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Original Article

Microbiology Section

Evaluation of Microscopy, Adenosine Deaminase and Lactate Dehydrogenase Levels as Diagnostic Methods of Tubercular Pleural Effusion

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ABSTRACT

Introduction: Pleural effusion has been associated with over 50 aetiologies, amongst which Tuberculous Pleural Effusion (TPE) is one of the most prevalent infection. TPE is highly infectious and easily transmitted. Pleural biopsy is currently, the most accepted diagnostic criteria. However, the procedure is invasive, tedious and time-consuming.

Aim: To bring about a better understanding of Adenosine Deaminase (ADA), Lactate Dehydrogenase Levels (LDH), LDH: ADA ratio and microscopy as screening and diagnostic methods of TPE.

Materials and Methods: The research was a hospital-based observational study which analysed 50 cases of pleural effusion. After obtaining informed consent, Pleural Fluid (PF) was collected and ADA and LDH levels were analysed. Ziehl-Neelsen (ZN) staining of PF was performed and pleural biopsy was obtained. Using appropriate statistical analytical tools, Receiver Operating Characteristic (ROC) curves were prepared to find the ideal cut-off point for each diagnostic criterion. The sensitivity, specificity and Area Under Curve (AUC) for each parameter were compared.

Results: In this study, 76% were males and 24% were females with a mean age of 41 years. Pleural biopsy was used as the gold standard for the positive tuberculosis case. Of the 50 cases analysed, 31 cases (62%) were reported with a positive biopsy report for TPE, while 19 (38%) presented a negative biopsy report. At a cut-off level of 26 IU/L, pleural ADA level reported sensitivity and specificity of 96.77% and 57.89%, respectively, while that of LDH was 93.55% and 68.42%, respectively at a cut-off of 326 IU/L. The LDH: ADA ratio was found to be most significant at 6.64 with a sensitivity of 93.55% and a specificity of 36.84%. The demonstration of AFB in ZN staining had 100% specificity but a mere 9.68% sensitivity.

Conclusion: According to the present study, ADA had maximum sensitivity and thus, is the most suitable screening criterion for suspected cases of TPE, followed by LDH and LDH:ADA ratio, while ZN staining has maximum specificity, amongst the various parameters evaluated.

Keywords: Pleural biopsy, Sensitivity, Specificity, Tuberculosis screening, Ziehl Neelsen staining

INTRODUCTION

Despite the isolation of Tuberculosis (TB) in 1882 by Robert Koch, as well as the availability of effective treatment and the availability of a live attenuated vaccine in most parts of the world, TB continues to be one of the deadliest communicable diseases. A total of 1.5 million people died from TB in 2018 (including 2,51, 000 people with HIV) [1]. Eight countries accounted for two thirds of the global total: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (6%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) [1].

Although, it primarily affects the lungs, extra-pulmonary organs such as the pleura, lymph nodes, abdomen, and genitourinary tract are also affected. Of these, TPE imposes a serious threat, as TB is a leading cause of pleural effusions in developing countries [2]. Characteristic symptoms of TPE include chronic non-productive cough, mild fever, pleuritic chest pain, dyspnea, loss of appetite, and significant weight loss [3,4]. Physical examination usually reveals reduced tactile fremitus, a stony dull note on percussion, and diminished or absent breath sound on auscultation.

The manifestation of paucibacillary mycobacterial infection within the pleural space results in an immunological response that both increases PF formation and decreases PF removal, leading to pleural effusion. It is characterised by an intense chronic accumulation of fluid and inflammatory cells. This fluid is rich in proteins and the specific leukocytes are recruited into the pleural space, following an increased vascular permeability due to inflammatory processes [5].

Adenosine Deaminase (ADA) level is an easy, non-invasive and inexpensive method of diagnosis. PF level of more than 40 IU/L is usually considered diagnostic [6,7]. Higher levels of ADA have been associated with other conditions such as malignancies, collagen vascular diseases, chylothorax and postcoronary bypass graft [8]. It is also found in patients with neutrophilic effusions such as parapneumonic effusions or empyemas [9].

