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RESEARCH ARTICLE

A PROSPECTIVE CLINICO-PATHOLOGICAL STUDY OF FLUID CYTOLOGY AND ADENOSINE DEAMINASE LEVELS IN PLEURAL EFFUSIONS

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ABSTRACT

Introduction: The use of biological markers in the diagnosis of tuberculous pleural effusion (TPE) is a breakthrough. Demonstration of elevated levels of Pleural fluid Adenosine deaminase (ADA), interferon-gamma (IFN-γ), tuberculous proteins/antibodies lysozme etc. have been proposed. Adenosine deaminase (ADA) estimation in pleural fluid has been shown as reliable biomarker specially when there is suspicion of tuberculosis. Detection of mycobacterium DNA by PCR is also a proposed test3,4. However India being a developing country with much of its people below poverty line cannot afford expensive tests like ELISA, PCR, IFN-γ. Hence, there is need for relatively cheaper and simple tests with feasibility and sensitivity going hand-in-hand5 TPE being proposed to be a delayed hypersensitive reaction and lymphocytes play a major role in the pathogenesis. With >50% lymphocytes in the pleural fluid, combined criterion of lymphocyte to neutrophil ratio of >0.75 with a raised ADA level increased the specificity of diagnosis in many recent studies10,12. **Aims and Objectives**: The aims and objectives of our study are - To analyse the cell count, protein and glucose levels in pleural fluid. To correlate cell counts with ADA levels of pleural fluid in order to confirm diagnosis of tuberculosis. To correlate cytomorphology of pleural fluid with clinical and other details available of patient in order to arrive at the diagnosis. **Material And methods:** The present institutional based prospective study was undertaken after obtaining Ethical clearance. 500 patients presenting with pleural effusion were studied. In all the patients, a detailed history was taken and physical examination of pleural fluid was done in which colour of fluid, amount and nature of fluid was noted. Pleural fluid was received for cytological examination. Total and differential count (TDLC) was done by manual method. Glucose and protein estimation in pleural was also done. Pleural fluid ADA levels wasmeasured by spectrophotometric method. Clinicocytological as well as ADA correlation was done in all cases for the diagnosis of tubercular pleural effusions. All cases except hemothorax and empyema were included in the study. Pleural fluid was collected in 2 separate containers one for biochemical analysis- protein, glucose and ADA estimation, another for TDLC and cytological examination. About 20 cc fluid was collected for the study. The pleural fluid was subjected to the above mentioned tests within 3-4 hours. -Pleural fluid Glucose, Protein, ADA level were estimated by spectrophotometeric method using (COBAS C311, (Hitachi-Roche) instrument.

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Tuberculosis is a major contributor of pleural effusion which is the second most common extra-pulmonary manifestation of tuberculosis following tubercular lymphadenitis (Basu, 2012). The use of biological markers in the diagnosis of tuberculous pleural effusion (TPE) is a breakthrough. Demonstration of elevated levels of Pleural fluid Adenosine deaminase (ADA), interferon-gamma (IFN-γ), tuberculous proteins/antibodies lysozme etc. have been proposed. Adenosine deaminase (ADA) estimation in pleural fluid has been shown as reliable biomarker specially when there is suspicion of tuberculosis. Detection of mycobacterium DNA by PCR is also a proposed test (Gupta, 2010; Kelam, 2013).

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However India being a developing country with much of its people below poverty line cannot afford expensive tests like ELISA, PCR, IFN-γ. Hence, there is need for relatively cheaper and simple tests with feasibility and sensitivity going hand-in-hand (Ungerer, 1994) TPE being proposed to be a delayed hypersensitive reaction and lymphocytes play a major role in the pathogenesis. With >50% lymphocytes in the pleural fluid, combined criterion of lymphocyte to neutrophil ratio of >0.75 with a raised ADA level increased the specificity of diagnosis in many recent studies (Burges, 1995; Shirish M Kawathalkar). The present study is aimed to observe the diagnostic correlation of cytological examination of pleural fluid and Adenosine deaminase levels (ADA) in cases of pleural effusions..

AIMS AND OBJECTIVES

The aims and objectives of our study are

- To analyse the cell count, protein and glucose levels in pleural fluid.
- To correlate cell counts with ADA levels of pleural fluid in order to confirm diagnosis of tuberculosis.

