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DIAGNOSTIC ROLE OF THE ANTIBODY RESPONSE TO THE 38kDa, 16kDa PROTEINS AND LIPOARABINOMANNAN OF MYCOBACTERIUM TUBERCULOSIS

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ABSTRACT

The antibody response to the 38kDa, 16kDa and Lipoarabinomannnan (LAM) antigens of *Mycobacterium tuberculosis* was evaluated using three different ELISAs based on these antigens. The study group included tuberculosis patients (n=52), patients with HIV and TB co-infection (n=10), other chest symptomatics (n=5), HIV infected individuals (n=10), leprosy cases (n=7) and healthy controls (n=75). The results indicate that the 38kDa and LAM based ELISA for IgM/IgG has a low specificity (ranging from 69-85%) and sensitivity (ranging from 55-78%). When three ELISAs are carried out on a single patient the probability of detection of tuberculosis was significantly increased to 95.2% indicating that a single ELISA test is of low sensitivity and that a combination of ELISA's may be needed to be of any value as a diagnostic test for tuberculosis. Additionally, a western blot assay of the serum antibody response to protein fraction of *M.tuberculosis* was analysed in 15 tuberculosis patients and five healthy controls. A multiple antibody response to various M. *tuberculosis* proteins was observed which varied from patient to patient as compared to controls who showed a single 38-39 kDa protein band positivity. These findings suggest that a western blot assay which determines the antibody response to a set of antigenic components of *M.tuberculosis* could be a better serological test for the diagnosis of tuberculosis in our population.

KEY WORDS

Tuberculosis, Serodiagnosis, ELISA, Western blot, 38kDa and 16kDa M.tb protein, Lipoarabinomannan.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (M.tb)* and has been a chronic problem in the developing countries. Its unexpected re-emergence in developed countries, recent out break of multidrug- resistant tuberculosis and the susceptibility of human immunodeficiency virus (HIV) infected individuals to the tubercle bacilli (1), enhances the need for a rapid and accurate detection of *M.tb* infection.

Presently the diagnosis of tuberculosis largely depends upon clinical, bacteriological, radiological and cytological examinations. Though the direct microscopy of sputum for acid-fast bacilli is reliable for pulmonary tuberculosis, it is not helpful in extra pulmonary TB and childhood tuberculosis where the cough may not be productive and the bacillary load less than 10^4. Culture has its importance, but it is cumbersome and requires 6-8 weeks for results. Other methods of diagnosis such as radiological examination and tuberculin skin test are non-specific. Newer techniques such as polymerase chain reaction, DNA Probes, restriction fragment length polymorphism (RFLP) and BACTEC culture system for diagnosis of tuberculosis are sensitive and help in rapid diagnosis, but do not find their way into routine diagnosis as they are not cost effective and practical in developing countries (2). Various serodiagnostic tests based on different M.tb antigens have been developed for the diagnosis of TB and a few have been evaluated in our population (3,4,5,6,7,8). The present study aims to

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investigate the diagnostic utility of the antibody responses to the 38kDa, 16kDa proteins and Lipoarabinomannan (LAM) of *M.tb* using ELISA and western blot.

MATERIALS AND METHODS

Patients: Informed consent was obtained from the patients included in the study. Blood samples were collected from TB patients attending three centers in Hyderabad, District TB hospital at Dabeerpura, Mahavir Hospital & Research Centre and from Blue Peter Research Centre. Blood was also collected from leprosy patients and individuals with chest symptoms other than tuberculosis. The basis for diagnosis of TB was mainly on the presence of acid-fast bacilli (by sputum microscopy) and patients diagnosed as TB on clinical or X-ray and who responded to anti-TB treatment. The clinical forms and stages of tuberculosis in the patients were as follows; pretreatment (n=13), under going treatment (n=19), post treatment (immediately after completion of treatment) (n=12), and confirmed extra pulmonary TB (n=8). The subjects in the study were of different age groups. The female age group was 16-55 years (n=22) and male age group was 15-70 years (n=40). Healthy blood donors (n=75) who were screened at the Blood Bank of Nizam's Institute of Medical Sciences were used as controls. Blood from 20 HIV positive individuals registered at our centre for outpatient based clinical management were also collected for the study, among whom 10 were of HIV/TB co-infection cases. Other chest symptomatics (n=5) were also screened. The serum was separated and stored at -20^{0}C until the tests were carried out.

