Markers for differentiation of tubercular pleural effusion from non-tubercular effusion

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ABSTRACT

BACKGROUND

The limitations of the conventional methods for diagnosing tuberculosis (TB) have spurred multi-faceted research activities throughout the world. This study aims to explore the levels of adenosine deaminase (ADA) and interleukins in pleural effusion of tuberculous, malignant, and miscellaneous origin for differential diagnosis of tubercular and non-tubercular effusion.

METHOD

Adenosine deaminase was estimated by kinetic method employing xanthine oxidase while interleukins were measured using commercially available ELISA kits in pleural fluids of tubercular and non-tubercular origin.

RESULTS

Pleural fluids INF- γ , sIL-2R, TNF- α and ADA were significantly higher in TB group ($n=48$) as compared to the non-TB group ($n=33$) (mean $\pm$ SD: INF- γ ; 1,958.7 $\pm$ 896.5 pg/mL vs 356.9 $\pm$ 733.6 pg/mL, sIL-2R; 6,101 $\pm$ 1,753.8 pg/mL vs 3,166 $\pm$ 2,611.1 pg/mL, TNF- α ; 195.5 $\pm$ 292.1 pg/mL vs 59.7 $\pm$ 128.9 pg/mL, ADA; 123.6 $\pm$ 81.8 IU/L vs 48 $\pm$ 48.5 IU/L, $P < 0.01$).

CONCLUSION

INF- γ is more sensitive and specific than ADA for the diagnosis of TB and should be added to the armamentarium of the diagnostic workup of pleural fluids for timely and accurate diagnosis of TB and differentiation of tubercular pleural effusion from non-tubercular effusion.

MJAFI 2011;67:338–342

Key Words: ADA; interleukins; non-tubercular pleural effusion; tubercular pleural effusion

INTRODUCTION

Tuberculosis (TB), with around 1,800 million people infected worldwide, two million deaths in 2004,¹ one death every 15 minutes,² and nine million new cases, is a leading cause of bacterial morbidity and mortality in world. In India, 33 million people are infected with mycobacteria with three million suffering from pulmonary TB (PTB).³

World Health Organization (WHO) and International Union against Tuberculosis and Lung Disease specify that the essential step in investigation of PTB should be the microscopic examination of at least three serial sputum specimens for acid-fast bacilli (AFB).^{4,5} Direct examination of pleural fluid (PF) and Ziehl-Neelsen staining requires bacillar concentrations of 10,000/mL.⁶ Diagnosis of PTB remains a challenge because of small numbers of bacilli in the PF.^{7,8} Culture is more sensitive,⁹ but requires 2–6 weeks to grow and a minimum of 10–100 viable bacilli. Sensitivity of pleural biopsy specimens is high but requires greater expertise.¹⁰ Failure to diagnose and treat PTB results in progressive disease and treatment based on clinical suspicion results in overtreatment.¹⁰ Limitations of conventional methods have spurred multi-faceted research activities globally.¹¹ Adenosine deaminase (ADA) activity in PF, has been widely used but sensitivity and specificity vary from laboratory to laboratory.¹² Variety of newer markers have been proposed, viz. interferon gamma (INF- γ), interleukin (IL)-12p40, IL-18, tumour necrosis factor alpha (TNF- α), and soluble IL-2 receptors (sIL-2R).¹³

To this end we estimated the levels of sIL-2R, TNF- α , INF- γ , and ADA in pleural effusion (PE) of tuberculous, malignant, and miscellaneous origin to determine the diagnostic utility of this marker to differentiate tubercular pleural effusion (TPE) from non-tubercular effusion (NTE).

