Original article

Evaluation of molecular and immunological methods for diagnosis of tuberculosis pleurisy

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Abstract

Objective: The inefficiency of conventional laboratory methods for diagnosis of Pleural tuberculosis (TBP) and the reliance on pleural biopsy have motivated the evaluation of alternative diagnostic strategies. Our goal was to evaluate different laboratory techniques Ziehl Neelsen, Mantoux skin test, determination of interferon gamma in serum and pleural fluid, polymerase chain reaction and serological study of specific IgG, IgM and IgA beside bacteriological culture by BACTEC 460 TB for rapid and accurate diagnosis of tuberculosis pleurisy. **Methods**: Patients presented with pleural effusions were subjected to study by ZN, PCR, serological study By specific IgG, IgM and IgA for A60 of tuberculosis compared to culture by BACTEC460 TB. Interferon gamma was determined both in serum and pleural fluid. **Results**: Mantoux skin test was positive in 19 TBP patients and four patients with exudative pleural effusion. Z. N staining results of pleural biopsy specimens were positive in only 1 of 23 patients (4.3%) in the tuberculous pleural effusion group. PCR was positive in 20 cases of group 1 (87%). Serum and pleural fluid interferon had significantly elevated levels ($P<0.000\ 1$) in TBP and both measurements had significant correlation in TBP ($P<0.000\ 1$). The serum IgA ELISA test was positive in 7/23 (30.4%), IgM was positive in 17/23 (73.9%) patients and IgG was positive in 16/23 (69%) patients. Non of the non TBP had either ZN, PCR or positive serum IgA, IgG, or IgM. When the positive results for IgG and IgM were combined together the serological tests correctly identified 20/23 (87%) of patients. **Conclusion**: We suggest that in TBP serological diagnosis by combined use of IgG and IgM for A60 antigen with serum determination of interferon gamma can provide rapid and non invasive diagnostic tool that can justify the starting of chemotherapy while awaiting the results of culture.

Keywords: Tuberculous pleurisy; Molecular diagnosis; Tuberculin; Serology; Interferon gamma

INTRODUCTION

Tuberculous pleuritis (TBP) is one of the most common forms of extra-pulmonary tuberculosis. The diagnosis of TBP can be established directly by: 1) demonstrating tubercle bacilli in the sputum, pleural fluid, or pleural biopsy specimen; 2) antibody or antigen detection in either serum or pleural fluid[1-5]; or 3) pleural fluid polymerase chain reaction (PCR) for *Mycobacterium tuberculosis* DNA [6]. Indirect evidence with reasonable certainty includes granuloma in the pleura without any clinical evidence of other granulomatous disease or increased levels of pleural fluid adenosine deaminase or interferon gamma [7,8].

Serodiagnosis, which has been demonstrated to be clinically useful [9], was attempted using crude and semipurified antigens, and was found to give different ranges of sensitivity and specificity [10]. Searching for IgG [11,12] and IgA [13] against the antigen 60 (A60) provided satisfactory results in terms of sensitivity and specificity in the diagnosis of pulmonary tuberculosis. However, the usefulness of A60-based serological tests in the diagnosis of extrapulmonary tuberculosis has been less extensively

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evaluated[14].

Many studies have investigated the usefulness of interferon (IFN)-gamma measurements in pleural fluid for the early diagnosis of tuberculous pleurisy[15]. Recently Quantiferon test was applied to diagnose active tuberculosis of pleura, however, this assay performed with peripheral blood or adapted for pleural fluid cells does not add diagnostic value [16].

The purpose of this study is to evaluate the diagnostic value of laboratory methods Zhiel Neelsen, PCR, Mantoux skin test, Interferon gamma assay both in pleural fluid and in serum and serological assay for IgA, IgG and IgM for antigen 60 compared to BACTEC 460 TB culture.

MATERIALS AND METHODS

Study population

Forty six patients who underwent diagnostic evaluation for pleural effusion consecutively at the diagnostic laboratory, Mansoura university Egypt were enrolled to this study. All patients gave written informed consent, as approved by Mansoura faculty of Medicine ethical committee. Mantoux skin test was performed for all patients and considered positive with diameter >10 mm.

