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Assessment of the IgA Immunoassay Diagnostic Potential of the *Mycobacterium tuberculosis* MT10.3-MPT64 Fusion Protein in Tuberculous Pleural Fluid[▽]

Leonardo Silva Araujo,[1] Renata Maciel Moraes,[1] Anete Trajman,[2] and Maria Helena Féres Saad[1]*

Laboratory of Cellular Microbiology, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, Brazil,[1] and Gama Filho University, Rio de Janeiro, Brazil[2]

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Pleural tuberculosis (PL-TB) remains difficult to diagnose. An enzyme-linked immunosorbent assay (ELISA) was developed based on a construction containing the fusion of the *Rv3019c* (MT10.3) and *Rv1980c* (MPT64) gene sequences, and its performance was evaluated in an area where TB is endemic. A total of 92 pleural fluid (PF) samples at serial dilutions of 1:50 to 1:800 were included in the ELISA IgA MT10.3-MPT64 evaluation: 70 from TB patients and 22 from patients with other pleurisies. Confirmation of the expression and subsequent purification of the protein was made by SDS-PAGE and Western blot assays, resulting in a 36-kDa protein. ELISA IgA MT10.3-MPT64 showed sensitivities of 61.4%, 58.6%, 62.9%, 67.1%, and 70% at each PF dilution, respectively. The cumulative results of all dilutions increased sensitivity to 81.4% without jeopardizing specificity. Similar results were also obtained at the combined dilutions of 1:50, 1:200, and 1:800 or 1:50 plus 1:800 dilutions (80%). The overall sensitivity of the reference test, i.e., histopathological examination, was 74%. But, via the ELISA IgA MT10.3-MPT64 test, sensitivity was high for specimens with a negative culture (23/27; 85.2%) or nonspecific histopathology (17/18; 94.4%). Our findings demonstrated the promising use of this test as an adjunct in PL-TB diagnoses, particularly in cases with lower bacterial loads and false-negative results in the reference tests, since the new test includes such important features as quick and easy application, high sensitivity and, perhaps most importantly, affordability, which is so crucial for its widespread use in developing countries.

Tuberculosis (TB) is the leading cause of preventable morbidity and mortality from infectious agents worldwide. A global plan has been outlined to eradicate the disease by 2050 (37). To reach this goal, both political commitments and increments in interdisciplinary research are needed; because the specific diagnostics, drugs, and vaccines available for this purpose have been insufficient in eradicating the disease, the development of more effective tools and strategies is urgently needed (7).

In Brazil, the TB prevalence rate is approximately 60 per 100,000 inhabitants (44). Generally speaking, the incidence of pleural tuberculosis (PL-TB) is closely related to the local disease prevalence. Moreover, PL-TB is the major cause of pleural effusions, responsible for approximately 50% of all related diagnoses in Brazil (25, 32).

TB diagnoses have traditionally relied on the identification of acid-fast bacilli (AFB) and the culture of clinical specimens. However, low sensitivities (5 and 47%, respectively) are achieved with PL-TB. Besides, the final culture results may take as long as 4 to 6 weeks to complete, delaying the initiation of specific therapy.

The histopathological examination of pleural biopsy specimens comprises the standard PL-TB reference test. Nevertheless, its sensitivity is highly variable (39 to 84%), it is expensive and time-consuming, and its proper administration requires skilled personnel. It is clear that there is an overriding need for the development of a simple, fast, effective, and affordable yet robust tool that would be readily available throughout the national public health care sector to aid in the diagnosis of PL-TB, with special emphasis on developing countries (12, 15, 30). What is most frequently used is an enzyme-linked immunosorbent assay (ELISA) together with commercial and in-house antibody tests, which have been developed using antigens in a cocktail-like format. The downside is that lower reactivity has been described for this format compared with the rates obtained after testing these components individually (9, 13, 22). In our previous studies, when the single proteins MPT64 and MT10.3 were tested individually for each patient, the combined results achieved a 76% sensitivity rate in a pleural fluid IgA ELISA, with 96% specificity. However, when the two proteins were mixed together and tested as one for each patient, sensitivity decreased (14).

