RAPID, EARLY AND SPECIFIC DIAGNOSIS OF TUBERCULOSIS AND OTHER MYCOBACTERIAL DISEASES IN BURUNDI

by

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Summary - The potential usefulness of ELISA based serological tests to assist in rapid, early and specific diagnosis of tuberculosis was investigated. The materials were selected, based on published data and on our preliminary findings. Initially screening tests were performed using crude antigens such as Purified Protein Derivate (PPD) and a BCG-filtrate. Unfortunately, the results with these antigens were not promising. The specificity of both antigens using sera from 94 healthy controls was 64%. As a consequence of these findings, the crude antigens were excluded from further tests, and the study was continued with purified antigens.

The work focused on 2 purified proteins: Antigen 6O (A60), a lipopolysaccharide-protein complex, and P32, a stress protein produced in zinc deprived cultures, identified as Antigen 85 A in the BCG reference system, both isolated from *Mycobacterium bovis* BCG. The commercial A60 based ELISA and our own P32 based ELISA were used to test a total of 300 sera from HIV positive, negative and unscreened individuals, mainly originating from Burundi. These sera were collected from clinical established cases of pulmonary TB, extrapulmonary TB, and patients with non-tuberculous tropical diseases such as salmonellosis, trypanosomiasis, malaria, etc. and healthy individuals.

The A60 based ELISA had a sensitivity of 76.8 % for the proven cases of active pulmonary tuberculosis and 61.9 % for the extrapulmonary tuberculosis cases. No difference was shown between HIV positive and HIV negative patients. Specificity reached 95.2 % for healthy individuals, but dropped to 68.1 % when persons with active non-tuberculous tropical diseases were included.

Eighty-six percent of the pulmonary cases and 87.7 % of the extrapulmonary cases were detected by the ELISA-P32. These findings suggest that this test might be useful as a confirmatory test for the diagnosis of extrapulmonary tuberculosis. Again no difference was noticed between HIV negative and positive patients. The main contraindication for the use of the ELISA-P32 for the diagnosis of tuberculosis is its low specificity: 70.2 % with sera from healthy controls and 22.2 % for hospitalised patients and persons with non-tuberculous tropical diseases.

In a small recent prospective study 4 out of 10 HIV+ persons with no evidence for TB yielded a positive result for the ELISA-P32. Two of them developed

pulmonary tuberculosis within 6 months, whereas 2 P32-positives and 6 P32-negatives remained up to now without any manifestations of tuberculosis. The difference was not significant, but the number of cases was limited.

To avoid false positive reactions, two specific lipid antigens isolated from *M. tuberculosis* were selected : Sulfolipid IV (SLIV), a diacyl trehalose sulfate, and Phenolic Glycolipid Tb1 (PGLTb1), a glycolipid. Most of the testing was done with SLIV, where a specificity of 81.2 % was reached among control sera. When only 'healthy' persons (HIV negatives and positives) were included, the specificity increased to 87%. The test detected 75.0 % of the pulmonary TB cases and 52.0% of the tuberculous lymphadenitis cases.

Our results are promising and further studies on the usefulness of the ELISA-P32, ELISA-SLIV and other antigens, in confirming active tuberculosis or predicting reactivation in HIV + persons, should be pursued on a larger number of subjects.

In view of the low specificity of the ELISA-P32 among patients with other diseases, research on the possible occurence of cross reactions with shared epitopes among other etiological agents is being studied in collaboration with the Erasmus Hospital in Brussels (Drowart and coworkers). Although a good diagnostic ELISA test (with <u>both</u> high sensitivity and specificity) for the detection of active tuberculosis still needs to be developed, our findings and those of others would suggest that it is possible to improve or modify existing tests by identifying specific epitopes and careful application of these tests to high risk groups.

INTRODUCTION

Worldwide tuberculosis remains one of the major health care problems due to infectious diseases, with an estimated incidence of 10 million new cases a year causing 3 million deaths (29). Where tuberculosis was thought to have become a minor problem in industrialised countries in the seventies, it again became one of the priorities in the early eighties due to the HIV-infection epidemic (24). In developing countries the existing primary health care problems caused by tuberculosis were enhanced by the HIV epidemic, where both infections are often associated (5) and nosocomial infections are not uncommon (12).