Patients were classified into three groups:

Group 1: Tuberculous Pleural Effusion: This group included 23 patients (14 men and 9 women) with a mean age of 43.15 ± 2.8 years. Diagnosis of pleural TB was confirmed by culture and/or histopathology of pleural biopsy specimens, supported by laboratory, radiographic, and clinical data[17]. There was no underlying parenchymal infiltration, cavitation, or lymphadenopathy.

Group 2: Exudative Pleural Effusion: This group included 13 patients (six men and four women) with a mean age of 47.3 ± 5.96 years. Within this group, malignant pleural effusion was diagnosed in seven patients (small cell carcinoma, two patients; lymphoma, one patient; mucoepidermoid, one patient; adenocarcinoma, two patients; and undifferentiated carcinoma, one patient), systemic lupus erythematous (SLE) was diagnosed in two patients, and parapneumonic effusion was diagnosed in one patient.

Group 3: Transudative Pleural Effusion: This group included ten patients (five men and five women) with a mean age of 52.78 ± 5.37 years. Bilharzial liver cirrhosis was diagnosed in five patients, and congestive heart failure was diagnosed in five patients.

Procedure

Thoracocentesis was performed under local anesthesia using sterile techniques. About 200 mL of pleural fluid was collected, 20 mL for routine cell and differential counts and chemistry tests, 50 mL for microbiological procedures, and 130 mL for cytological preparations. At least three pieces of pleural tissue were harvested by percutaneous pleural biopsy using an Abrams´needle; these were sent for histological studies. Serum and effusion samples used for serology were collected and stored at −70°C for further assay.

Processing of biopsy samples

The pleural biopsy was performed using Abram needles, and 4 to 5 punches of parietal pleura were divided equally into two parts. One part of the specimen was fixed in formalin for histopathologic examination, while the other part was put into sterile normal saline solution, then homogenized and used for ZN smear and culture.

ZN smear of biopsy

Smears of pleural fluid sediments or pleural biopsy specimens were stained by ZN stain and examined according to the method of Jenkins[18].

Loewenstein-Jensen culture of biopsy specimen

Ready to use bottles of Loewenstein-Jensen (LJ) medium (Hispan Lab; Medicopharmatrade Co; Madrid, Spain) were inoculated with 0.1 mL concentrated pleural fluid or biopsy specimen and were kept in a CO_2 incubator for 8 weeks. Resulting growth was left in light for 2 h and was examined for yellow pigments to identify photochromogen species.

Pleural Biopsy Culture Using Radiometric BACTEC 460 System

The BACTEC 460 system and BACTEC 12B vials (Becton Dickinson Microbiology Systems; Cockeysville, MD) were used for radiometric culturing and identification of *M tuberculosis* according to manufacturers directions.

Serology for T. B (1gG, 1gA, 1gM) was detected by ELISA Technique by Anda. TB (France)

The antigen used was A60 antigen complex which an interspecific antigen found in the cytosol of typical and atypical mycobateria. It reacts with antibodies

created during mycobactrial infection (tuberculosis, leprae)

PCR of pleural biopsy specimen

PCR was used for the detection of DNA (*IS*986) specific for the mycobacterium complex on 33 of the studied pleural biopsy samples. The size of the amplification product was 123 base pairs (bp). A PCR reaction was performed using an *M. tuberculosis* kit (code H.02; Experteam; Venice, Italy) according to the manufacturer's instructions.

Interferon gamma assay

It was measured in serum and supernatant fluids for patients in group 1 and 3 (patients in group 2 were excluded due to their medical conditions) by sandwich enzyme-linked immunosorbant assay (ELISA) according to instructions of manufacturers (Immunotech).

Statistical analysis

Sensitivity, specificity, accuracy and receiver operative curves were calculated by standard methods [19].

RESULTS

The diagnosis of tuberculosis was confirmed by bacteriological culture and/or histopathological examination of pleural biopsies in 23 patients. One sample (3.8%) of pleural fluid was positive with culture and ZN staining in the tuberculous pleural effusion group, and was negative in both the exudative and transudative pleural effusion groups. Tuberculin skin test was positive in 19 cases, and was positive in 4 cases of the control group 2.

ZN staining results of pleural biopsy specimens were positive in 1 of 23 patients (4.3%) in the tuberculous pleural effusion group, and were negative in other groups.