MPT64, a well-characterized antigen located in the region of difference 2, which is the second region missing from the original *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) Pasteur strain, is highly immunogenic. It is also absent from nontuberculosis mycobacteria and, although present in some BCG strains, including the one used in Brazil, MPT64 renders good reactivity in extrapulmonary TB (14, 27, 40, 42). On the other hand, MT10.3, a member of a large family of mycobacterial proteins (CFP10/ESAT6 family) encoded by 23 genes, *esxA* to *-W*, has been detected in culture supernatants and is recognized by T cells in TB patients and infected animals. Like ESAT6 and CFP10, MT10.3 is also conserved within the

* Corresponding author. Mailing address: Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Microbiologia Celular, Av. Brasil, 4365, Rio de Janeiro 20045-360, Brazil. Phone and fax: 55-21-25984346. E-mail: saad@ioc.fiocruz.br.

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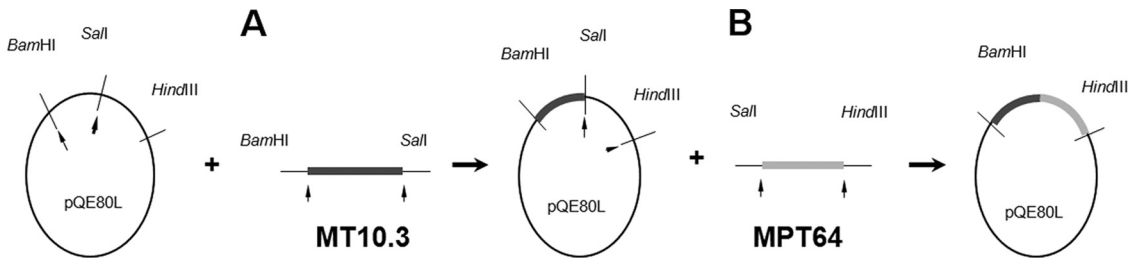

FIG. 1. Cloning scheme of MT10.3-MPT64. (A) Digestion of the plasmid vector and amplicon of the *Rv3019c* gene (MT10.3) with the enzymes BamHI and SalI and subsequent ligation into the pQE80L vector. (B) Digestion of the pQE80L (*Rv3019c*) and *Rv1980c* (MPT-64) gene amplicon with SalI and HindIII, followed by enzyme linkage to generate MT10.3-MPT64-pQE80L.

Mycobacterium leprae genome. Since the clinical evolution of leprosy is different from that of TB, these antigens can be considered highly specific to *Mycobacterium tuberculosis* (8, 19, 35, 39).

The protein fusion approach has been useful in fusing two or more epitopes into a single molecule. It is noteworthy that a number of recent studies have demonstrated the diagnostic and vaccine potentials of a number of fused proteins (5, 20, 21). As such, the objective of the present study was to simplify the IgA ELISA by using the MT10.3 and MPT64 antigens as a fusion protein while evaluating its ability to maintain an efficient humoral diagnostic response to PL-TB and improve immunoreactivity by serial pleural fluid (PF) dilutions as an alternative to overcoming the individual antibody heterogeneic responses.

MATERIALS AND METHODS

Construction, expression, and purification of the recombinant protein. Coding sequences of MT10.3 (*Rv3019c*) and MPT64 (*Rv1980c*) were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR with specific endonuclease restriction sites and then inserted into plasmid ZERO Blunt (Invitrogen, Carlsbad, CA). With the expression vector pQE80L (Qiagen Valencia), the encoding MT10.3-MPT64 protein was first generated by the linkage-coding regions of MT10.3 and then of MPT64, using the BamHI, SalI, and HindIII sites of the vector and inserts, as can be seen in the scheme outlined in Fig. 1. The hybrid MT10.3-MPT64 was linked to a region encoding an N-terminal histidine tag.