As Collins and coworkers say : "The best way to prevent the spread of tuberculosis in any community is to identify those people who are excreting the tubercle bacillus and to render them non-infectious to others." (6), meaning that infected persons should be diagnosed and treated as soon as possible. Diagnosis of tuberculosis is difficult and often based on the clinical signs (fever, cough, weight-loss), supported by subjective confirmation through the microscopic detection of acid fast bacilli and rarely by X-ray of the chest and/or culture of the bacilli. The sampling for extrapulmonary TB and pulmonary TB from children is particularly difficult and diagnosis of HIV positive patients with active pulmonary TB is complicated because they may present normal radiographs (17). These short comings in diagnosis require development of additional tools to detect infection and disease.

Alternative approaches such as the detection of tuberculostearic acid (TSA) by combined gas-chromatography-mass spectrometry (15), detection of bacilli by polymerase chain reaction (PCR) (22,28) and radiometric determination of bacterial growth (13) have been developed but their use is still limited for financial and practical reasons. Many efforts have been made to develop a serodiagnostic test for the rapid, early and specific detection of tuberculosis (1, 9).

In this study we examine the potential usefulness of some newer ELISA antigens to assist in the diagnosis of tuberculosis.

OBJECTIVES OF THE STUDY

The objectives of this study were :

(1) To compare the suitable mycobacterial antigens in ELISA tests for the rapid, early and specific detection of IgG anti-tuberculous antibodies
(2) To analyze the antibody response in patients with pulmonary and extrapulmonary tuberculosis
(3) To analyze the antibody response in HIV negative and HIV positive patients

MATERIALS AND METHODS

Antigens

Different classes of mycobacterial antigens were analyzed for their ELISA antigenic activity. The following antigens were used :

- *Crude antigen* : *M. bovis* BCG filtrate : prepared by J. De Bruyn
- *Semi purified antigen* : Purified Protein Derivate (PPD) : a reagent for cutaneous tests, prepared from *M. bovis* BCG and kindly provided by Dr. I De kantor (Centro Panamerica de Zoonosis, Buenos Aires, Argentina)
- *Purified protein* : Antigen 60 (A 60) : a lipopolysaccharide-protein complex, prepared from *M. bovis* BCG by ANDA biologicals (Strasbourg, France)
- *Purified protein* : Protein 32 (P32) : a fibronectin binding 32 kDa component identified as Antigen 85 A in the BCG reference system, prepared by J. De Bruyn
- *Purified lipid* : Sulfolipid IV (SLIV) : a trehalose sulfate isolated from *M. tuberculosis* and kindly provided by Dr. H.L. David (IPP, Paris, France)
- *Purified lipid* : Phenolic Glycolipid (PGL-Tb1) : a glycolipid isolated from *M. tuberculosis* and kindly provided by Dr. H.L. David (IPP, Paris, France)

Serum samples

For the initial screening tests with crude antigens, well documented sera of patients from Europe, Honduras, Burundi and Zaïre with pulmonary tuberculosis, and healthy controls from the same population were used. More sera from Burundi became available as the study progressed. A review of the serum samples that were used for the testing of the ELISA-A60, ELISA-P32 and ELISA-SLIV is presented in Table 1.

