account of biological variability.



Figure 1: chest X ray PA showing massive pleural effusion-left side

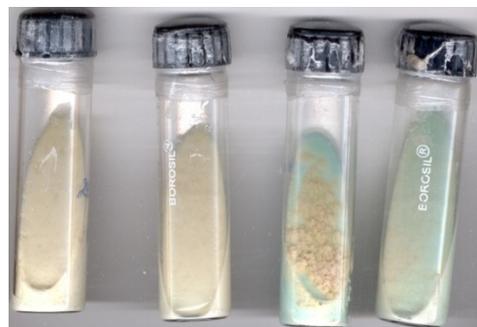


Figure 2: LJ Media showing classical growth characteristics of *M. Tuberculosis* isolated from pleural Fluid



Figure 3: Colony Morphology of *M.tuberculosis* - Dry wrinkled warty growth, Eugonic

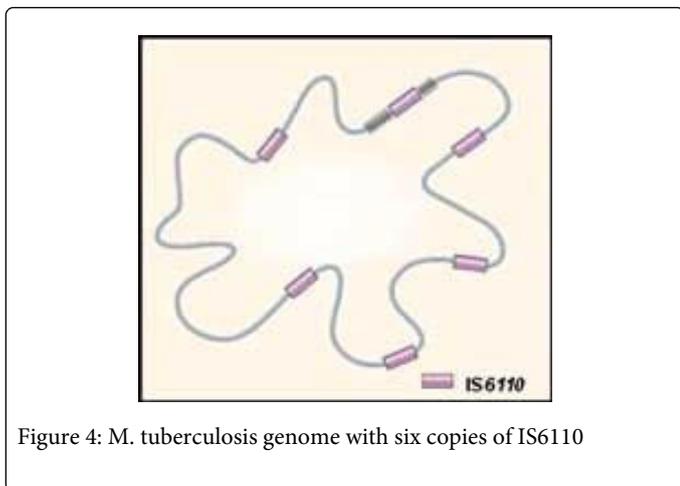


Figure 4: M. tuberculosis genome with six copies of IS6110

Observations and Analysis

Out of 100 patients enrolled in the study, 77 were males and 33 were females. The mean age of presentation was 45 ± 17.83 years. Majority of the patients were in the age group 21-50 years (52%), minimum age of 13 years and the maximum 82 years. 7% had past history of tuberculosis and 14% had family history of being treated for tuberculosis. Most common symptom was chest pain (98%) followed by cough (93%), fever (72%), dyspnea (71%), expectoration (37%). Commonest side involved was right (61%), in 4% of the cases the effusion was bilateral whereas 96% had a unilateral pleural effusion. 9% of the patients had a sputum smear positive for tubercular bacilli. 59 patients (59%) of the patients had a positive Mantoux test, among which 50 (50%) patients had a positive DNA PCR result and 19 (19%) patients had a positive pleural fluid culture for mycobacterium tuberculosis. 52 (52%) patients had lymphocytes greater than 50% in their pleural fluid.

	CULTURE +VE	CULTURE -VE	TOTAL
PCR +VE	26	48	74
PCR -VE	2	24	26
	28	72	100

(chi square 7.18,DF=1, p value<0.01)
Sensitivity=92.86% Specificity=33.33%
Positive Predictive Value=35.13% Negative Predictive Value=92.30%

Table 1: Comparing MTBPCR (NAAT) results with pleural fluid MTB Culture

	ADA IN CULTURE POSITIVE N (MEAN \pm SD)	ADA IN CULTURE NEGATIVE N (MEAN \pm SD)	P VALUE
PCR (n=74) +VE	26 (66.32 \pm 34.96)	48 (76.28 \pm 56.46)	>0.4

Table 2: Comparison of ADA levels in pleural fluid in PCR positive cases

	ADA IN CULTURE POSITIVE N (MEAN \pm SD)	ADA IN CULTURE NEGATIVE N (MEAN \pm SD)	P VALUE
PCR (n=26) -VE	2 (45.35 \pm 6.15)	24 (66.35 \pm 45.06)	<0.05

Table 3: Comparison of ADA levels in pleural fluid in PCR negative cases

PARAMETERS	YIELD (N)	PERCENTAGE
PLEURAL FLUID AFB SMEAR	03	03%
SPUTUM FOR AFB	09	09%
PLEURAL FLUID CULTURE	28	28%
PCR	74	74%
PLEURAL FLUID ADA >40 UNITS/LITER	85	85%
PLEURAL FLUID LYMPHOCYTES >50%	87	87%
LN RATIO 0.75	91	91%
PLEURAL FLUID CULTURE+PCR+ ADA+PLEURAL FLUID LYMPHOCYTE >50% +LN RATIO>0.75	98	98%
PLEURAL FLUID CULTURE+PCR+ ADA+LN RATIO+PLEURAL BIOPSY	100	100%

Table 4: Yield of various diagnostic procedures

PCR was positive in 74 of the total 100 cases with tuberculous pleural effusion. The sensitivity of the PCR was 92.86% and the specificity was 33.33% with a positive predictive value of 35.13% and

the negative predictive value of 92.30%. This shows that PCR is highly sensitive in diagnosing tubercular pleural effusion (Table 1).