The recombinant plasmid MT10.3-MPT64-pQE80L was transformed into *Escherichia coli* BL21(DE3) and grown overnight in LB medium with penicillin at 37°C under shaking induction, performed by adding 1 mM isopropy-β-D-thiogalactoside (IPTG) until the optical density at 600 nm (OD_{600}) reached 0.6. Cells were then grown for an additional 4 h. Thereafter, the cells were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C and suspended with 60 ml of EDTA-Tris-potassium buffer (50 mM Tris [pH 8], 100 mM KCl, 1 mM EDTA, 1 mM phenylmethyl sulfonylfluoride [PMSF]), which were then disrupted by sonication for 8 min (pulse on, 1 min; pulse off, 1 min; amplitude, 40%) on ice. The lysate was centrifuged at 27,000 × *g* for 15 min at 4°C, and the pellet containing insoluble recombinant fusion protein (inclusion bodies) was washed four times with EDTA-Tris-potassium buffer. The inclusion bodies were resuspended in buffer (20 mM Tris [pH 8.5], 2.5 mM EDTA, 5 mM imidazole, 1% Triton X-100), centrifuged, and solubilized in buffer (0.3% *N*-laurylsarcosine, 50 mM *N*-cyclohexyl-3-aminopropanesulfonic acid [CAPS; pH 11], 1 mM dithiothreitol [DTT]) for 30 min accompanied by stirring at room temperature, followed by centrifugation at 1,000 × *g* for 15 min at 4°C. The collected supernatant was dialyzed twice for 4 h and then overnight in 5 liters of dialysis buffer (34.25 mM NaCl, 0.67 mM KCl, 2.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4; pH 7.4) at 4°C. Purification of His-tagged fusion protein MT10.3-MPT64 was made under imidazole competition conditions in a metal chelate column (HisLink protein purification resin; Promega, Madison, WI) containing Ni-nitrilotriacetic acid (NTA) affinity resin, in accordance with the manufacturer's instructions. The resin was incubated with the supernatant for 30 min with stirring at 4°C, transferred to a column, and washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8.0). The bound protein was then eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole; pH 8.0). As described above, the purified fractions were collected and dialyzed overnight. Recombinant protein was analyzed by SDS–12% polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the Bradford method (Coomassie Plus: The Better Bradford; Pierce, Rockford, IL) using bovine serum albumin (BSA standard; Pierce, Rockford, IL) as the protein standard.

Patients and clinical setting: study population. PF was collected from patients with pleural effusion, as described elsewhere (14). A total of 92 samples were included and classified into two groups according to patient diagnosis. The PL-TB group consisted of 70 patients diagnosed with pleural tuberculosis by positive AFB staining in the PF or sputum, by a positive culture in sputum, PF, or tissue, by the finding of granuloma in pleural tissue, or by a pleural TB diagnosis based on clinical findings (defined as fever, night sweats for 15 days, lymphocytic exudative pleural effusion with a nondiagnostic pleural biopsy specimen [with a positive or negative culture], and a good response to specific treatment). The group with other, non-TB pleural diseases (PL-NTB group) included 22 patients with other pleural diseases, diagnosed as follows: cancer (9 patients), cirrhosis (1 patient), parapneumonic effusion (2 patients), empyema (2 patients), heart failure (5 patients), renal failure (2 patients), and systemic lupus erythematosus (1 patient). None of the patients had any information regarding their tuberculin skin tests or BCG vaccinations. Patients underwent blood and PF tests for lactic dehydrogenase (LDH), glucose, cholesterol, amylase, protein and albumin concentrations, and total and differential cell counts, together with AFB and Gram staining (data not shown). Human immunodeficiency virus (HIV) infection status was determined using an ELISA (Genscreen HIV1/2; Bio-Rad, Hercules, CA). A sample of spontaneous or induced sputum was obtained for AFB staining and culture in Löwenstein-Jensen (LJ) medium. Four fragments of parietal pleura were obtained with a Cope needle: three for histopathological examination and one for LJ culture. The supernatants of the centrifuged PF samples were maintained, aliquoted, and stored at −70°C until use for the immune assay. The PF samples with notable hemolysis were excluded from the study.