MATERIALS AND METHOD

The study subjects (SS) included 95 patients with PE due to any cause admitted to the Cardio-thoracic Centre, Military Hospital (CTC), Pune. The SS were classified into group I or TB group (subjects confirmed of TB) and group II or non-TB (subjects confirmed of disease other than TB). Group II was further subdivided into group IIa which comprised non-TB patients with malignancy and group IIb which comprised non-TB, non-malignancy or miscellaneous group. Diagnosis of tubercular,

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Received: 26.11.2010; Accepted: 16.07.2011

doi: 10.1016/S0377-1237(11)60080-4

malignant, and miscellaneous PE was done on the following basis. Tubercular effusion was diagnosed when any of the following criteria is met:

- Isolation of mycobacterium from PF or tissue, granuloma in tissue showing staining for AFB.
- Granulomas in pleural tissue that did not stain for AFB, but which responded to antituberculous treatment.
- A sputum culture positive for AFB.

Malignant PE was diagnosed in patients with the following:

- (i) Positive PF cytology and/or positive histology of pleural biopsy.
- (ii) A known malignant disease, after the exclusion of alternative causes of PE.

All PEs caused by less common underlying condition (e.g. post-bypass surgery syndrome, trapped lung, and pulmonary embolism) not fitting in either TB or malignant disease were classified as a group of miscellaneous PEs. Out of the 95 SS, 81 were finally included in the study for statistical analysis, while the remaining were excluded due to incomplete data, etc.

Samples of PF were collected in a sterile syringe during thoracentesis from patients of PE. The PE samples were subjected to lactate dehydrogenase (LDH), glucose, albumin, total protein, etc. as a routine procedure at MH CTC and about 3 mL was kept in refrigerator at about 4°C for this study. Samples were immediately brought in cold container to Department of Biochemistry, AFMC, where they were centrifuged at 2,000 rpm for five minutes and supernatant separated into micro-centrifuge vials/Eppendorf tube and frozen at -70°C in deep freezer at the Department of Transfusion Medicine, AFMC until analysed for ADA, INF- γ , sIL-2R, and TNF- α .

Adenosine deaminase was estimated by kinetic method employing xanthine oxidase peroxidase (XOD-POD) employing fully automated clinical chemistry analyser XL 600 from Erba

Manheim, using ADA assay kit from Diazyme Laboratories, USA. TNF- α , INF- γ and sIL-2R was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits from Diaclone, France as per the instructions given with the kits.

Calculation of Results

Results were calculated by generating a linear standard curve by plotting the average absorbance on the vertical axis vs the corresponding TNF- α , INF- γ , and sIL-2R standard concentration on the horizontal axis. The amount of TNF- α , INF- γ , and sIL-2R in each sample is determined by extrapolating the optical density (OD) values to TNF- α , INF- γ , and sIL-2R concentrations using the standard curve. Controls for TNF- α , INF- γ , sIL-2R, and ADA were used, respectively, during each batch of estimation to ensure the accuracy of results.

RESULTS

Levels of INF- γ , sIL-2R, TNF- α , and ADA were compared between the study groups using unpaired *t*-test and results are presented in Table 1.

Levels of INF- γ , sIL-2R, TNF- α , and ADA were also compared between the sub-groups of group II and results are presented in Table 2. Using the receiver operating characteristics (ROC) curve optimal cut-off points of INF- γ , sIL-2R, TNF- α , and ADA for diagnosing TB were determined and are presented in Table 3.

DISCUSSION

Tuberculosis a major killer worldwide, is recognised as the world's leading bacterial cause of death, yet 95% of infection is believed to exist in an asymptomatic form defined not by the

Table 1 Comparison of levels of biomarkers between study groups.

Biomarker	Group I (n=48)			Group II (n=33)			<i>t</i>	<i>P</i>
	Mean	SD	Range	Mean	SD	Range		
INF- γ (pg/mL)	1,958.6	896.5	33–3,516	356.9	733.6	0–2,229	8.490	<0.01
sIL-2R (pg/mL)	6,101	1,753.8	431–8,495	3,166.1	2,611.1	127–8,741	5.641	<0.01
TNF- α (pg/mL)	195.5	292.1	0–1,344	59.7	128.9	0–578	2.843	<0.01
ADA (IU/L)	123.6	81.8	33.4–395.5	48.0	48.5	1–187	5.209	<0.01

INF- γ : interferon gamma, sIL-2R: soluble IL-2 receptors, TNF- α : tumour necrosis factor alpha, ADA: adenosine deaminase, SD: standard deviation.