Table 1
Sera tested with ELISA-A60, ELISA-P32 and ELISA-SLIV

Group	Infection	A60 n	A60 HIV +	A60 HIV -	A60 HIV NT	P32 n	P32 HIV +	P32 HIV -	P32 HIV NT	SLIV n	SLIV HIV +	SLIV HIV -	SLIV HIV NT
I	PULMONARY TB												
	- confirmed	112	25	43	44	74	46	27	1	16	14	0	2
	- suspected	64	31	24	9	0	0	0	0	0	0	0	0
II	EXTRAPULMONARY TB												
	lymphadenitis												
	- confirmed	21	3	18	0	57	11	4	42	25	3	1	21
	- suspected	13	0	10	3	1	0	0	1	1	0	0	1
	other forms												
	- confirmed	0	0	0	0	0	0	0	0	15	5	2	8
	- suspected	0	0	0	0	6	4	2	0	4	0	1	3
III	CONTROLS												
	healthy	62	0	51	11	104	9	29	66	78	22	48	8
	hospitalized	0	0	0	0	12	9	3	0	25	4	3	18
	tropical inf.												
	- Cryptococcus sp	0	0	0	0	12	7	2	3	0	0	0	0
	- Onchocerca sp	0	0	0	0	12	0	0	12	0	0	0	0
	- Plasmodium sp	16	9	6	1	15	9	6	0	0	0	0	0
	- Salmonella sp	4	0	0	4	12	0	0	12	1	0	0	1
	- Schistosoma sp	8	0	0	8	15	1	0	14	14	0	0	14
	- Toxoplasma sp	0	0	0	0	16	0	0	16	0	0	0	0
	- Treponema sp	0	0	0	0	15	0	0	15	15	0	0	15
	- Trypanosoma sp	4	0	2	2	0	0	0	0	0	0	0	0
Subtotal		94				213				133			
TOTAL		304	68	154	82	351	96	73	182	194	48	55	91

Table 2
Results obtained with ELISA-A60, ELISA-P32 and ELISA-SLIV

Group	Infection	A60 +	(%)	A60 -	(%)	P32 +	(%)	P32 -	(%)	SLIV +	(%)	SLIV -	(%)
I	PULMONARY TB												
	- confirmed	86	<u>(76.8)</u>	26	(23.2)	64	<u>(86.5)</u>	10	(13.5)	12	<u>(75.0)</u>	4	(25.0)
	- suspected	25	(39.1)	39	(60.9)								
II	EXTRAPULMONARY TB												
	•*lymphadenitis*												
	- confirmed	13	<u>(61.9)</u>	8	(38.1)	50	<u>(87.7)</u>	7	(12.3)	13	<u>(52.0)</u>	12	(48.0)
	- suspected	6	(46.2)	7	(53.8)	1	(100.0)	0		1	(100.0)	0	(0.0)
	•*other forms*												
	- confirmed									3	(20.0)	12	(80.0)
	- suspected					3	(50.0)	3	(50.0)	0		4	(100.0)
III	CONTROLS												
	•*healthy*	3	(4.8)	59	<u>(95.2)</u>	31	(29.8)	73	<u>(70.2)</u>	10	(12.8)	68	<u>(87.2)</u>
	•*hospitalized*					11	(91.7)	1	(8.3)	9	(36.0)	16	(64.0)
	•*tropical inf.*												
	- <u>Cryptococcus sp</u>					10	(83.3)	2	(16.7)				
	- <u>Onchocerca sp</u>					9	(75.0)	3	(25.0)				
	- <u>Plasmodium sp</u>	15	(93.7)	1	(6.2)	8	(53.3)	7	(46.7)				
	- <u>Salmonella sp</u>	4	(100.0)	0		10	(83.3)	2	(16.7)	0		1	(100.0)
	- <u>Schistosoma sp</u>	4	(50.0)	4	(50.0)	13	(86.7)	2	(13.3)	6	(42.9)	8	(57.1)
	- <u>Toxoplasma sp</u>					13	(81.3)	3	(18.78)				
	- <u>Treponema sp</u>					14	(93.3)	1	(6.7)	0		15	(100.0)
	- <u>Trypanosoma sp</u>	4	(100.0)	0									
	Subtotal controls	30	(31.9)	64	<u>(68.1)</u>	119	(55.8)	94	<u>(44.1)</u>	25	(18.8)	108	<u>(81.2)</u>

blanc space = not tested

ELISA procedures

ELISA-BCG and ELISA-PPD : The peroxidase based IgG antibody capture assay was performed as described by Ritacco *et al.* (25) with slight modifications (20).

ELISA-A60 : An ELISA diagnostic kit, prepared by Anda Biologicals (Strasbourg, France) was used to measure the IgG level directed against A60. Based on the optical densities (OD) of the reference sera, the OD values of the samples were recalculated as described in the manual. The results were interpreted as follows : <100 units = negative; 101 - 124 units = doubtful; >125 units = positive. Doubtful results were considered as positives.