Demographic, clinical, and laboratory characteristics are described in Table 1. Only one PL-TB patient specimen tested positive for the AFB stain. Culture for *M. tuberculosis* was positive in 12.9% (9/36) patients, and only 1 of those 9 patients showed a nonspecific histopathological result. Six PL-TB (8.6%) and one PL-NTB (4.5%) subject showed positive HIV serology. Patients with other pleurisies were generally older than the PL-TB group ($P < 0.002$), with age intervals ranging from 15 to 85 years and 27 to 79 years, respectively. As to gender, the majority of TB patients were male ($P < 0.0001$).

Pleural fluid IgA MT10.2-MPT64 determination by ELISA. Flat-bottom polystyrene microtiter plates (C96 Maxisorp; Nunc, Swedesboro, NJ) were coated for 2 h at 37°C with 50 μl/well of fusion protein MT10.3-MPT64 solution (0.5 μg/ml) in carbonate-bicarbonate buffer (pH 9.6; Na_2CO_3 at 15 mM and $NaHCO_3$ at 15 mM). Nonspecific binding was blocked by adding 100 μl/well of phosphate-buffered saline (pH 7.4) with 0.1% Triton X-100 (PBST 0.1%) and containing 5% bovine serum albumin (BSA-PBST; US Biological, MA) and incubated, as described above. The plates were washed three times with 200 μl PBST 0.1% and used immediately or stored at 4°C for no more than 2 weeks. The PF samples were added as serial 2-fold dilutions (1:50 to 1:800) with PBS plus 0.3 M NaCl and incubated at 37°C for 1 h. The plates were washed again and incubated for 1 h with horseradish peroxidase-conjugated goat anti-human IgA monoclonal antibodies (Pierce, Rockford, IL) at a 1:5,000 dilution in PBS–0.3 M NaCl and then washed, as described above. Enzyme activity was assayed with 50 μl/well of 3,3′,5,5′-tetramethylbenzidine substrate solution (TMB; Pierce, Rockford, IL)

TABLE 1. Clinical, laboratory, and demographic characteristics of patients with pleural effusion whose specimens were used in this study

Characteristic	Total no. (%) of patients per group with characteristic[a]	
	PL-TB	PL-NTB
Total	70 (76.1)	22 (23.9)
Mean age ± SD (yrs)	40.56 ± 18.24*	57.14 ± 13.73
Gender		
Female	11 (15.7)	10 (45)
Male	59 (84.3)**	12 (54.5)
Acid-fast bacilli in sputum		
Positive	1 (1.4)	0 (0)
Negative	64 (91.4)	20 (90.9)
ND[b]	5 (7.1)	2 (9.1)
Culture		
Positive	9 (12.9)	0 (0)
Negative	27 (38.6)	13 (59.1)
ND	34 (48.6)	9 (40.9)
Histopathology of pleural biopsy specimen		
Positive	51 (7.8)	0 (0)
Negative	0 (0)	2 (9.1)
Nonspecific	18 (25.7)	16 (72.7)
ND	1 (1.3)	4 (18.2)
HIV		
Positive	6 (8.6)	1 (4.5)
Negative	49 (70)	12 (54.5)
ND	15 (21.4)	9 (4.1)

[a] Based on χ^2 test: *, $P = 0.002$ (PL-TB versus other pleural disease); **, $P < 0.0001$ (within the PL-TB group). Data shown for age are mean years (± SD) rather than the number (and percentage) of patients in each category.
[b] ND, not done.

and incubated in the dark at room temperature for 20 min. Reactions were stopped by adding 50 μl of 2 M H_2SO_4, and absorbance was read at 450 nm on an ELISA plate reader (ELx800; Biotek, VT). All tests were performed in duplicate, and in each set of experiments, pooled positive and negative controls (1:50 to 1:400) were used as references.