ELISA: ELISA's were performed using commercially available kits and the procedures were as per the manufacturers guidelines. The kits used were;

1. 'Pathozyme Myco' kit for detection of IgG using recombinant 38kDa and LAM derived from *M.tb* (ELISA I) (9).
2. 'Pathozyme Myco' kit for detection of IgM, using recombinant 38kDa and LAM from *M.tb* (ELISA II) (9).
3. 'Pathozyme TB complex plus' for the detection of IgG using recombinant 38kDa and 16kDa proteins (ELISA III) (10).

The ELISA results were interpreted according to the manufacturer's guidelines and the sensitivity and specificity were calculated:

Sensitivity: The capacity to correctly identify in a population, those individuals who have the disease. 100% sensitivity is that everyone who is infected tests positive. In the present study confirmed TB patients and HIV/TB co-infected patients were included in order to evaluate the sensitivity of the ELISA.

Specificity: The capacity to correctly identify in a population, those individuals who are free from the disease. 100% specificity is that everyone who is not infected tests negative. Healthy blood donors with no history of tuberculosis were included in order to evaluate the specificity of the ELISA.

Western Blot: The following procedure was followed for the western blot analysis. H37Rv was obtained from National Tuberculosis Institute (Bangalore) and was cultured on LJ medium (11) at 37^{0}C for 6-8 weeks. Further processing of the bacteria, from collection to protein extraction was carried out in the TB laboratory at our centre. Briefly, H37Rv was scraped and collected from the LJ slope and suspended in 20mmol/l Trishydroxymethylaminomethane (Tris) (Sigma Chemical Company, St. Louis, U.S.A) (pH 7.4) containing 145mmol/l sodium chloride (buffer A). The bacteria was pelleted by centrifugation and washed thrice with buffer A. The protein was precipitated with 10% Trichloroacetic acid (TCA) (Sigma) (12). The precipitated protein was dissolved in a buffer of 20mmol/l Tris-HCl (pH 7.4), containing 1mg/l benzamidine-HCl (Sigma), 0.2mmol/l phenyl methyl sulphonyl fluoride and 1gm/l Triton X –100 (Sigma). Protein concentration was determined by the method of Lowry et al (13) and a constant amount of protein (approx 75 µg) was electrophoresed. Electrophoresis was carried out on 12% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (14). The protein was blotted from the gel on to nitrocellulose membrane according to Towbin et al (15) in a BIO-RAD blotting apparatus. The nitrocellulose membrane was blocked in 2% BSA for 2 hours, air dried under the laminar air flow, cut into strips which were interacted with the sera of tuberculosis patients (1:100) for one hour. The strips were then washed and interacted with goat anti-human IgG biotin (GENEI, Bangalore, India (GBI)) (1:500) for 30 minutes, washed and interacted with Streptavidin Alkaline phosphatases conjugate (GBI) (1:500) for 15 minutes and washed. These strips were developed with the BCIP/NBT i.e., bromochloroindolylphosphate/ nitro blue tetrazolium (GBI) till the bands appeared. The molecular weights of the unknown proteins were estimated by comparing them to the relative mobilities of known molecular weight proteins in SDS-PAGE, that were phosphorylase b (97.4kDa), bovine serum albumin (66kDa), ovalbumin (43kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1kDa) and lysozyme (14.3kDa). [Genie, Bangalore, India]

HIV Test: Commercially available HIV kits Tridot (16) (BIO-RAD, USA) and ELISA (Lab Systems, Finland) were used to carry out HIV screening. The sera of the TB patients was coded and screened in an anonymous

unlinked method for HIV I and II antibodies using one rapid and two ELISA tests.