Lactate Dehydrogenase (LDH) level has been used as a pathophysiological marker in numerous opportunistic infections, as its level rises upto 500 IU/L from its normal range of 135 to 250 IU/L [10]. Levels higher than 1000 IU/L suggest empyema, malignant effusion, rheumatoid effusion, *Pneumocystis jiroveci* pneumonia or pleural paragonimiasis [11]. LDH and ADA levels are individually reliable factors to distinguish between TPE and Parapneumonic Pleural Effusion (PPE). However, LDH level varies from normal to severely increase in PPE and a significantly elevated ADA is frequently measured in both conditions. A recent study had found that an LDH: ADA ratio of 16.20 was specific for TPE [12].

Due to its simplicity and high sensitivity, ZN staining is a highly recommended diagnostic method [13]. The demonstration of Acid-Fast Bacillus (AFB) in the sample is considered as a positive result. However, *Mycobacterium tuberculosis* is rarely observed on direct examination due to its very poor yield in extrapulmonary TB and less than 30% of cultured PF are usually positive [14].

The detection of *Mycobacterium tuberculosis* in pleural biopsy or culture or histological demonstration of caseating granulomas in pleura along with AFB is currently considered to be the gold standard diagnostic method [14]. However, the procedure is invasive, tedious, time-consuming, and not available in all clinical settings. Moreover, culture takes up to eight weeks to yield positive results.

Hence, more practical diagnostic methods are required, especially in high burden, developing countries like India. So, the overall aim of the study was to compare the sensitivity and specificity of ZN staining, pleural ADA, LDH levels and LDH: ADA ratio as diagnostic methods for TPE with the results of pleural biopsy, which is the gold standard, using a ROC curve for each diagnostic method.

MATERIALS AND METHODS

This was a hospital-based observational study, carried out in the Microbiology Department of Dr DY Patil Medical College, Hospital and Research Centre, Pune, Maharashtra, India for a period of two months between August and September, 2019. Total 50 patients of suspected TPE having symptoms such as dyspnoea, pleuritic chest pain, evening rise of temperature, weight loss and dry cough, were selected irrespective of their age and sex [15]. Known cases of empyema, haemothorax, and patients on chemotherapy were excluded. Patients of pleural effusion in whom fluid could not be aspirated were also excluded. The study was initiated after approval was obtained from the Institutional Ethics Committee, Dr DY Patil Medical College, Hospital and Research Centre in letter number IESC/C-105/2019, dated 10/07/2019.

After enrolment, the procedure was explained to the patients in their vernacular language and informed consent was obtained. Patient data such as age and sex were noted and general and systemic examination was performed. Mild to moderate rise in temperature, increased respiratory rate, and diminished movements of affected side of chest were most commonly observed. On percussion, stony dull note was heard over affected area, along with diminished vesicular breath sounds and decreased vocal resonance while auscultating.

Samples Collected

A pleural biopsy sample and a minimum of 40 ml of PF were collected in sterile, leak-proof, screw-capped containers, using standard thoracocentesis procedure [16]. PF was collected in a plain tube for ADA and LDH analysis, and smear for ZN staining.

Investigations Performed

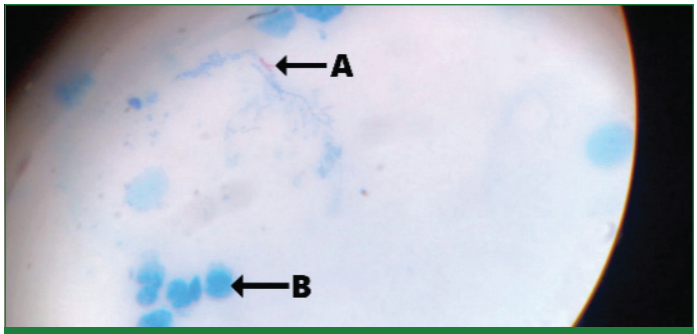
A pleural biopsy was performed and submitted to the histopathology laboratory. The sample was considered as tuberculous, if granulomatous epitheloid caseating cells were identified on histological examination. A positive microbiological result was declared if tuberculous bacilli were identified in a sample of PF.

Total ADA and LDH were measured by the colorimetric method of Guisti G and Galanti B [17]. The supernatant was analysed in semi-automated chemical analyser (Swing TwinSampler, Biorad), and biochemical measurements were performed on a clinical chemistry analyser (Siemens, Dimension EXL-200 I) using the standard laboratory method.