In PCR positive cases of tuberculous pleural, there was no significant association between ADA levels in pleural fluid culture for MTB positive and negative ($P > 0.4$) (Table 2).

In PCR negative cases of tuberculous pleural; there was statistically significant association between ADA levels in pleural fluid culture for MTB positive and negative results ($P < 0.05$) (Table 3).

The Table 4 shows the usefulness of combining various tests in diagnosing tubercular pleural effusion. 09% cases were sputum positive for AFB. In the pleural fluid samples examined 3% were positive for AFB, 28% were culture positive, 74% were DNA PCR positive, and 85% cases had pleural fluid ADA > 40 units/liter, 87% cases had a LN Ratio greater than 0.75. Combined yield of pleural fluid culture, ADA > 40 units/liter, DNA PCR and LN ratio > 0.75 gave a positive diagnostic yield in 98% of cases, 2% with diagnostic dilemma were diagnosed by pleural biopsy.

Discussion

Role of pleural fluid DNA PCR

We subjected all the pleural fluid samples for DNA PCR and observed positive in 74 (74%) of the cases, among which 26 (26%) were culture positive, 50 were MT positive, 8 were sputum positive for AFB, 63 (63%) had an ADA greater than 40 units/liter. 64 of the PCR positive patients had a lymphocyte count greater than 50% in their pleural fluid. 65 patients had protein greater than 3.5 gm/dl. Sensitivity of PCR in our study was 92.85% and Specificity of 33.33%, Positive predictive value of 28.57%, Negative Predictive Value of 77.77%, this low specificity can be explained by cross contamination during the procedure which is a common problem in laboratories using in house protocol.

Reechaipichitkul et al. [9] mentioned a sensitivity of 50% and specificity of 61% and had PCR positive in 100% of culture positive TB effusion and only in 30-60% of culture negative pleural fluid. Bahador et al. [10] reported a PCR positive in 66 (84%) of 78 patients studied. Chakravarthy et al. [11] found 40 PCR positive cases out of 53 patients studied (75.47%), with a sensitivity of 75.5% and specificity of 93.8% PPV 97.6% and NPV 53.6%. Handojo et al. [12] reported a PCR positive in 26 out of 62 patients (41.19%), sensitivity of 53.35% and specificity 93.75% PPV (Positive Predictive Value) 96.15% and NPV (Negative Predictive Value) 41.67%. Khusboo et al. [13] reported PCR positive in 68.18%, with a sensitivity of 68.6% and specificity of 66%. Rhoda Lynn et al. [14] observed 50.70% PCR positive cases (36 PCR positive in 71 patients), sensitivity 61% and specificity 75%.

In this study, 2 cases with culture positive report were found to have MTB PCR negative and this can be caused by technical error in PCR processing, presence of inhibition enzyme DNA polymerases (internal inhibition), destruction of DNA in extraction process and sample containing no specific or low amount of DNA. In our study 72 patients had PCR positive and 28 patients had culture positive, is the result of high sensitivity of PCR compared with the culture which needs 50-100 bacilli/ml sample, dead bacilli, bacilli not having active metabolism (dormant) or contamination and inflammation reaction to part of mycobacterium TB another possible reason was uneven distribution/ mycobacterium forms a group at the infective site.

Role of Pleural Fluid ADA in our Study

85% of the patients had ADA levels > 40 units /liter and the mean ADA was of the patients studied was 70.69 ± 48.29 . Among them 25 (25%) were culture positive and 60 (60%) were culture negative and the mean ADA in culture positive cases and negative cases were 64.82 ± 34.11 and 72.96 ± 52.82 units/liter with no significant difference between the values. All 9 sputum ZN positive patients had an ADA > 40 units/litre, 63 of the 85 cases with ADA > 40 units/liter had positive PCR DNA for mycobacterium tuberculosis. 76 of the 85 patients with ADA > 40 units/liter had a LN ratio > 0.75 . 75 of the 85 patients with ADA > 40 units/liter had a protein level > 3.5 g/dl in pleural fluid. Sensitivity of ADA in our study was 89.28%.

In PCR positive cases of tuberculous pleural, there was no significant association between ADA levels in pleural fluid culture for MTB positive and negative ($P > 0.4$). In PCR negative cases of tuberculous pleural; there was statistically significant association between ADA levels in pleural fluid culture for MTB positive and negative results ($P < 0.05$).