RESULTS

Table 1 gives the results of three ELISA tests, which were carried out for the IgG and IgM responses to the 16kDa, 38kDa proteins and LAM of *M.tb.* The first ELISA (ELISA I) measured the IgG response to the 38kDa protein and LAM and the specificity of the test was 80%. The overall sensitivity of this test in tuberculosis is 56.4%. The patients under going treatment showed highest sensitivity (78.95%) followed by post treatment group (66%), pretreatment (46%), extra pulmonary (37%) and the lowest sensitivity was observed in HIV-TB co-infection (30%). ELISA positivity was also observed in 10% of the HIV patients, 28% of the leprosy patients and 40% of the chest symptomatic (Table1, row1).

The second ELISA (ELISA II) was carried out to detect IgM response to the 38kDa protein and LAM of M.tb. The specificity of the test was 64% and the overall sensitivity in tuberculosis was 69.3%. When sensitivities in the various clinical forms of tuberculosis were compared the highest sensitivity was in the extra pulmonary group (87%) followed by pretreatment (84%), post treatment group (75%), group under going treatment (73.68%) and the lowest sensitivity was in HIV-TB co infection (20%). The ELISA positivity was also observed in all the leprosy patients and chest symptomatic and in 60% of HIV cases. (Table1, row 2).

Table1. Sensitivities of 3 ELISA test in various categories of subjects

S.No.	ELISA	Tuberculosis patients n=62						Leprosy n=7	Chest Symptomatics n=5	Normal n=75
		Pre Treatment n=13	Undergoing Treatment n=19	Post treatment n=12	Extra Pulmonary n=8	HIV/TB n=10	HIV n=10			
1	I IgG 38kDa LAM	6 (46%)	15 (78.95%)	8 (66%)	3 (37%)	3 (30%)	1 (10%)	2(28%)	2(40%)	15 (20%)
2	II IgM 38kDa LAM	11(84%)	14 (73.68%)	9 (75%)	7 (87%)	2 (20%)	6 (60%)	7 (100%)	5 (100%)	27 (36%)
3	III IgG 16kDa 38kDa	7 (53%)	12 (63.16%)	8 (66%)	2 (25%)	8 (80%)	4 (40%) 0(0%) 1 (20%) 10 (13%)			

Table 2. Western blot analysis in the TB patients

Mol Wt. kDa	Individual TB patients sero reactivity															% Positivity
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
29-33		+					+								+	20.00
38-39	+	+				+	+			+		+	+	+		53.33
41-43	+	+		+	+			+	+		+				+	53.33
47-52	+	+	+	+				+		+		+	+	+	+	66.66
58-60				+	+	+		+	+	+	+	+		+		60.00
68					+	+			+	+						26.66
76-80			+	+	+	+	+	+	+	+	+		+			66.66
84-89		+	+	+	+	+	+		+	+		+	+	+		73.33
101								+			+					13.33

Note: Healthy controls (n=5) showed a single 38-39kDa protein band.

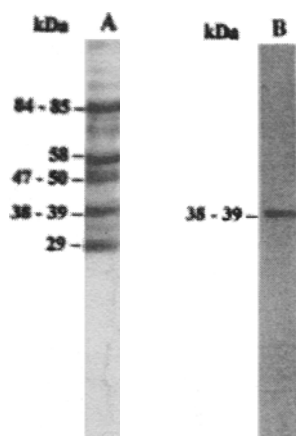

Fig 1. Western blot of *M. tuberculosis* proteins separated on 12% polyacrylamide gel showing interaction with TB patient's serum (lane A) and normal serum (lane B).

The third ELISA (ELISA III) was carried out to detect the IgG response to a combination of the 16kDa and 38kDa proteins of M.tb. The specificity of this ELISA was 87%. The overall sensitivity in tuberculosis patients was 59.6%. When sensitivities in the various clinical forms of tuberculosis were compared the highest sensitivity was in the HIV- TB co-infection cases (80%) followed by post treatment (66%), group under going treatment (63.16%), pretreatment group (53%) and the lowest sensitivity was in the extra pulmonary group (25%). The ELISA positivity was also observed in 40% of the HIV infected individuals and 20% of the chest symptomatic patients. Leprosy patients did not show ELISA positivity (Table 1, row 3).

Strips of western blot of *M.tb* proteins were interacted with serum collected from 15 TB patients and five normal individuals. Western blot results showed that an antibody response was obtained to *M.tb* proteins in the range of 150kDa to 16kDa (fig.1). Analysis was carried out for the prominent protein bands (Table 2).