Statistical analysis. Results were expressed as the means ± standard deviations (SD), and groups were compared using the nonparametric Mann-Whitney or Kruskal-Wallis test. The cutoff value was chosen according to a receiver operating characteristic (ROC) curve analysis for each PF dilution, using a specificity of 95.5%. The diagnostic value of the ELISA was evaluated in terms of sensitivity and specificity in addition to the positive and negative likelihood ratios (LR+ and LR−). McNemar's test was used to compare different diagnostic methods. A P value of <0.05 was considered statistically significant. Data processing was performed using the SPSS 13.0 (SPSS Inc., Chicago, IL), MedCalc 11.0 (MedCalc Software, Mariakerke, Belgium), and Prism4 (Graph-Pad Software Inc., San Diego, CA) programs.

RESULTS

Expression and purification of the MT10.3-MPT64 fusion protein. The designed fusion gene encoding MT10.3-MPT64 was produced and expressed in *E. coli*, generating a protein with an apparent molecular mass of 36 kDa. Although expressed, it was not possible to purify it from the cytosolic lysis supernatant. Thereafter, cells expressing the recombinant MT10.3-MPT64 fusion protein were made soluble by the inclusion body method. Most of the purified protein obtained

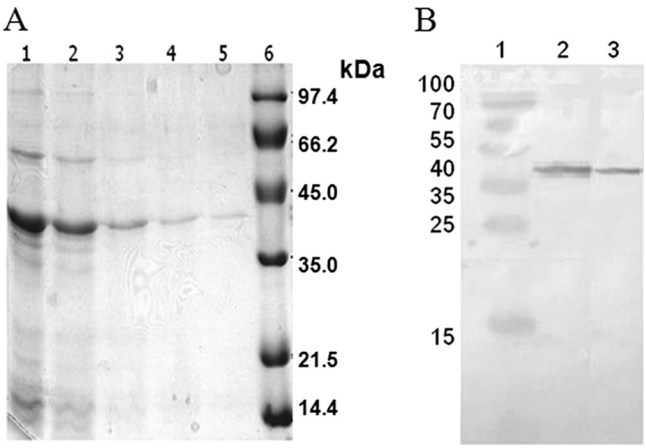

FIG. 2. (A) Coomassie-stained 12% SDS-PAGE gel of the purified fusion protein MT10.3-MPT64. Lanes 1 to 5, eluates collected at different times; lane 6, molecular mass marker. (B) Western blot of MT10.3-MPT64 fusion protein from a mixture of eluates 1 and 2 incubated with mouse IgG anti-His. Lane 1, prestained protein molecular ladder; lane 2, mixed eluates 1 and 2; lane 3, mixed eluates 3, 4, and 5.

was slightly contaminated with other proteins, as shown in Fig. 2. Eluted fractions 1 and 2 were mixed and dialyzed for 16 h in dialysis PBS buffer. Then, after being submitted to anti-His Western blotting to confirm the presence of the MT10.3-MPT64 recombinant fusion protein (Fig. 2B), the protein was quantified and used for all immunological tests.

ELISA IgA MT10.3-MPT64. Figure 3 details individual reactivities in the pleural fluid specimens. The IgA MT10.3-MPT64 mean level was higher in PL-TB samples at all tested dilutions ($P < 0.0001$), reflecting a positivity that decreased from 1:800 to 1:50 and 1:100 while registering sensitivities of 70%, 67.1%, 62.9%, 61.4%, and 58.6%, respectively, at a