PCR was positive in 20 cases of group 1 (87%) and in none of other patients. The serum IgA, IgM and IgG was positive in 30.4%, 73.9%, and 69.0% respectively. When the positive results for IgG and IgM were collected together the serological tests correctly identified 20/23 (87.0%) of TBP patients, table 1.

The mean ± SD of serum IFNγ of positive cases was 11.36 ± 5.86 and for negative serum IFNγ was 0.36 ± 0.24 with statistically significant difference $P < 0.000\ 1$. The mean ± SD of positive pleural fluid IFNγ was 71.11 ± 18.30 and the negative pleural fluid was 2.80 ± 0.65 with $P < 0.000\ 1$, table 2.

There was positive correlation between serum and pleural fluid IFNγ $P < 0.000\ 1$, Figure 1.

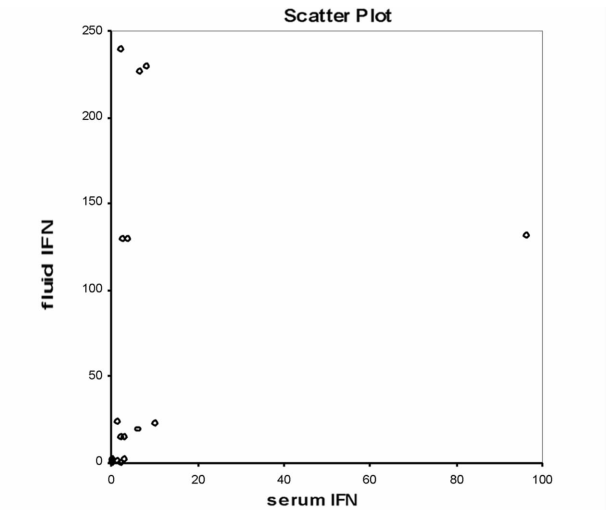

Figure 1 Correlation between serum and pleural fluid in diagnosis of positive tuberculosis cases

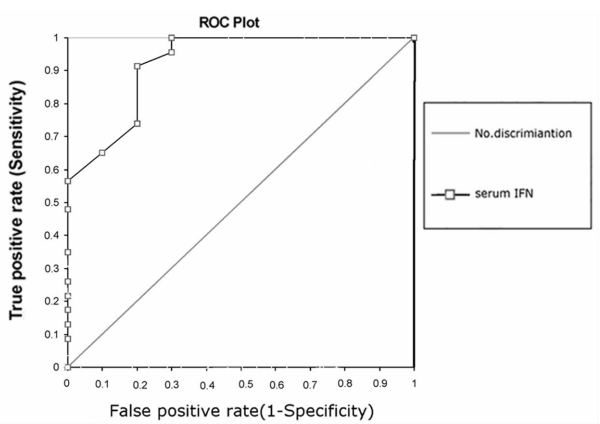

Figure 2 Serum interferon gamma assay receiver operative curve

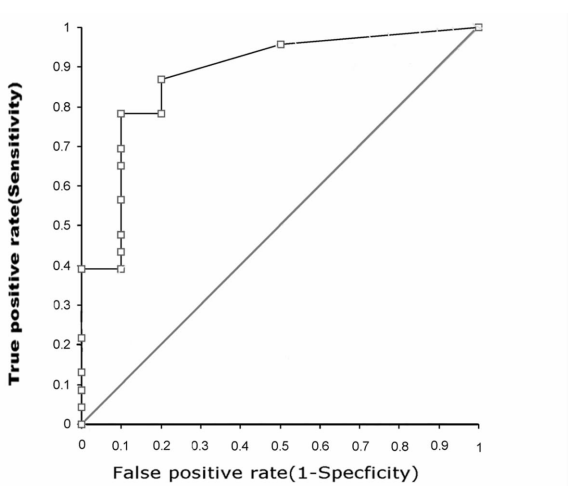

Figure 3 Receiver operative curve for fluid IFN gamma

Table 1 Positive results for ZN, serology, Mantoux skin test and PCR compared to culture in tuberculosis pleurisy ($n=23$)