Table 2 Comparison of levels of biomarkers between groups I and IIa and groups I and IIb.

Biomarker	Group I (n=48)			Group IIa (n=6)			Group IIb (n=27)		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
INF- γ (pg/mL)	1,958.6	896.5	33–3,516	191	440	0–1,089	394	786	0–2,229
sIL-2R (pg/mL)	6,101	1,753.8	431–8,495	3,557	2,479	1,057–7,200	3,079	2,677	127–8,741
TNF- α (pg/mL)	195.5	292.1	0–1,344	55	113	0–281	61	134	0–578
ADA (IU/L)	123.6	81.8	33.4–395.5	34	21	8–61	51	52	1–187

INF- γ : interferon gamma, sIL-2R: soluble IL-2 receptors, TNF- α : tumour necrosis factor alpha, ADA: adenosine deaminase, SD: standard deviation.

Table 3 Sensitivity and specificity of biomarkers for optimal cut-off points and predictive values.

Statistical parameter	Biomarkers			
	INF- γ	sIL-2R	TNF- α	ADA
Optimal cut-off	1,090 pg/mL	4,257.4 pg/mL	10 pg/mL	71 IU/L
Sensitivity (%) (95% CI)	88 (80–92)	88 (79–93)	65 (56–72)	80 (71–86)
Specificity (%) (95% CI)	85 (74–92)	70 (58–78)	67 (54–78)	76 (63–85)
Area under curve (95% CI)	0.90 (0.82–0.98)	0.80 (0.70–0.91)	0.71 (0.59–0.82)	0.83 (0.73–0.93)
Significance for area	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
+ve Predictive value (95% CI)	89 (82–94)	81 (67–90)	74 (63–83)	83 (74–89)
–ve Predictive value (95% CI)	82 (72–89)	79 (60–91)	56 (45–66)	71 (60–80)

Note: Biomarkers levels greater than or equal to optimal cut-off point is labelled as TB.

INF- γ : interferon gamma, sIL-2R: soluble IL-2 receptors, TNF- α : tumour necrosis factor alpha, ADA: adenosine deaminase.

identification of bacteria, but by the host immune response in the form of reactivity to tuberculosis proteins.

Detection of TB and differentiation of PE from non-tubercular effusion still poses diagnostic challenges. Instead of traditional and conventional methods for diagnosis of TB, research is now being focused on alternative methods and biomarkers for diagnosis and differentiation of TPE from NTE.

In this study, we have attempted to evaluate the significance of PF levels of INF- γ , sIL-2R, TNF- α , and ADA in the diagnosis and differentiation of TPE from NTE.

The PF levels of INF- γ , sIL-2R, TNF- α , and ADA were significantly higher in TB group ($n=48$) as compared to non-TB group ($n=33$) (INF- γ ; $1,958.7 \pm 896.5$ pg/mL vs 356.9 ± 733.6 pg/mL, sIL-2R; $6,101 \pm 1,753.8$ pg/mL vs $3,166 \pm 2,611.1$ pg/mL, TNF- α ; 195.5 ± 292.1 pg/mL vs 59.7 ± 128.9 pg/mL, ADA; 123.6 ± 81.8 IU/L vs 48 ± 48.5 IU/L, $P < 0.01$; Table 1). This result is in agreement with studies of Kim YK et al¹⁴ who reported INF- γ and sIL-2R concentrations in TPE higher than those from NTE (INF- γ ; $1,154.4 \pm 252.4$ pg/mL vs 292.0 ± 68.9 pg/mL, sIL-2R; $9,805.2 \pm 978.9$ pg/mL vs $3,426.7 \pm 695.6$ pg/mL, $P < 0.05$) and the studies of Sharma SK et al¹⁵ who reported PF ADA levels significantly higher in TB ($n=48$; mean 95.8 ± 57.5 IU/L) compared with non-TB group ($n=27$; mean 30.7 ± 27.2 IU/L, $P < 0.001$).