ZN staining of PF was performed using the conventional method on all 50 samples collected. The demonstration of AFB was recorded as a positive result [Table/Fig-1].

STATISTICAL ANALYSIS

Data was entered into Microsoft Excel document.The sensitivity and specificity of these criteria for detecting TB were calculated using Epi Info Statistical Analysis and WinPepi, with the level of statistical significance set at p-value <0.05.



[Table/Fig-1]: ZN staining of Pleural Fluid (PF) smear.
A: Pink acid fast bacilli; B: Blue nonacid fast pus cells

A ROC curve was prepared using MedCalc for ADA, LDH, LDH: ADA ratio and ZN staining and used to determine the optimal cut-off points for the diagnosis. Youden's index which reflects the overall capacity of an early warning model to detect outbreaks was also calculated.

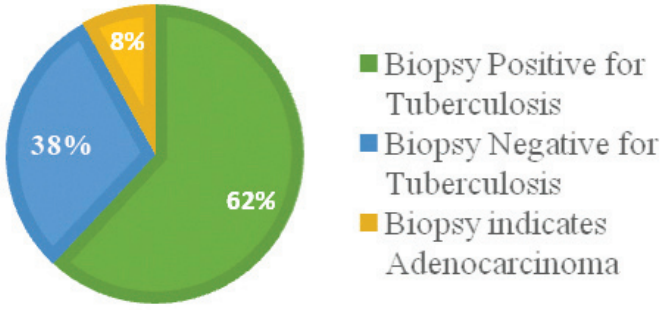
RESULTS

A total of 50 patients between the ages of 15 years to 80 years were analysed, with a mean age of 41 years. There were 38 males and 12 females [Table/Fig-2]. Maximum patients were between the age group of 21 to 40 years and a male preponderance (76%) was seen.

Age distribution (years)	Male	Female	Total
11 to 20	6	2	8
21 to 30	8	4	12
31 to 40	10	2	12
41 to 50	3	2	5
51 to 60	3	1	4
61 to 70	3	1	4
70 to 80	5	0	5
Total	38	12	50

[Table/Fig-2]: Age and sex distribution of the 50 participants evaluated in the study.

Of the 50 cases studied, 31 cases presented with a positive biopsy report for TPE, while 19 presented with a negative biopsy report, amongst whom 4 cases were positive for adenocarcinoma of the lung [Table/Fig-3].



[Table/Fig-3]: Results of biopsy.

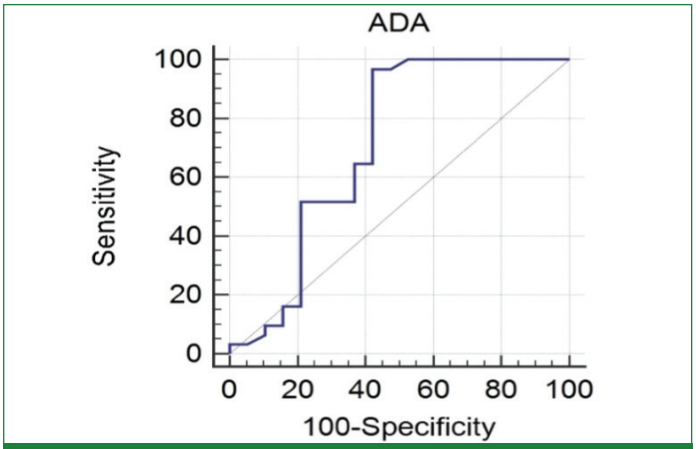
Analysis of Pleural ADA Levels

The maximum level of ADA in pleural effusion level was 126 IU/L while the minimum was 7.3 IU/L with a mean value of 53.52 IU/L [Table/Fig-4].

	In positive cases	In negative cases	In total cases
Maximum (IU/L)	126	112	126
Minimum (IU/L)	24	7.3	7.3
Mean	61.63	40.27	53.52

[Table/Fig-4]: Range of ADA levels.

Using the ROC curve of pleural ADA levels [Table/Fig-5], for the cut-off value of >26 IU/L, sensitivity and specificity are 96.77% and 57.89%, respectively [Table/Fig-6].



[Table/Fig-5]: ROC curve of pleural ADA levels.