ELISA-P32 : The peroxidase based IgG antibody capture assay was performed as described by Turneer *et al.* (30). The cut-off value was arbitrarily chosen at an OD of 0.500 units.

ELISA-SLIV and ELISA-PGL-Tb1 : The galactosidase based IgG antibody capture assay was performed as described by Cruaud *et al.* (7). The cut-off value was arbitrarily chosen at an OD of 0.500 units.

RESULTS

The screening tests for serodiagnosis of active tuberculosis using the crude and semi-purified antigens suffered from low specificities (64.2 % for healthy controls with BCG-filtrate and 63.6 % with PPD).

The results of the ELISA-A60 tests are shown in Table 2. The proven cases of active pulmonary TB and lymphadenitis resulted in a sensitivity of 76.8 % and 61.9 % respectively, whereas the suspected cases resulted in lower sensitivities (39.1 % and 46.2 % respectively). Sixty-four out of 94 control sera were negative in the A60 serological test, giving a specificity of 68.1 %. The positive sera found in the control group came from hospitalized and out-patients with active infections different from *M. tuberculosis* : *Plasmodium sp* (15/16), *Salmonella sp* (4/4), *Tryponasoma cruzi* (4/4) and *Schistosoma sp* (4/8).

The results of the ELISA-P32 are presented in Table 2. Pulmonary TB and different forms of proven tuberculous lymphadenitis reached sensitivity of 86.5 % and 87.7 % respectively. One case of suspected lymphadenitis and 3 cases suspected of other forms of extrapulmonary TB (50 %) reacted positively. Specificity with healthy controls was 70.2 %, but dropped to 44.1 % upon inclusion of patients hospitalized without known tuberculosis and out-patients with other tropical infections. In this last group 79.4 % (77/97) were positive.

In view of HIV serology in 'healthy' controls we observed a significant (X^2 = 6.12; $p < 0.05$) difference in the P32 seroreactivity between HIV+ en HIV- cases: 8 out of 9 (88.8 %) HIV+ subjects were positive in the ELISA-P32, whereas 10 out of 39 (34.5 %) HIV- cases remained negative in the ELISA-P32. A clinical follow-up of the patients could not be performed for practical reasons.

In a small prospective study, ten serum samples of HIV+ persons with no evidence for TB or any other opportunistic infection, were collected in Burundi and tested in Antwerp by the ELISA-P32. Four of the ten sera were ELISA positive. During a six months clinical follow up in Burundi, 2 out of 4 patients with a positive TB serology developed pulmonary tuberculosis, whereas 2 P32-positives and 6 P32-negatives remained up to now without any manifestations of tuberculosis disease. The difference was not significant (Fischer Exact Test).

The results of the ELISA-SLIV are summarized in Table 2. Twelve out of 16 proven pulmonary TB cases gave a positive reaction with the ELISA-SLIV, resulting in a sensitivity of 75.0 %. Only 37.8 % of the sera from confirmed extrapulmonary cases responded with positive reactions (52.0 % for lymphadenitis, 20.0 % for other forms) and one of the suspected cases was positive. Specificity of 87.2 % was obtained with sera from healthy controls, dropping to 81.2 % when non TB hospitalized patients were included. Remarkable was the high number of positive reactions of schistosomiasis patients (6/14). None of the 22 'healthy' HIV-seroconverted persons reacted positive for the ELISA-SLIV.

No difference was noticed between the seroreactivity capacity of HIV positive and negative TB patients for the three TB tests.

DISCUSSION AND CONCLUSION

Our results confirm that the A60, P32 and SLIV based ELISA tests might be useful as a confirmatory and suggestive test for the diagnosis of pulmonary TB for both HIV positive and negative patients, giving sensitivities of 76.8, 86.5 and 75.0 % respectively. These sensitivities are comparable to the findings of Zatla et al. (84 % with A60) or higher than the reports of Turneer *et al*. (56 % with P32) and Cruaud *et al*. (59 % with SLIV) (7, 30, 32). The lower sensitivities obtained by Turneer and Cruaud mainly resulted from their option to define a high cut-off value, giving high specificities in healthy controls (95 % and 100 % respectively).