The 84-89kDa protein was the major protein band in 73.3% of the cases, followed by two groups of proteins with mol.wts 76-80kDa and 47-52kDa in 66.6% each; 58-60kDa protein in 60% of the cases and 38-39 kDa and 41-43kDa proteins in 53.3% cases. Normal sera interaction showed only a single band of 38-39 kDa in all the controls.

The sera obtained from the TB patients were screened for HIV I and II antibodies and the results showed that 3/52 (5.7%) were reactive for HIV I (excluding the 10 known HIV/TB coinfected patients).

DISCUSSION

M.tb possesses a wide variety of antigenic components, which include proteins, lipids and polysaccharides. The 14kDa, 16kDa, 19kDa, 23kDa, 35kDa, 38kDa and 47kDa proteins of *M.tb* have been characterized and used in various immuno diagnostic tests (2). In the present study the test used was based on the 38kDa lipoprotein, which is a well characterized phosphate specific transporter, the 16kDa immunoprotective extra cellular protein and LAM which is a glycolipid specific to Mycobacteria.

When the specificities of the three ELISAs were compared the IgG response to 38kDa and LAM was 80% whereas the IgM response was 64%. A combination of 38kDa and 16kDa gave 87% specificity. Similar observation was made in a study from Tamil Nadu (South India) (7). An ELISA based on two antigens 16kDa and 38kDa proteins did not significantly increase the specificity of the test. In the category of leprosy the cross reactivity to 38kDa and 16kDa proteins of *M.tb* was obtained in an average of 42.5% cases. This could be due to known antigenic similarities between *M.tb* and *M.leprae* (17) as well as to host proteins (18).

When the three ELISA's were evaluated together for the different categories of TB patients the average sensitivities decrease in the following order; under going treatment (71.89%), post treatment (69%), extra pulmonary (49.6%) and HIV/TB co-infection (43.3%). The pretreatment group showed lower detection level when compared to the group under going treatment. This may be due to a suppression or escape of *M.tb* from the host immune response before treatment and an immune activation during treatment due to increased release of bacterial antigen (19). ELISA positivity to 16kDa and 38kDa in chest symptomatic patients, HIV patients and in leprosy patients suggests that our population in general are exposed to mycobacterial antigens and may mount a significant and sustained antibody response.

When three ELISAs are carried out on a single tuberculosis patient the probability of a single ELISA to be positive was 69.3% (ELISA II). The probability of diagnosing tuberculosis by carrying out two ELISAs (ELISA II & III) was highest at 93.5% followed by ELISA I & II and ELISA I & III with 87.09% and 69.35% respectively. It was observed that when three ELISAs were carried on a single TB patient the probability of any of the three ELISAs to be positive is 95.16% (results not shown). Therefore a single ELISA test to detect TB is not sensitive however two or more ELISA's increase the sensitivity significantly.

The Western blot analysis of TB patients showed a higher specificity and sensitivity for a diagnosis of tuberculosis. Similar observations were made in other studies (20, 21, 22). When three or more protein bands were considered, a 100% sensitivity was observed. However western blot positivity to the 38kDa protein band in 53.33% (8/15) of TB patients and 100% (5/5) of normal individuals decreased the specificity. In general, in all categories of TB patients three or more antigens showed an antibody response though these antigenic sets are not the same in all the patients. This suggests that *M.tb* has a varied host immune response that could be influenced by ethnic variations (23), BCG vaccination (24) variation in the strain of *M.tb* (25) and exposure of the population to environmental mycobacteria (26). In our study, positivity to the 38-39kDa protein in normal individuals by western blot was 100% and by ELISA 23%. Further work needs to be carried out on normal individuals as to know how their past exposure to *M.tb*, BCG vaccination and other factors (leprosy) could influence the antibody response and western blot patterns.