MATERIALS AND METHODS

The present institutional based prospective study was undertaken after obtaining Ethical clearance. 500 patients presenting with pleural effusion were studied. In all the patients, a detailed history was taken and physical examination of pleural fluid was done in which colour of fluid, amount and nature of fluid was noted. Pleural fluid was received for cytological examination. Total and differential count (TDLC) was done by manual method. Glucose and protein estimation in pleural was also done. Pleural fluid ADA levels was measured by spectrophotometric method. Clinicocytological as well as ADA correlation was done in all cases for the diagnosis of tubercular pleural effusions. All cases except hemothorax and empyema were included in the study. Pleural fluid was collected in 2 separate containers one for biochemical analysis- protein, glucose and ADA estimation, another for TDLC and cytological examination. About 20 cc fluid was collected for the study. The pleural fluid was subjected to the above mentioned tests within 3-4 hours. -Pleural fluid Glucose, Protein, ADA level were estimated by spectrophotometeric method using (COBAS C311, (Hitachi-Roche) instrument.

METHODS

TDLC: Total leucocyte count (TLC) of pleural fluid was performed after dilution, with WBC diluting fluid (Turk's fluid) in a improved Neubauer's chamber and the cells were counted. For Differential leucocyte counts (DLC) -pleural fluid was centrifuged at 3000 rpm for 15 minutes. The slides were prepared from the sediment and stained with Field's stain or Giemsa stain and examined.

Pleural fluid glucose estimation:

Automated Method: The glucose oxidase reagent was used and tests were performed in fully automated analyser of Hitachi/ Roche- model-902.

Pleural fluid protein estimation

Automated Method: The colour intensity is directly proportion to the protein concentration which can be determined photometrically. The entire tests were performed by Biuret method in fully automated analyser of Hitachi/ Roche- model-501.

4.PLEURAL FLUID ADA was determined by spectrophotometric method using. (COBAS C311, Hitachi-Roche) instrument by Galanti and Giusti 1974.

Measurement Of ADA

Principle:

The adenosine deaminase assay is based on the enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to uric acid and hydrogen peroxide (H2O2) by xanthine oxidase (XOD) H2O2 is further reacted with N-ethyl-N- (2-hydroxyl-3-sulfopropyl) 3-methylaniline (EMSPT) and 4-amino antipyrine (4-AA) in the presence of peroxidase (POD) to generate quinine dye which is monitored in a kinetic manner.

5.PLEURAL FLUID CYTOLOGY:

Method of preparation of smear-

About 20 mL of pleural fluid was taken for cytological examination.

The fluid was centrifuged in a conical tube at 1500 rpm for 5-10 minutes. The supernatant was discarded and smears were made from the sediment, rapidly wet fixed in 95% ethanol, for Papanicolou stain.

- Papanicolaou Staining Method

- Papanicolaou staining method is the routine staining procedure used in cytopathology laboratory. This technique is named after Dr. George N. Papanicolaou, the father of exfoliative cytology.

The Papanicolaou stain results in well stained nuclear chromatin, differential cytoplasmic counterstaining and cytoplasmic transparency.

STATISTICAL ANALYSIS

Sample size:

Sample size determination

$$n = \frac{22\sigma Z}{E^2}$$

Where σ is Standard Deviation found to be 0.57

Absolute error, E was considered to be 5% and Standard Normal variate z at 5% level of significance was 1.96

$$N = \frac{(0.57)^2(1.96)^2}{(0.05)^2}$$

$$= \frac{1.248}{0.0025}$$

$= 499.25 \approx 500$ sample Sample size = 500

For further analysis of data, various suitable statistical test were used so as to fulfill the objects of study.

Duration of study = 1 year

For statistical analysis chi-square test was used

$$\chi^2 = \left[\frac{(O - E)^2}{E}\right.$$

χ = chi square value

O = observed value

E = expected value

OBSERVATIONS

A clinicopathological prospective study was done in patients who presented with pleural effusion at Geetanjali Medical College and Hospital, Udaipur during a period of one and half years from January 2016 to June 2017. A total of 500 cases were studied in the present study.

The pleural fluid physical examination, cell count, ADA estimation and cytological examination were done to diagnose them as tubercular effusion, malignant effusion, suppurative or transudative effusion.