There was significant difference between TB group and malignant group as well as between TB group and miscellaneous group in the PF levels of INF- γ , sIL-2R, TNF- α , and ADA (Table 2).

For each biomarker a ROC curve was generated. Area under curve (AUC) for INF- γ , sIL-2R, TNF- α , and ADA was found to be 0.898, 0.804, 0.709, 0.827, respectively. From the AUC optimal cut-off point determined for each biomarker INF- γ , sIL-2R, TNF- α , and ADA was found to be 1,090, 4,257.4, 10 pg/mL, and 71 IU/L, respectively. At this cut-off level for the respective biomarker PF INF- γ offers the highest sensitivity and specificity of 88% and 85% which is followed by sIL-2R with sensitivity and specificity of 88% and 70%, respectively, while sensitivity and specificity of TNF- α was lowest of all four biomarkers investigated in this study and was found to be only 65% and 67%, respectively. Also the PF INF- γ offers the highest positive and negative predictive value (89% and 82%, respectively) while that of TNF- α was lowest (74% and 56%, respectively) of all four biomarkers (Table 3). The sensitivity and specificity

of ADA which in this study was found to be 80% and 76%, respectively, at a cut-off of 71 IU/L was comparatively lower than that reported in the other studies. The sensitivity and specificity of tuberculous PF ADA in the studies¹⁶ has been reported in the range of 91–100% and 81–94%, respectively, while Goto et al¹⁷ in his meta-analysis of 40 studies reported sensitivity range of 47.1–100%, and specificity range of 0–100%, and also reported the difference due to different methods of estimation. Most frequent method for the measurement of total ADA has been the colorimetric method¹⁷ described by Guisti et al¹⁸ because of its low cost and being readily availability. The diagnostic cut-off value for PF ADA in various studies has been reported in the range of 40–60 U/L.^{16,18,19} Choosing a lower value increases sensitivity at the expense of specificity. From the ROC curve which indicates sensitivity and specificity at different cut-off levels, our study showed that at a cut-off value of 41 IU/L PF ADA the most widely used biomarker was found to exhibit sensitivity of 96% but at reduced specificity of 66%. This is in total agreement with the study of Sharma et al¹⁵ who reported sensitivity and specificity of PF ADA in the diagnosis of TB to be 83.3% and 66.6%, respectively, at a cut-off of 35 IU/L. The optimal cut-off point of 71 IU/L determined for ADA in our study was found to be in total agreement with study of Aggarwal et al.²⁰

The sensitivity and specificity of 88% and 85% for INF- γ in this study is slightly lower than those reported in the various published studies. Various existing studies reported the sensitivity of an elevated level of INF- γ in the range of 78–100% and specificity from 95% to 100%.^{10,21–27} Ribera et al and Gao and Tian in a study conducted on 80 and 66 patients, respectively, reported both sensitivity and specificity of 100% at a cut-off level of 2 IU/L by RIA and 60 pg/mL by ELISA, respectively. Similarly Aoki et al²² in a study conducted on 39 patients also reported both sensitivity and specificity of 100%. In this study detectable or not detectable by ELISA was considered positive or negative, respectively (thus, almost zero level was the cut-off). Thus, comparison between different studies is not possible, as the method of estimations, units and cut-off used were different. Moreover, it is well mentioned in few studies that other diseases can also cause elevated INF- γ levels in PF such as haematologic malignancies and empyema.^{24,25} The higher

sensitivity and specificity of INF- γ as compared to ADA in this study was found to be in total agreement with the existing studies which reported PF INF- γ levels to be more sensitive and specific than ADA²⁸ and suggested INF- γ as a better marker than ADA for the diagnosis of tuberculous pleuritis.

Considering the ROC, sensitivity, and specificity INF- γ and sIL-2R definitely adds to the diagnosis. This study also, in agreement with Sharma and Banga²⁹ opined that IFN- γ levels are more sensitive and specific than ADA.