A 2% HIV positivity rate is present in the general population in the state of Andhra Pradesh in India (27,28). In the present study, HIV seroprevelance in TB affected individuals was 5.7% which is moderately higher than in general population (29). The susceptibility of immuno-compromised HIV patients to TB is known and is due to either a new infection or activation of a dormant TB infection. Diagnosis of TB in patients with HIV is more difficult by routine sputum microscopy and the sensitivity is only 50-70% whereas by culture it is about 90%. The sensitivity and reliability of the tuberculin test is only 30-50% and chest X-ray abnormalities can be even more non-specific in HIV infected cases than in HIV negative cases (29). In the present study, among the three ELISAs tested to detect TB in HIV infected individuals the 38 and 16 kDa antigens in combination had a sensitivity of 80%. When three ELISAs are carried out on a HIV-TB co infected individual the sensitivity of detection increased to 90%. Further work is necessary on larger numbers to assess the utility of serodiagnostic tests to detect TB in HIV co infected individuals.

In conclusion, the antibody response to the 38kDa, 16kDa and LAM antigens of M.tb when applied independently are not useful as diagnostic tests for the diagnosis of TB in our population; however two or three ELISAs significantly enhance the sensitivity of detection. A western blot assay which evaluates the antibody response to multiple antigens of M.tb could be a better diagnostic test. Recently, ELISA based on ES-31 antigen and a new capture ELISA assay based on the whole pathogen were found to be highly sensitive and specific for the diagnosis of TB (3,4,30). Hence, multicentric evaluation of these methods and development of newer methods based on multiple antigens could throw light on whether there are any antigens to which there is a common antibody response in different populations around the world. Post genomic research, sequencing of different M.tb strains and characterization of epitope specific antibodies in TB patients may further clarify the common antigens for serological diagnosis.

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REFERENCES

1. World Health Organization. Tuberculosis fact sheet. WHO Tuberculosis site (cited 2000 Sept 15); 1(3screens), Available from; URL; http:// www.who.int/gtb/ publications/factsheet/ index.htm.
2. Davies, P.D.O. (1997) Clinical tuberculosis. (Ed 1st). Chapman & Hall Medical Publishers.
3. Bhatia, A.S., Kumar, S. and Harinath, B.C. (2003) Immunodiagnosis of tuberculosis: An update. Indian.J.Clin.Biochem. 18(2), 1-5.
4. Banerjee, S., Gupta, S., Shende, N., Kumar, S. and Harinath, B. C. (2003) Serodiagnosis of tuberculosis using two ELISA systems. Indian.J.Clin.Biochem. 18(2), 48-53.
5. Kaur, J., Arora D.R., Sikka, R. and Gupta, K.B. (1999) Evaluation of ELISA test using antigen 60 in the serodiagnosis of TB. Indian J. Med. Microbiol. 17(2), 72-73.
6. Chander, I., Subrahmanayam, S. and Gupta, R. (1960) Sensitivity of EIA in the diagnosis of tuberculosis using 38kDa antigen. J.Ind.Med.Assoc. 94, 376-378.
7. Mathai, E., Rajkumari, R., Kuruvilla, P.J., Kirubakaran, H., Brahmadathan, K.N., Inbamalar, & Abraham, O.C. (2002) Evaluation of serological tests for the diagnosis of tuberculosis. Indian J. Pathol. Microbiol. 45(4), 439-441.
8. Papiha, S.S., Wentzel, J., Behjati, F. and Agarwal, S.S. (1985) HLA and circulating immunoglobulin levels in Indian patients with pulmonary tuberculosis. Tubercle 66, 25-33.
9. Sada, E., Brennan, P.J., Herrera, T. & Torres, M.

(1990) Evaluation of lipoarabinomannan for the serological diagnosis of tuberculosis. J. Clin. Microbiol. 28 (12), 2587-2590.

10. Wilkinson, R.J., Haslov, K., Rappuoli, R., Giovannoni, F., Narayanan, P.R., Desai, C.R., Vordermeier, H.M., Paulsen, J., Paslov, G., Ivanyi, J. & Singh, M. (1997) Evaluation of the Recombinant 38kDa antigen of *Mycobacterium tuberculosis* as a Potential Immunodiagnostic Reagent. J. Clin. Micro. 35(3), 553-557.