Comparison of the optical densities of individual serum samples, revealed that all the sera of TB patients that were tested with both the ELISA-P32 and the ELISA-SLIV, were positive in at least one of these tests, suggesting a 100 % sensitivity if both antigens were to be assembled in the same test. Indeed, so far, no real dominant specific Ag for TB has been found, and patient to patient variations in the Ag to which an individual responds, were noticed. Therefore use of a pool of purified antigens might augment the sensitivity. Indeed, Bothamley et al. demonstrated that a combination of a 38 kDa and 19 kDa antigen, liporabinomannan and Hsp65 gave good seroreactivities for smear positive and smear negative pulmonary tuberculosis cases, and that these tests were useful for monitoring therapeutic outcomes (4). Similar results were obtained for the serodiagnosis of leprosy patients, for which it was shown that lepromatous and tuberculoid patients had (class) specific antibody responses to a broad panel of protein antigens (9, 10, 18, 19, 26).

Although a better specificity (81.2 %) is obtained with ELISA-SLIV compared to ELISA-P32 (44.2 %) and ELISA-A60 (68.1 %), the lower sensitivity for confirmed pulmonary tuberculosis (76.8 %) does not allow to recommend this antigen alone for the serological diagnosis of tuberculosis. Ridell and co-workers found that the use of a combination of highly purified native lipid antigens from *M. tuberculosis* gave good results for the diagnosis of tuberculosis in Swedish patients (23).

The benefit of synthetic glycolipids to augment the sensitivity of the lipid based ELISA tests is still being discussed. Daffé *et al.* found a disappointing seroreactivity rate in TB patients with a synthetic Ag resembling the triglycosyl phenolic glycolipid, though there was a good correlation with the native Ag (8). However, Laszlo and coworkers reported an enhanced serodiagnostic power of a synthetic glycolipid resembling sulfolipid IV, compared to the native mycobacterial antigens (16). The synthetic antigens are thought to resemble more to phagosome-lysosome modified antigens, giving a better epitope presentation.

In cases of extrapulmonary tuberculosis, a good sensitivity of 87.7 % was obtained with the ELISA-P32, whereas 61.9 % and 52.0 % of the lymphadenitis cases were detected with the ELISA-A60 and ELISA-SLIV respectively. This means that the test might be useful as a confirmatory and suggestive test for the serodiagnosis of lymphadenitis. Examination of sera from other forms of extrapulmonary TB have yielded less promising results, but the number of patients tested was limited and not always bacteriologically confirmed. Conclusions on the use of these serodiagnostic tests for these cases are premature.

The main contra-indication for the use of the ELISA-P32 or ELISA-A60 for the diagnosis of tuberculosis was their low specificity, as seen in the reactions of patients with non-tuberculous tropical diseases. The occurence of similar reactions with A6O has been reported by Zatla and coworkers (32). The research on the possible occurrence of cross reactions with shared epitopes among the microorganisms causing non tuberculous diseases in our control group, is being carried out at the Erasmus Hospital in Brussels (Drowart *et al.*). Preliminary immunoblotting results of sera from patients with *Schistosoma* infections have shown positive reactions against P-32 antigen (Drowart *et al.*, personal communication). Further investigations are necessary to confirm these findings.

Another preliminary study on the possible cross-reaction of fibronectine with the control sera which gave positive reactions in the ELISA-P32, yielded no definite results (21).

The results of the small prospective study on IgG anti-TB antibody levels in HIV + persons, suggests that the ELISA-P32 might have been effective as a rapid and early diagnosis of TB for 2 cases. Similar findings were reported by Berlie and coworkers, who used PGL-Tb1 glycolipid as antigen (2). We found no such reactions with the ELISA-SLIV so far. Although the statistical difference was not significant, the total number of cases in our study was a limiting factor in the evaluation. A follow-up study of a larger HIV+ population seems warranted to investigate whether the higher IgG antibody levels detected with the ELISA-P32 in HIV+ persons result from an aspecific polyclonal B cell activation induced by the Human Immunodeficiency Virus (27) or from a TB-specific B cell activation, predicting the onset of tuberculosis.