	Positive	
	No.	%
ZN	1	4.3
IgM serum	17	73.9
IgG serum	16	69.0
IgA serum	7	30.4
Combined IgM &IgG	20	87.0
Tuberculin skin test	19	86.0
PCR	20	87.0

Table 2 Interferon gamma assay in serum and pleural fluid(Mean ± SD)

Test of significance	IFN gamma	Sample
Positive serum Negative serum	11.36 ± 5.86 0.36 ± 0.24	$P<0.000\ 1$
Positive Fluid Negative Fluid	71.11 ± 18.30 2.80 ± 0.65	$P<0.000\ 1$

Table 3 Sensitivity, specificity and accuracy of Tuberculin, serology, ZN and IFN gamma assay(%)

	Sensitivity	Specificity	Accuracy
Tuberculin skin test	86.0	86.0	82.6
IgA	30.4	91.3	69.6
IgG	69.0	74.0	73.0
IgM	74.0	91.3	82.6
Combined IgM&IgG	87.0	100.0	93.5
ZN	4.3	100.0	50.0
Cut off serum IFN 0.10	91.0	80.0	75.7
Cut of fluid IFN 0.1	87.0	80.0	84.4

The sensitivity, specificity and accuracy ELISA tests in serum are shown in Table 3. Interesting finding that the use of combined serological tests IgG and IgM correctly identified 20/24 of cases with sensitivity 87.0%, specificity 99.0% and overall accuracy 93.5%. The best Cut off value for IFN gamma was 0.1pg/mL in serum and pleural fluid. The sensitivity for serum and pleural IFN was 91% and 87% respectively and specificity for serum and IFN was 86.0% and 80.0% respectively, Figures 2, 3.

DISCUSSION

The pathogenesis of TBP involves a limited number of bacilli, with the main mechanism being delayed hypersensitivity[20-22]. In TBP patients there was only one case diagnosed by ZN of pleural biopsy which reflects a small load of tubercle bacilli this could influence the stimulation power of humoral system to produce local antibodies.

PCR was positive in 20 cases of group 1 (87.0%). The sensitivity of nucleic amplification techniques depends on the number of mycobacteria, their homogenous distribution in the sample, the presence of the amplification inhibitor in the sample[23] and type of the primer[24-26]. In our study, we select the primer pair amplifying 123 bp, which is specific for members of the *M tuberculosis* complex[25].

Interferone gamma level was found to be sensitive marker for diagnosis of TBP Similar finding was reported in metanalysis study for value of interferon gamma in diagnosis of TBP[15]. The measurement of IFN-gamma levels in pleural effusions is thus likely to be a useful tool for diagnosing tuberculous pleurisy.

Measurement of IgG and IgM provided good characteristics of sensitivity and specificity with lower diagnostic value for IgA. The combined use of the two tests allowed an increase in sensitivity with specificity 100.0%.

Gupta *et al*[14] have reported a sensitivity of 73.8%, for the anti-A60 IgG tests in Indian population with extrapulmonary tuberculosis; the corresponding values of specificity were 92.4%, while the study had reported higher diagnostic value for IgA. The difference of results suggests that some factors may influence the diagnostic value of A60 based serological tests, such as different prevalence of tuberculosis, immunological status of the host, and phase and duration of disease before admission to hospital.

Detection of IgG and IgM against A60 in patients with TBP was negative in a small percentage of cases. As already suggested[13, 27] this may be a result of immunosuppression due to the disease as well as to the presence of immune complexes that are quickly removed from the bloodstream.

Results of sensitivity and specificity were determined by using the of Mycobacterium tuberculosis culture. Thus, our values of specificity detect already infected patients with active tuberculosis. As suggested for the serological test based on P90 antigen [28].

Tuberculin skin test had valuable results in the present study. Sensitivity and specificity were (86%). Lower sensitivity and specificity were reported in other study[29] with sensitivity and specificity of TST was 65% and 68%, respectively. This can be attributed to the role of previous vaccination with BCG vaccine.

Cut of value was determined for pleural and serum interferon gamma with good sensitivity, specificity and accuracy. Similar findings were reported by Jiang *et al*[15], 2007.

Thus for rapid diagnosis of TBP can depend on combined use of serum IgG and IgG combined with serum determination of interferon gamma with accurate results and low cost compared to high cost PCR especially in less developed countries.

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