Variables	Values
Area under the ROC curve (AUC)	0.710
Standard error	0.0901
Youden index J	0.5467
Associated criterion	>26
Sensitivity	96.77%
Specificity	57.89%

[Table/Fig-6]: Statistical analysis of ADA Level.

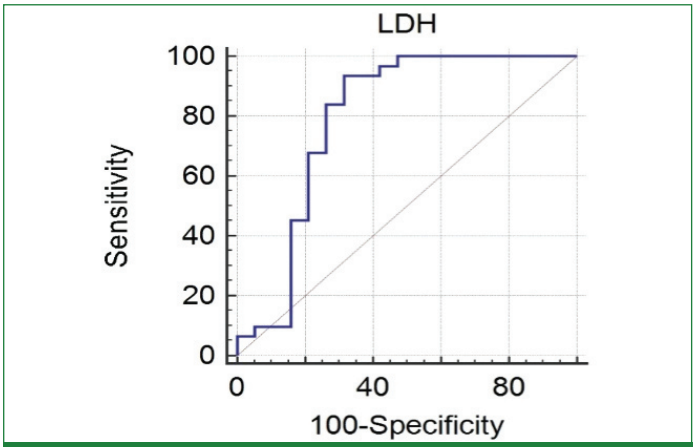
Analysis of Pleural LDH Levels

The maximum level of LDH in pleural effusion level was 4726 IU/L, while the minimum was 74 IU/L with a mean value of 791.42 IU/L [Table/Fig-7].

	In positive cases	In negative cases	In total cases
Maximum (IU/L)	4726	115	4726
Minimum (IU/L)	293	74	74
Mean	994	460.89	791.42

[Table/Fig-7]: Range of LDH levels.

Using the ROC curve of pleural LDH levels [Table/Fig-8], for the cut-off value of >326 IU/L, the sensitivity and specificity are 93.55% and 68.42%, respectively [Table/Fig-9].



[Table/Fig-8]: ROC curve of pleural LDH levels.

Analysis of Pleural LDH: ADA Ratio

The maximum ratio of LDH: ADA in pleural effusion level was 141.3 while the minimum ratio was 1.02 with a mean value of 23.7 [Table/Fig-10].

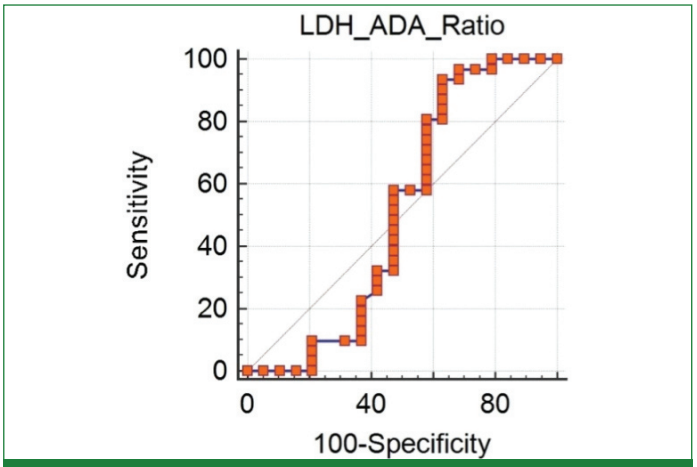
Using the ROC curve of pleural LDH: ADA ratio [Table/Fig-11], for the cut-off value of LDH: ADA ratio >6.64, the sensitivity and specificity are 93.55% and 36.84%, respectively [Table/Fig-12].

Variables	Values
Area under the ROC curve (AUC)	0.793
Standard error	0.0810
Youden index J	0.6197
Associated criterion	>326
Sensitivity	93.55%
Specificity	68.42%

[Table/Fig-9]: Statistical analysis of LDH Level.

	In positive cases	In negative cases	In total cases
Maximum	49.74	141.3	141.3
Minimum	3.13	1.02	1.02
Mean	16.69	35.14	23.7

[Table/Fig-10]: Range of LDH: ADA ratio.



[Table/Fig-11]: ROC curve of pleural LDH: ADA ratio.

Variables	Values
Area under the ROC curve (AUC)	0.510
Standard error	0.102
Youden index J	0.3039
Associated criterion	>6.64
Sensitivity	93.55%
Specificity	36.84%

[Table/Fig-12]: Statistical analysis of LDH: ADA ratio.