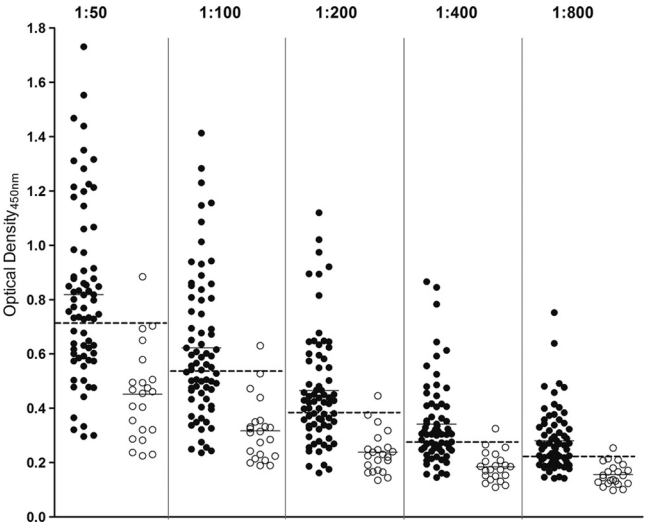

FIG. 3. Distribution of individual humoral responses of the IgA MT10.3-MPT64 fusion protein in patients with pleural tuberculosis (●) or other pleural diseases (○) tested in serial PF dilutions of 1:50, 1:100, 1:200, 1:400, and 1:800. Short bars, mean ODs; longer dashed bars, cutoffs.

TABLE 2. Validity parameters for detection of IgA against MT10.3-MPT64 in different dilutions of PF and their combinations by ELISA

Pleural fluid dilution	Cutoff (OD_{450})	No. of positive specimens/total tested (%); mean ± SD		Likelihood ratio	
		Sensitivity	Specificity	Positive	Negative
1:50	0.703	43/70 (61.4); 0.819 ± 0.316	1/22 (95.5); 0.451 ± 0.172	13.51	0.40
1:100	0.527	41/70 (58.6); 0.622 ± 0.264	1/22 (95.5); 0.316 ± 0.115	12.89	0.43
1:200	0.375	44/70 (62,9); 0.465 ± 0.207	1/22 (95.5); 0.238 ± 0.078	13.83	0.39
1:400	0.266	47/70 (67.1); 0.341 ± 0.151	1/22 (95.5); 0.185 ± 0.054	14.77	0.34
1:800	0.214	49/70 (70); 0.281 ± 0.113	1/22 (95.5); 0.155 ± 0.041	15.40	0.31
All		57/70 (81.4)	1/22 (95.5)	17.9	0.19

specificity of 95.5%. Sensitivity improved after combining the results of two (1:50 and 1:800; 80%), three (1:50, 1:200, and 1:800; 81,4%), and all five dilutions (81.4%) without jeopardizing specificity. The highest LR+ (17.91) and LR− (0.19) values were likewise achieved for dilution combinations (Table 2).

The mean immunoreactivity level of PL-TB-positive versus -negative cultures ($P > 0.323$) as well as the presence or absence of granuloma consistent with TB in the histopathology of pleural biopsy specimens ($P > 0.110$) were not significantly different between groups, although slightly higher reactivity was observed among those with negative cultures and nonspecific histopathological results (Table 3). The group of PL-TB/HIV^+ patients showed a lower mean reactivity than the HIV^- group, but with no significant differences by the Kruskall-Wallis test. By comparing results according to the conventional diagnostic methods used in pleural TB cases, the McNemar test showed no significant statistical difference between the histopathological and ELISA IgA MT10.3-MPT64 results of combined dilutions in the PL-TB group ($P = 0.458$). More than half of the PL-TB patients (39/69; 56.5%) were positive in both tests. The majority of these cases were confirmed by clinical criteria, while the patients' histopathological examinations were nonspecific and their ELISA IgA MT10.3-MPT64 results showed positivity (17/18; 94.4%). Enhanced ELISA IgA fusion protein positivity was also observed among negative culture specimens (85.2%) (Table 3). Reactivity decreased toward concentrated dilutions, even though only one PL-TB patient diagnosed by clinical criteria tested positive at the 1:50 dilution. In the PL-NTB group, there was only one false-positive result in all five dilutions tested, for a patient diagnosed with empyema.