We may conclude that although a good diagnostic ELISA test (with high sensitivity and specificity) for the detection of active tuberculosis is not yet available, the results are promising and further studies on the usefulness of ELISA-tests with P32, SLIV and other antigens, in confirming active tuberculosis or predicting reactivation in HIV + persons should be pursued on a larger number of subjects.

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REFERENCES

1. Ashraf AF, Elsaghier MD, Edmund GL, Wilkins MD, Praveen K, Mehrota MVSC, Satish J, Ivanyi J. Elevated antibody levels to stress protein Hsp70 in smear-negative tuberculosis. Immunol. Infect. Dis., 1991; 1: 323-328.

2. Berlie HC, Petit JC, David HL. Use of the SL-IV and the PGL-Tb1 glycolipid antigens in ELISA for the diagnosis of tuberculosis in AIDS patients. Zbl. Bakt., 1991; 275: 351-357.

3. Bothamley G, Beck JS, Britton W, Elsaghier A, Ivanyi J. Antibodies to *Mycobacterium tuberculosis*-specific epitopes in lepromatous leprosy. Clin. Exp. Immunol., 1991; 86: 426-432.

4. Bothamley GH, Rudd R, Festenstein F, Ivanyi J. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. Thorax, 1992; 47: 270-275.

5. Broekmans JF. Tuberculosis and HIV infection in developing countries. Trop. Geogr. Med., 1991; 43: S13-S21.

6. Collins CH, Grange JM, Yates MD. Organization and practice in tuberculosis bacteriology. Preface p.v. London, Butterworth, 1985.

7. Cruaud P, Berlie C, Torgal Garcia J, Papa F, David HL. Human IgG antibodies immunoreacting with specific sulfolipids from *Mycobacterium tuberculosis*. Zbl. Bakt. A, 1989; 271: 481-485.

8. Daffé M, Cho SN, Chatterjee D, Brennan PJ. Chemical synthesis and seroreactivity of a neoantigen containing the oligosaccharide hapten of the *Mycobacterium tuberculosis*-specific phenolic glycolipid. J. Infect. Dis., 1991; 163: 161-168.

9. Daniel TM. Antibody and antigen detection for the immunodiagnosis of tuberculosis: Why not? What more is needed? Where do we stand today? J. Infect. Dis., 1988; 158: 678-680.

10. Das PK, Rambukkana A, Bass JG, Groothuis DG, Kok A, Halperin M. Identification of mycobacterial antigens for "ELISA" serology in the diagnosis of leprosy and tuberculosis. Acta Leprol., 1989; 7(Suppl.1): 117-120.

11. Das PK, Rambukkana A, Bass JG, Groothuis DG, Halperin M. Enzyme-linked immunosorbent assay for distinguishing serological responses of lepromatous and tuberculoid leprosies to the 29/33-kilodalton doublet and 64-kilodalton antigens of *Mycobacterium tuberculosis*. J. Clin. Microbiol., 1990; 28: 379-382.

12. Dooley SW, Villarino ME, Lawrence M, Salinas L, Amil S, Rullan JV, Jarvis WR, Bloch AB, Cautchen GM. Nosocomial transmission of tuberculosis in a hospital unit for HIV-infected patients. JAMA, 1992; 267: 2632-2635.

13. Ellner PD, Kiehn TE, Cammarata R, Hosmer M. Rapid detection and identification of pathogenic mycobacteria by combining radiometric and nucleic acid probe methods. J. Clin. Microbiol., 1988; 26: 1349-1352.

14. Espitia C, Sciutto E, Bottasso O, Gonzalez-Amaro R, Hernandez-Pando R, Mancilla R. High antibody levels to the mycobacterial fibronectin-binding antigen of 30-31 kD in tuberculosis and lepromatous leprosy. Clin. Exp. Immunol., 1992; 87: 362-367.

15. Franotovic M, Todoric A, Petrovic J. Gas chromatographic analysis of fatty acids and identification of tuberculostearic acid in *Mycobacterium tuberculosis* by using gas chromatography-mass spectrometry. Period. Biol., 1991; 93: 397-403.

16. Laszlo A, Baer HH, Goren MB, Handzel V, Barrera L, de Kantor IN. Evaluation of synthetic pseudo cord-factor-like glycolipids for the serodiagnosis of tuberculosis. Res. Microbiol., 1992; 143: 217-223.