Analysis of ZN Staining

Of the 50 total samples stained and the 31 tuberculous positive cases as determined by biopsy, only three presented with a positive ZN staining result, which involved the demonstration of AFB.

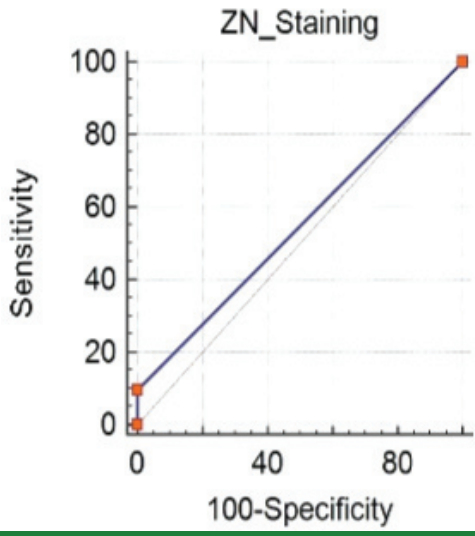
Using the ROC curve [Table/Fig-13], ZN staining has a sensitivity of 9.68% and a specificity of 100% for the 50 cases analysed [Table/Fig-14].

[Table/Fig-15] compares the diagnostic accuracy of ADA, LDH, LDH: ADA ratio and ZN staining.

LDH has the maximum area under curve (0.793), followed by ADA (0.791), ZN Staining (0.548) and LDH: ADA ratio (0.541), making it a better diagnostic criterion as compared to the others. [Table/Fig-16] shows the comparison of significant cut-off, sensitivity and specificity of ADA,LDH, LDH:ADA and ZN staining. Thus, in the current study ADA level proves to have the highest sensitivity (96.77%) at the cut-off value of 26 IU/L while ZN Staining has the highest specificity at 100% for the 50 patients evaluated.

DISCUSSION

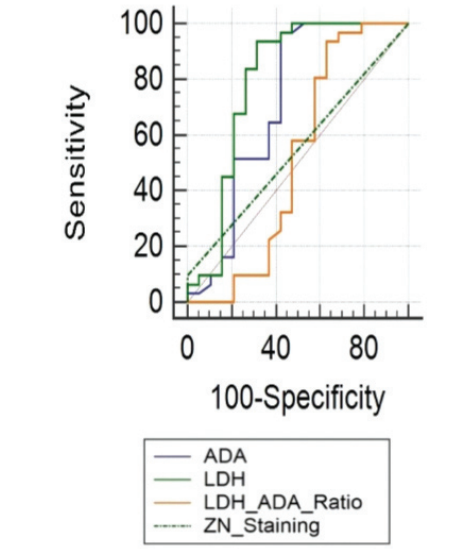
Tuberculosis is often referred to as Koch's disease, in light of the social dilemma attached, it is highly prevalent in India. Despite a reasonable understanding of the underlying cause and pathology



[Table/Fig-13]: ROC curve of ZN staining.

Variables	Values
Area under the ROC curve (AUC)	0.548
Standard error	0.0270
Youden index J	0.09677
Sensitivity	9.68%
Specificity	100.00%

[Table/Fig-14]: Statistical analysis of ZN staining.



[Table/Fig-15]: ROC curve comparing various parameters evaluated.

Parameter	Significant cut-off	Sensitivity (%)	Specificity (%)
ADA	26 IU/L	96.77	57.89
LDH	326 IU/L	93.55	68.42
LDH: ADA	6.64	93.55	36.84
ZN Staining	-	9.86	100.00

[Table/Fig-16]: Comparison of significant cut-off, sensitivity and specificity of various parameters.

of the disease as well as the availability of cheap clinical treatment, it still remains one of the 10 most dangerous infectious diseases worldwide, second only to HIV [1]. The highly communicable nature of TB and India's population density contribute to making India the world's TB capital. While extra-pulmonary TB can manifest in almost every organ, one of the most common forms is TPE [18].

Common manifestations of pleural effusion include coughing, sharp chest pain and breathlessness. Even though the diagnosis of pleural effusion is clinically easy, finding the underlying cause proves to be a cumbersome task, as more than 50 aetiologies of pleural effusion have been reported, such as TB, congestive cardiac failure, pericarditis, pancreatitis, and malignancies [18]. Of these, TPE presents as chronic cough, mild fever, pleuritic pain, dyspnoea, loss of appetite and weight loss [3,4]. Physical examination reveals reduced tactile fremitus, a stony dull note on percussion and diminished or absent breath sound on auscultation.