Comparing reactive results of the single proteins (data not shown) with the newly fused protein, the ELISA IgA found that 11 PL-TB samples tested positive for the newly fused protein and negative when the antigens were tested separately. On the other hand, while only four PL-TB samples did not react to the fused protein, they were positive for the single proteins (Fig. 4).

DISCUSSION

The second most frequent presentation of TB is extrapulmonary PL-TB. In this regard, to date the most sensitive diagnostic procedure at our disposal has been the histopathological examination of pleural biopsy specimens. Some of the drawbacks commonly associated with this method are its invasive nature and the requirement of highly specialized professionals to correctly process and interpret the specimen, making it overly time-consuming and expensive. In our set, sensitivity of histopathology was 74% while those for sputum and culture were 1.4% and 12.9%, respectively. At this time, the antibody-based diagnosis of TB is close to the ideal point-of-care test, but it is a difficult task because its antigen-related sensitivity and specificity results along with those of geographic endemicity and the genetic condition of the patient are so highly variable.

Kaisermann et al. described high sensitivity in an in-house IgA ELISA in PF against the single antigens MPT64 and MT10.3. But, when these two antigens were mixed in the same well, a loss of sensitivity and specificity occurred, possibly due to epitope overlapping or, perhaps, to competition for the adsorption sites (14, 16). Lyaschenko et al. reported that pooling different antigens may decrease sensitivity in comparison to somewhat opposite results when using them separately (22). In an attempt to simplify the Kaisermann et al. immunoassay system and increase its speed (one test per person), cost-effectiveness, and perhaps even improve its sensitivity and/or spec-

TABLE 3. Reactivities of PF dilutions in the ELISA IgA MT10.3-MPT64 according to tuberculosis reference test

PF dilution	Mean no. and SD of specimens with indicated result; no. with result/total no. tested (%) based on[a]:			
	Histopathology of pleural biopsy specimens		Culture	
	Positive	Nonspecific	Positive	Negative
1:50	0.791 ± 0.297; 29/51 (56.9)	0.902 ± 0.368; 13/18 (72.2)	0.904 ± 0.354; 7/9 (77.8)	0.810 ± 0.354; 16/27 (59.3)
1:100	0.603 ± 0.256; 28/51 (54.9)	0.681 ± 0.291; 13/18 (72.2)	0.678 ± 0.237; 6/9 (66.7)	0.474 ± 0.222; 16/27 (59.3)
1:200	0.449 ± 0.197; 30/51 (58.8)	0.516 ± 0.236; 14/18 (77.7)	0.511 ± 0.178; 6/9 (66.7)	0.350 ± 0.167; 20/27 (74.1)
1:400	0.332 ± 0.152; 32/51 (62.7)	0.374 ± 0.151; 15/18 (83.3)	0.365 ± 0.121; 7/9 (77.8)	0.283 ± 0.107; 20/27 (74.1)
1:800	0.277 ± 0.117; 34/51 (66.7)	0.296 ± 0.102; 15/18 (83.3)	0.301 ± 0.103; 7/9 (77.8)	0.283 ± 0.107; 20/27 (74.1)
All	39/51 (76.5)	17/18 (94.4)	7/9 (77.8)	23/27 (85.2)

[a] Results were not significantly different, based on the χ^2 test at a P level of >0.05.