11. Zaher, F. & Marks, J. (1977) Methods and medium for the culture of tubercle bacilli. Tubercle. 58,143-5.

12. Lodam, A.N., Reddy, M.V., Narang, P., Gupta, O.P., & Harinath, B.C. (1996) Fractionation, analysis and diagnostic utility of *Mycobacterium tuberculosis* H37Ra excretory-secretory antigen in pulmonary tuberculosis. Indian. J. Biochem. Biophys. 33(1), 66-71.

13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) Protein measurement with folin phenol reagent. J.Biol. Chem. 193, 265-275.

14. Laemmli, U.K. (1970) Cleavage of proteins during the assembly of head of bacteriophage T4. Nature 227, 680-685.

15. Towbin, H., Staehelin, T., & Gordon, J. (1979) Electrophorectic transfer of protein from polyacrylamide gels to NCM sheets: Procedures and some applications. Proc Natl.Acad. Sci. USA. 76, 4350.

16. World Health Organization. (1994) Operational characteristics of commercially available assays to detect antibodies to HIV-1 and or HIV-2 in human sera. Geneva: WHO. GPA/RID/CRD/94.4.

17. Cole, S.T., Eigilmeier, K., Parkhill, J., James, K.D. et al. (2001) Massive gene decay in the leprosy bacillus. Nature. 409, 1007-1011.

18. Van den Akker, T.W., Naafs, B., Kolk, A.H., De Glopper-van der Veer, E., Chin, R.A., Lien, A., & Van Joost, T. (1992) Similarity between mycobacterial and human epidermal antigens. Br. J. Dermatol; 127 (4), 352-358.

19. Harboe, M. (1994) Overview of host-parasite relations. In: Robert C. Hasting, editor. Leprosy. (Ed 2nd) London: Churchill Livingstone. pp 87-111.

20. Rovatti, E., Corradi, M.P., Amicosante, M., Tartoni, P.L., Panini, W., Ancora, A., Cenci, A.M., Zucchi, L., Monno, L., Angarano, G., & Saltini, C. (1996) Evaluation of a western blot serum test for the diagnosis of Mycobacterium tuberculosis infection. Eur. Respir .J. 9(2), 288-92.

21. Franco, J., Camarena, J.J., Nogueira, J.M., Blanquer, R., Ruiz, M.J., & Marin, J. (2001) Serological response (Western blot) to fractions of Mycobacterium tuberculosis sonicate antigen in tuberculosis patients and contacts. Int. J. Tuberc. Lung. Dis. 5(10), 958-62.

22. Lu, X.D., Sun, H.P., & Zhang, Y.H. (1993) Application of western blotting technique in the serodiagnosis of Mycobacterium tuberculosis infection. Zhonghua Jie He He Hu Xi Za Zhi; 16(6), 357-9, 375-6.

23. Stead, W.W., Sender, J.W., Reddick, W.T. & Lofgren, J.P. (1990) Racial differences in susceptibility to infection by *Mycobacterium tuberculosis.* New Engl J. Med. 322, 422-427.

24. Smith, P.G. (1994) Clinical tuberculosis. In: Davies PDO, editor. Prevention: BCG vaccination. (Ed 1st). London: Chapman Hall; pp: 297-320.

25. Hoal-van Helden, E.G., Hon, D., Lewis, L.A., Beyers, N., & van Helden, P.D. (2001) Mycobacterial growth in human macrophages: variation according to donor, inoculum and bacterial strain. Cell. Biol. Int. 25(1), 71-81.

26. Fine, P.E.M., Floyd, S., Stanford, J.L., Nkhosa, P., Kasunga, A., Chaguluka, S., Warndorff, D.K., Jenkins, P.A., Yates, M., & Ponnighaus, J.M. (2001) Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy. Epidemiol. Infect. 126, 379-387.

27. UNAIDS/AIDS.AIDS epidemic update. UNAIDS, Geneva, December 2000.

28. www.naco.nic, www.unaids.com.

29. Walia, K. (2002) Current issues in HIV/TB co-infection. Ind. J. Tub. 49,21-26.

30. Farajollahi, M.M. & Moghaddamnia, H. (2002) Development of a new capture ELISA assay of whole pathogen for diagnosis of tuberculosis in body fluids. Int. J. Tuberc. Lung Dis. 6(10), 165.