Tuberculous infection leads to rupture of subpleural caseous foci which results in mycobacterial invasion of the pleural space. The current gold standard for the diagnosis of TPE is pleural biopsy, due to its high specificity [13]. The presence of caseating granulomas containing AFB is considered a positive biopsy for the diagnosis of TB [14]. However, pleural biopsy has low sensitivity and culturing may take up to eight weeks, making it time-consuming which also delays medical treatment. Moreover, it is invasive, lengthy and requires skilled labourers, making it a suboptimal diagnostic criterion for a high-burden country like India.

Pleural ADA Levels

Numerous biomarkers in the PF have been analysed in an attempt to find a reliable method. Amongst those studied, ADA and Interferon-gamma have been found the most useful [19]. Due to its low cost and easy access, ADA is widely used and accepted.

ADA is an enzyme that catalyses the conversion of adenosine and deoxyadenosine to inosine and deoxyinsosine in the purine degradation pathway, As its level rises in the immature and non-differentiated T-lymphocytes following mitogenic and antigenic stimulation in conditions such as mycobacterium infection of alveolar macrophages, it can act as a reliable biomarker. Several studies have been performed which report a wide range of cut-off values, most commonly varying from 40 to 60 IU/L [19,20]. A lower specificity may be accounted for, by a rise in ADA levels in various other conditions such as lymphoma, collagen vascular diseases (rheumatoid arthritis, systemic lupus erythematosus), adenocarcinoma of lung and empyema [21]. Fungal infections such as coccidiodomycosis and histoplasmosis have also been identified as a cause of a rise in pleural ADA levels, especially in immunosuppressed patients [22].

Porcel JM et al., reported a value of ADA in PF of >35 IU/L with a sensitivity and specificity of 93% and 90%, respectively [23]. This study compared the values of ADA for various aetiologies and was not specific for TB. In another study conducted by Verma SK et al., a similar value of pleural ADA level of >36 IU/L was found at a sensitivity and specificity of 100% and 77.7%, respectively [7].

The current study found ADA levels of >26 IU/L in the PF to be the optimal cut-off with sensitivity and specificity of 96.77% and 57.89%, respectively for the diagnosis of TPE, as seen in [Table/Fig-6]. This study was comparable to that performed by Helmy NA et al., with a sample size of 30 of which 19 cases were TB positive and reported an optimal cut-off point at 30 IU/L where the sensitivity of ADA was (80%) and specificity was (85%), with values most similar to the results of the present study [24]. A similar small sample size can be the reason for such similarity.

This study also revealed that age may be used along with ADA for the diagnosis of TPE, as it increases the diagnostic value of ADA. A higher diagnostic value was achieved with the same cut-off value of 30 IU/L when patients under 50 years were compared against those above 50 years, as reported by Helmy NA et al., [24]. Similarly, Tay TR and Tee A, proposed a value of 72 IU/L as a cut-off criteria for pleural ADA levels in patients above 55 years of age and 26 IU/L in patients below 55 years, with a sensitivity of 95% in both cases [21]. The present study, however, does not analyse age due to the limitations of a small sample size. Further studies should be performed taking into account the age, which will help increase the specificity of the test for various age groups. Vorster MJ et al., reported a high negative predictive value in countries with low prevalence of TB [10]. As the present study has been conducted

in a high burden setting, a high value of ADA is considered to be a reliable diagnostic criterion.

There are two isoenzymes of ADA, amongst which ADA2 levels rise in TPE whereas an increase in ADA1 levels have been reported in other conditions such as empyema. Various different studies have reported specificity for TB from 91% to 96% and 92.1% to 98.6% with the use of the ADA-2 isoenzyme measurement [10,25,26]. The present study utilises total ADA as opposed to ADA1, despite a slightly lower sensitivity and specificity (96.77% and 57.89%, respectively) as the former is significantly cheaper and widely available.

When the tubercular infection is controlled, there is a decrease in leucocytes and hence, a corresponding decrease in ADA level, due to which it could also be used as a marker to monitor response to treatment of TB and to detect the development of resistance in Tubercle bacilli. Large-scale studies need to be conducted to evaluate its role as a prognostic method.