This study in total agreement with the meta-analysis by Qiu-Liang et al³⁰ who finally concludes that the results of ADA assays should be interpreted in parallel with the clinical findings, and INF- γ being more sensitive and specific than ADA for the diagnosis of TB should be added to the armamentarium of the diagnostic workup of PFs for timely and accurate diagnosis of TB, and differentiation of TPE from NTE.

Intellectual Contributions of Authors

Study concept: Vivek Ambade

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ACKNOWLEDGEMENTS

I am extremely grateful to the office of the DGAFMS for sanctioning the project. I am grateful to Col RS Sarkar, Professor and Head, Department of Transfusion Medicine, AFMC, for permitting me to store sample at -70°C and Dr S Naveen and Lt Col AK Harith who helped me in setting the ELISA reader. I thank Hav Mukesh, Lab Attendant, Department of Biochemistry for carrying out the analysis of ADA by fully automated clinical chemistry analyser.

I appreciate the efforts taken by PG trainees of Respiratory ward for providing the required data of the patients in the proper format and laboratory staff specially Hav Rajesh of Military Hospital (CTC), Pune who monitored the collection and preservation of PFs.

CONFLICTS OF INTEREST

This study has been financed by the research grants from the office of the DGAFMS.

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Journal scan

Balu V, Gayathri S, Sinimol TM, Karimassery RS, Karukappilly KRW, Raman KK. Clinical screening for congenital heart disease at birth: a prospective study in a community hospital in Kerala. *Indian Pediatr* 2011; 48:25–30.

Congenital heart diseases (CHD) account for about 10% of infant deaths and about half of deaths due to congenital malformations in developed countries. The prevalence of CHD diagnosed in the first 12 months is estimated to be 6–8 per 1,000 live births. About 25% of CHDs are life-threatening and may manifest before the first routine clinical examination. Failure to identify these critical lesions after birth leads to delay in referral and increased risk of mortality and morbidity. Challenges for CHD management in developing countries include early diagnosis, transport of a sick neonate with CHD to a tertiary cardiac centre, and limited availability of state-of-the-art paediatric cardiac centres. With this background, this study was conducted to develop a clinical strategy for the detection of CHD in newborns through a combination of clinical signs and pulse oximetry. This study was a prospective longitudinal study conducted in a community hospital in Kochi (Kerala). All consecutive newborns born between June 2006 and February 2009 were prospectively screened for CHD, 48 hours after birth. The on-site paediatrician performed the clinical screening. A study nurse recorded pulse oximetry in a lower extremity (a value of <94% was defined as abnormal). Echocardiography was performed on-site by a trained research officer. A six-week clinical follow-up evaluation was done for all. Of 5,487 babies screened, 425 (7.75%) had CHD. In depth, 17 babies (0.31%) had major CHD, two of them (one, anomalous left coronary from the

pulmonary artery and the other, large ventricular septal defect) were missed during the initial evaluation. Rest of the babies had minor CHD (408 infants, 7.44%), most of which normalised by six weeks. First univariate analysis was done using χ^2 method and variables with $P < 0.2$ were analysed by using multiple logistic regression model in which murmur, central cyanosis, abnormal precordial pulsations, and abnormal pulse oximetry emerged as significant predictors of CHD. The sensitivity of the clinical evaluation and pulse oximetry combined was 19% for all CHDs and 20% for major CHD; the specificity was 88%. The authors concluded that clinical evaluation and pulse oximetry after birth had a very low sensitivity for the detection of CHD. Though an abnormal screening warrants prompt echocardiography, a six-week clinical evaluation is recommended to ensure that major CHD is not missed.

While it is a noble type of study done in an Indian setting, the authors concluded that more training (how much) is required to get a better result which raises the question of the internal validity. This study also suffers from certain typographical errors such as major is being used instead of minor and multivariate analysis is being used instead of multiple logistic regression analysis. Moreover, 95% OR for murmur and abnormal precordial pulsations seems to be labelled wrong. Lastly, the authors conclude that pulse oximetry may emerge as a useful screening tool in future.

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