Total and differential leucocyte count in pleural fluid: The mean total WBC count in the cases of tuberculous pleural effusion was 1761.82 cells/mm^3 among which majority of cells (73.32%) were lymphocytes. Lymphocyte predominance was also seen in malignant pleural effusion (68.83%) but total leucocyte count was much lower (mean 995.56/cmm) than that in tubercular effusion. Suppurative effusions showed Neutrophil predominance (95.00%) and total leucocyte count in range of 1000 to 10,000/cmm with mean of 2366/cmm. 350 of 370 i.e. 92.59% of cases of tuberculous pleural effusion and 70 of 90 i.e. 77.78% of malignant cases showed lymphocyte predominance (>50%) in pleural fluid cytology. In transudative pleural effusion, the total leucocyte count was low (mean 4 cells/cmm) and all were lymphocyte on differential leucocyte count. (Table1). The ADA levels ranged between was in the range of (13-157 U/L) among the tuberculous cases with a mean value of 76.29±30.91 U/L. The mean value of ADA among non tuberculous (malignant, acute suppurative, taransudative) cases was 26.77±19.09 U/L (Table 11).

Pleural Fluid ADA at 40 U/L Cutoff Values: A specificity of 83.08% was obtained at a cut off value of 40U/L, and the sensitivity of the test was 88.92% (Table 12). A specificity of 100% was obtained at a cut off value of 63U/L, of pleural fluid ADA levels but the sensitivity of the test dropped to a low 68.38% in tubercular pleural effusion. A definitive diagnosis of tuberculous pleural effusion can thus be made at ADA level above 63U/L. (Table 13).

Table 1.Total and differential White Blood Cell Count in Pleural fluid

Mean Values	TB	NTB		
		Malignant	Suppurative	Transudate
Total WBC count (cells/mm)				
	1761.82	995.56	2366.67	2.50
Neutrophils (%)	20.50	24.67	77.00	3.50
Lymphocytes (%)	93.32	80.83	17.00	2.55

(By Student's 't' test 'p' value of lymphocyte% : TB V/s Malig p>0.05; TB V/s SP p<0.001)

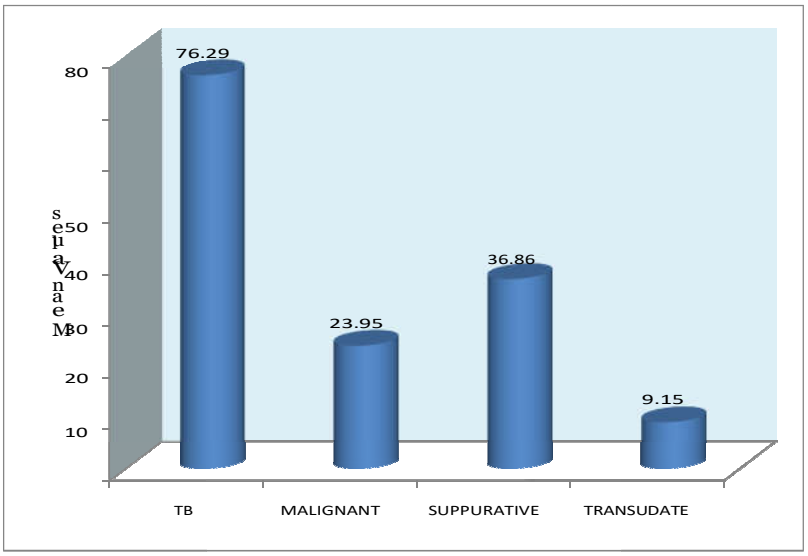
The pleural fluid Biochemistry

2. Protein and glucose in pleural effusion

Table 2. Levels of Protein and Glucose in Pleural Effusion

Mean Values	TB	NTB		
		Malignant	Suppurative	Transudate
Fluid Glucose (mg/dl)	30.16±11.16	25.12±18.49**	15.22±21.34**	74.26±19.37**
Fluid Protein (g/dl)	3.96±1.32	11.49±1.17**	8.94±2.11**	2.8±2.07**

**P<0.001 (HS)



Graph 1. ADA Levels in pleural effusion cases

Table 11. Pleural Fluid ADA Levels in pleural effusion cases

	Mean (U/L)	Minimum (U/L)	Maximum (U/L)
TB	76.29±30.91	13.00	157.00
MALIGNANT	23.95±14.71**	7.20	62.40
SUPPURATIVE	36.86±10.52**	20.00	50.00
TRANSUDATE	9.15±5.30**	5.40	12.90

**p<0.001 (HS); when TB V/s M; TB V/s SN & TB V/s T

Table 12. Significance of Pleural Fluid ADA at 40 U/L Cutoff Value

ADA levels	TB	NTB	Total
Positive (ADA ≥ 40)	330	20	351
Negative (ADA ≤ 40)	40	110	149
Total	370	130	500

Sensitivity- 88.92%
Specificity- 83.08%

Positive Predictive Value- 93.73%
Negative Predictive Value- 72.48%
Percentage of False Negatives- 11.08%
Percentage of False Positives - 16.92%