Pleural LDH level

LDH, an intracellular enzyme having five isoenzymes, of which isoenzyme 3 is associated with lung-related infections along with lymph tissue, platelets and pancreas and is used as a pathophysiological marker in numerous opportunistic infections, as its level rises in infectious mononucleosis, hepatitis, pancreatitis, sepsis and septic shock. The low specificity can be accounted for various aetiologies. Measurement of pleural LDH level is a non-invasive, repeatable, inexpensive and easily available test that provides rapid results.

The current study confirms that LDH levels are raised in TPE cases. According to the analysis presented in [Table/Fig-16], for the cut-off value of >326 IU/L, the sensitivity and specificity are 93.55% and 68.42% respectively. This was similar to the results of the study performed by Yang J et al., which found a pleural LDH level of 332 IU/L to be an ideal cut-off value for the diagnosis of TPE [27]. However, very few studies have assessed the diagnostic value of LDH for TPE [7,27]. One study performed by Young-Chul K et al., reported a mean value of 964.9IU/L in tuberculous patients [28]. This was comparable to the mean value of 994 IU/L reported in the current study. However, they also concluded that LDH was a poor diagnostic criterion for the differential diagnosis of tuberculous and malignant pleural effusions.

LDH:ADA Ratio

Wang J et al., reported LDH: ADA ratio to be highly predictive of TPE at a cut-off level of 16.20 with a sensitivity and specificity of 93.62% and 93.06% [12]. In the current study, the ratio was significantly different as for the cut-off value of 6.64 the present study reported a sensitivity and specificity of 93.55% and 36.84%, respectively [Table/Fig-16]. The sensitivity was comparable to that of ADA and LDH individually. However, its low specificity indicates that this ratio is a poor diagnostic criterion. This disparity between the study performed by Wang J et al., [12] and the current study may be due to a significant difference in sample size, as the former study used a sample size of 119, whereas the present study only evaluated 50 patients. Another cause for this difference could be because the present study was conducted in the Indian population which is different compared to the Chinese population, as analysed by Wang J et al., [12].

Numerous other aetiologies have been associated with an increased LDH: ADA ratio such as empyema, malignant effusion, parapneumonic and rheumatoid effusions. Possible contributing aetiologies should be ruled out.

ZN Staining

The demonstration of AFB in PF smears was considered as a positive result [Table/Fig-1]. The present study reported a mere sensitivity 9.86% with a specificity of 100%, as aforementioned in [Table/Fig-16]. This low sensitivity could be accounted for the fact that the hypersensitivity reaction to the mycobacterial infection causes TPE rather than the invasion of the pleura by the bacteria itself. Pleural effusion is hence, the result of accumulation of fluid enriched in proteins and the recruitment of specific leukocytes into the pleural space, following an increased vascular permeability due to inflammatory processes.

As a result, in very few cases, only 3 of the 31 biopsy-positive cases showed presence of mycobacteria on ZN staining. This result was similar to the 18% positive culture rate obtained by Bays A and David JP both of which are significantly lower figures than the 63% reported by Ruan SY et al., [29,30]. One cause for this disparity may be the difference in sample size.

Limitation(s)

Due to the small sample size evaluated for the study, a definitive criterion cannot be established. In the future, a study may be carried out in a larger population to obtain more accurate mean values and to decrease the margin of error.

CONCLUSION(S)

The present study analysed various parameters to determine the diagnostic reliability for the diagnosis of TPE, using the demonstration of granulomatous epitheloid caseating cells on pleural biopsy as the gold standard. Pleural ADA level appears to be an ideal screening method, especially in high burden settings, due to the highest sensitivity, along with numerous benefits such as low cost, rapid result and being a minimally invasive and less tedious procedure. Pleural LDH levels and LDH:ADA ratio has high sensitivity, but low specificity. Even though ZN staining has the highest specificity for the patients evaluated, making it reliable for an accurate diagnosis; it has very low sensitivity and thus, cannot be employed as a reliable screening method.

Further studies may be carried out analysing various combinations of these diagnostic criteria and may be used alone or in groups. Comparing and contrasting the diagnostic criteria will help determine a composite index with a balance between cost-effectivity and diagnostic accuracy of TPE.

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