Patel et al. [15] observed mean ADA was greater than 40 units/liter in their patients. Swamy et al. [16] reported a mean ADA of 100 ± 19.48 . Basu et al. [17] reported a mean ADA 100.05 and had 93.7% patients with ADA more than 70 units/liter. Soe et al. [18] reported a mean ADA 73.90 ± 33.95 , and took a cutoff of 42.5 units/liter and reported a sensitivity of 87%. Verma et al. [19] reported that 68% of patients had a ADA > 36 units/liter (range 36-229.7), with a sensitivity of 100% and states that if ADA is > 100 units/liter then it is seen only in TB. Maldhure et al. [20] observed 80 out of 83 patients studied had an ADA > 40 units/liter with a mean ADA 77.20 ± 32.63 units/liter. Krenke et al. [21] documented mean ADA 75.1 ± 39.1 unit/liter and sensitivity of 100% if cutoff for ADA was fixed at 40.3 units/liter. Moon et al. [22] observed 46 (80.70%) patients with an ADA > 45 units/liter. Before considering the level of ADA > 40 units/liter other conditions like lymphoma, brucellosis, Rheumatoid arthritis and empyema should be ruled out.

Correlation of all Parameters

The parameters used in our study to diagnose the tubercular pleural effusion were pleural fluid culture on LJ media, DNA-PCR for MTB in pleural fluid, pleural fluid ADA, pleural fluid lymphocyte greater than 50%, LN ratio greater than 0.75, pleural fluid AFB smear, sputum for AFB positive.

Culture was considered as Gold standard in our study and all the comparisons were made based on pleural fluid culture positivity and negativity. Considering various parameters for diagnosing the tubercular pleural effusion 3 were positive by pleural fluid AFB. Sputum for AFB was positive in 9 cases. 28 cases were confirmed by pleural fluid culture and 74 cases were diagnosed with the help of DNA PCR. 85 patients had an ADA greater than 40 units/liter and all these patients had an LN ration > 0.75 . In our study, 87 patients pleural sample had a lymphocyte greater than 50% and 91 had a LN ratio of > 0.75 .

When all the above parameters were combined, we were able to diagnose 98 patients with Tubercular pleural effusion, leaving a diagnostic confusion in two cases, where pleural biopsy and histopathology confirmed the tuberculous etiology for the pleural effusion.

Key note regarding pleural fluid MTB DNA PCR (NAATs) in evaluating tuberculous pleural effusion in our study

Although NAATs are widely used in western setting where prevalence of tuberculous pleural effusion is not remarkable, and results of these nucleic acid amplification tests are used for confirmation of tuberculosis as compared to conventional gold standard pleural fluid culture which is time consuming. In setting with high burden of tuberculous pleural effusion sensitivity and specificity of NAATs is not up to mark because of false positive and false negative issues.

False negative due to technical error in PCR processing, presence of inhibition enzyme DNA polymerases, destruction of DNA in extraction process and sample containing no specific or low amount of DNA, and false positive due to dead bacilli, bacilli not having active metabolism (dormant) or contamination and inflammation reaction to part of mycobacterium TB.

Important fact that needs to be considered is that, NAATs should be considered in cases where clinical and radiological spectrum is suggestive of tuberculosis, biochemical analysis shows exudative pleural effusion, cytology showing lymphocytic predominance (>50%), L/N ratio >0.75 and ADA is < 40 unit/litre.

Thus for the diagnosis of tubercular pleural effusion the following can be a valuable diagnostic utility-ADA >40 units/liter, Lymphocytes >50%, LN ratio greater than 0.75 and DNA PCR positivity gives a good diagnostic yield in case of non availability of pleural biopsy procedure and limitation of culture of pleural fluid, in a resource limited settings.

We specifically recommend MTB DNA PCR (NAATs) in cases with clinical-radiological features, biochemical and cytology analysis including ADA<40 units/liter are in clinical dilemma of tuberculosis as a etiology for pleural effusion. In these cases we observed additional yield in aiding diagnosis of tuberculous pleural effusion and we confirmed it with conventional gold standard pleural fluid culture for MTB.

Conclusion

Commonest cause of pleural effusion in India is tuberculosis (TB) and poses a diagnostic difficulty because of the low sensitivity of culture technique and many cases were diagnosed on the basis of pleural fluid ADA>40 units/liter and cytology analysis showing lymphocyte predominance with lymphocyte/neutrophil (L/N) ratio >0.75%.

In cases with exudative pleural effusion with Lymphocyte in pleural fluid >50% and L/N ratio>0.75 with ADA <40 units, MTB DNA PCR (NAATs) will be very useful in confirming tuberculosis as a cause for pleural effusion. Results of NAATs in this situation are very useful, sensitive, less time consuming and comparable to pleural fluid culture. We observe complete therapeutic response to antituberculosis chemotherapy in all the study cases. Hence we recommend MTB DNA PCR in these cases.

Additionally Pleural fluid NAATs will have good place in routine evaluation of Tuberculous pleural effusion where resources of pleural fluid culture are limited, case burden is high and is time consuming where results of NAATs are available in same day with acceptable sensitivity of these tests.

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