Table 13. Significance of Pleural Fluid ADA at 63 U/L Cutoff Values

	TB	NTB	Total
Positive (ADA ≥ 63)	253	0	253
Negative (ADA ≤ 63)	117	130	247
Total	370	130	500

Sensitivity- 68.38%
Specificity- 100%
Positive Predictive Value- 100%
Negative Predictive Value- 52.63%
Percentage of False Negatives- 31.62%
Percentage of False Positives - 0%

Table 16. Comparison of Various Tests and Their Combinations in the Diagnosis of Tuberculous Pleural Effusion

Test/Criteria	Sensitivity	Specificity	PPV	NPV
ADA >40 U/L	88.92%	83.08%	93.73%	72.48%
ADA >63 U/L	68.38%	100%	100%	52.63%
ADA >40 U/L & >50% lymphocytes in pleural fluid cytology	84.32%	94.62%	97.81%	67.96%

PPV- positive predictive value; NPV- negative predictive value

S. No.	Reference	No of patients	TB	NTB	Sensitivity	Specificity	Cut off level
1.	Ocana et al (1983)	182	46	136	100%	97%	45 U/L
2.	Martinez et al (1986)	74	38	36	100%	97%	50 U/L
3.	Strankinga et al (1987)	86	10	76	100%	87%	53 U/L
4.	Rajendra Prasad et al (1992)	47	21	26	100%	100%	30 U/L
5.	O.P. Mishra et al (1993)80	55	31	24	81%	75%	38 U/L
6.	Valdes et al (1993)	405	91	314	100%	95%	47 U/L
7.	Burgess L.J. et al (1996)	303	143	160	90%	89%	50U/L
8.	Pedro et al (1997)	254	254	-	100%	93%	47 U/L
9.	S.K. Sharma et al (1997)	75	48	27	83.3%	66.6%	35 U/L
10.	Maria Virginia et al (2000)	140	61	70	88.1%	85.7%	47 U/L
11.	Mo Lung Chen et al (2004)	210	63	147	87.3%	91.8%	55.8 U/L
12.	Present study (2017)	500	370	170	89.18%	84.61%	40 U/L

Pleural fluid ADA with good parameters emerged as a single best diagnostic test for tuberculous pleural effusion. Combined use of pleural fluid ADA and lymphocyte predominance was found to increase the specificity to 94.62% (Table 16). Table16: Comparison of Various Tests and Their Combinations in the Diagnosis of Tuberculous Pleural Effusion. In settings like ours where tuberculosis still remains the commonest cause of pleural effusion, we need a test which is more sensitive rather than specific. As we know untreated TPE, though may resolve spontaneously only to present as active TB elsewhere in future. Hence it's better to fix the cut off value at a lower range like 40U/L. An effort was made to correlate pleural fluid ADA levels reported by other workers with our study 57,78,79 and has been tabulated in table number 17

Summary and Conclusion

The present study was conducted on 500 cases of pleural effusion presenting to the Department of Pathology, GMCH, Udaipur between Jan 2016 to June 2017. On the basis of history and clinical examination, various investigations were done on the aspirated pleural fluid. Pleural fluid Cell count, Glucose, protein, ADA and cytology was carried out in all the cases. Results were observed, analysed and were summarised as follows.

- Tuberculosis was the most common cause (74%) of pleural effusion followed by malignancy (17.40%).
- A definitive tuberculous aetiology was established in 55(14.86%) cases and the rest 315 (85.14%) cases were defined by suggestive criteria for tuberculous origin.
- A specificity of 100% was obtained at a higher cut off value of 63U/L but the sensitivity decreased to 67.57%. With a positive predictive value of 100% at this level, thus pleural fluid ADA serves as a definite cofirmatory test.
- On combining the criteria of ADA > 40 U/L and lymphocyte predominance (>50%) in TPE cases, though the sensitivity decreased to 83.78%, the specificity increased to 96.15%. Positive Predictive Value was a high 98.41% and Negative Predictive Value was 67.57%.

At the end of this study, pleural fluid ADA emerged as a single best sensitive test with fair specificity as well. It being a simple and less time consuming test is an added advantage. Tuberculosis still remains the leading cause of pleural effusion and ADA with good positive predictive value is a valuable time sparing diagnostic tool. But since it can still

give both false positive and false negative results caution is advised. The use of comibation of pleural fluid Glucose, protein, ADA and differential cell counts and cytology will help in further increasing the specificity.

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