17. Pedro-Botet J, Gutierrez J, MirallesR, Coll J, Rubies-Prat J. Pulmonary tuberculosis in HIV-infected patients with normal chest radiographs. AIDS, 1992; 6: 91-93.

18. Pessolani MCV, Rumjanek DFD, de Melo Marques MA, de Melo FSF, Sarno EN. Serological response of patients with leprosy to 28- to 30- kilodalton protein doublet from early cultures of *Mycobacterium bovis* BCG. J. Clin. Microbiol., 1989; 27: 2184-2189.

19. Pessolani MCV, Peralta JM, Rumjanek FD, Gomes HM, de Melo Marques MA, Cerqueira Almeida EC, Feres Saad MH, Sarno EN. Serology and leprosy : immunoassays comparing immunoglobulin G antibody responses to 28- and 30- kilodalton proteins purified from *Mycobacterium bovis* BCG. J Clin Microbiol., 1991; 29: 2285-2290.

20. Portaels F, De Bruyn J, Bosseloir Y, d'Hoop MH, Rigouts L. Rapid, early and specific diagnosis of tuberculosis and other mycobacterial diseases. First periodic progress report contract, 1990, n° TS2-0136-B, personal communication.

21. Portaels F, Rigouts L, Barihuta T, De Bruyn J. Rapid, early and specific diagnosis of tuberculosis and other mycobacterial diseases. Fourth periodic progress report contract, 1992, n° TS2-0136-B, personal communication.

22. Ralphs NT, Garrett S, Morse R, Cookson JB, Andrew PW, Boulnois GJ. A DNA primer/probe system for the rapid and sensitive detection of *Mycobacterium tuberculosis*-complex pathogens. J. Appl. Bacteriol., 1991; 70: 221-226.

23. Ridell M, Wallerström G, Minnikin DE, Bolton RC, Magnusson M. A comparative serological study of antigenic glycolipids from *Mycobacterium tuberculosis*. Tuberc. Lung Dis., 1992; 73: 101-105.

24. Rieder HL, Cauthen GM, Comstock GW, Snider DE. Epidemiology of tuberculosis in the United States. Epidemiol. Rev., 1989; 11: 79-98.

25. Ritacco V. Assessment of the sensitivity and specificity of enzyme-linked immunosorbent assay (ELISA) for the detection of mycobacterial antibodies in bovine tuberculosis. J. Vet. Med. B, 1987; 34: 119-125.

26. Rumschlag HS, Shinnick TM, Cohen ML. Serological responses of patients with lepromatous and tuberculoid leprosy to 30-, 31-, and 32- kilodalton antigens of *Mycobacterium tuberculosis*. J. Clin. Microbiol., 1988; 26: 2200-2202.

27. Shirai A, Cosentino M, Leitman-Klinman SF, Klinman DM. Human Immunodeficiency Virus infection induces both polyclonal and virus-specific B cell activation. J. Clin. Invest., 1992; 89: 561-566.

28. Sjöbring U, Mecklenburg M, Andersen AB, Miörner H. Polymerase chain reaction for detection of *Mycobacterium tuberculosis*. J. Clin. Microbiol., 1990; 28: 2200-2204.

29. Styblo K. Overview and epidemiologic assessment of the current global tuberculosis situation with an emphasis on control in developing countries. Rev. Infect. Dis., 1989; 11: S339-S346.

30. Turneer M, Van Vooren JP, De Bruyn J, Serruys E, Dierckx P, Yernault JC. Humoral immune response in human tuberculosis: immunoglobulins G, A and M directed against the purified P32 protein antigen of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG). J. Clin. Microbiol., 1988; 26: 1714-1719.

31. Wilkins EGL, Ivanyi J. Potential value of serology for diagnosis of extrapulmonary tuberculosis. Lancet, 1990; 336: 641-644.

32. Zatla F, Petithory JC. L'ELISA avec l'antigène A6O dans le diagnostic de la tuberculose. Réactions croisées dans la leishmaniose viscérale. Techn. Biol., 1989; 5: 220-225.