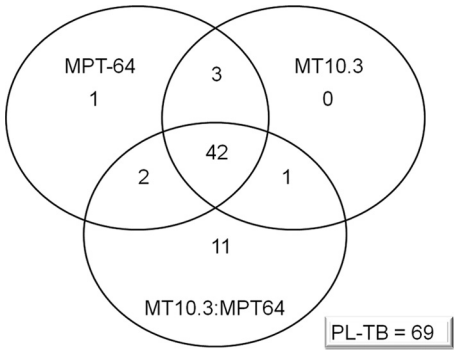

FIG. 4. Venn diagram showing the number of PF samples from patients with PL-TB in which IgA was detected via ELISA to single MT10.3 and MPT64 antigens and to the fusion protein MT10.3-MPT64 (combination of PF dilution results).

ificity measurements, the MT10.3-MPT64 fusion protein was expressed and purified, followed by an evaluation of IgA pleural fluid immune reactivity assayed in serial PF dilutions. The analyses by SDS-PAGE and Western blotting showed a protein with the expected molecular mass of 36 kDa (14). All ELISAs were performed using mixed eluates 1 and 2 after Ni-NTA purification. In this connection, even though there are other components in the fraction of the constructed chimera, no specificity impairment in the test could be found.

As expected, the histopathological examination demonstrated greater sensitivity than the conventional microbiological methods for TB diagnosis. Nonetheless, among the patients with nonspecific results, the ELISA IgA fusion protein MT10.3-MPT64 was able to identify all PL-TBs except one. The discovery of specific antibodies to mycobacterial antigens in PL-TB in the absence of histopathological changes could be supportive of the hypothesis that, in some cases, pleural effusion may also be caused by the possible entry of mycobacterial antigens into the pleural space, thereby stimulating the presence of IgA at this site (34). Conversely, only 23.5% (12/51) of the PL-TB patients diagnosed via histopathology had false-negative ELISA IgA fusion protein MT10.3-MPT64 results.

Some of the BCG vaccine strains, including the one used in Brazil, contain the gene coding for the MPT64 antigen. It has also been shown that tumor cells and the BCG strain may share antigens (23, 24). However, none of the nine cancer patients evaluated in this study showed reactivity to the IgA ELISA MT10.3-MPT64 fusion protein. It is important to note that despite the unavailability of BCG vaccination patient information for this study, in Brazil BCG vaccination at birth is mandatory, so we expected a homogeneous rate of vaccinated individuals on both studied groups.

Cross-reactivity was observed in a patient diagnosed with empyema solely as the result of a clinical examination and negative sputum for the AFB smear, since the culture and histopathological examinations had not been carried out. Furthermore, it is known that empyema also has its genesis in the rupture of a lung injury, with a significant burden of the *M. tuberculosis* bacillus in the pleural space representing active disease with pleural contamination by caseous material (34). It is possible that this patient had tuberculous empyema but was wrongly included in the PL-NTB group because all the available resources for TB diagnosis were not utilized. Further studies in a different setting in which all specimens can be accurately investigated must be done to confirm these data.

Among the tested PF dilutions, the 1:800 dilution demonstrated the highest accuracy (70%), similar to that obtained for the combined results of the single proteins MPT64 and MT10.3 (76%) (14). Kunter et al., after evaluating IgM anti-A60 in PF, reported similar sensitivity (77%) and specificity (94%) (17). However, when the combined results of all dilutions were analyzed, the sensitivity was higher ($\geq$80%) than that obtained in the histopathological biopsy examinations (74%), which in this particular case is a decided advantage. These results suggest that the test developed with the MT10.3-MPT64 fusion has the potential to aid in diagnosing PL-TB. The increased sensitivity attained by combining the results of various dilutions may be related to a reduction in antibody competitiveness in the struggle for certain epitopes of the antigen at different dilutions, making the test even more advantageous. The IgA ELISA with the fusion protein MT10.3-MPT64 was able to homogeneously detect patients with PL-TB regardless of their culture or histopathological results, perhaps ultimately suggesting that bacterial load is not directly related to the production of IgA against the epitopes present in the fusion protein.

In the present study, there was no interference of HIV serostatus on reactivity result, and we also found that serological immunodiagnostic tests appear to be less affected by HIV infection than those based on cell immune response, such as the tuberculin skin test. This assertion has merit, since studies in HIV-infected patients have shown that PL-TB can be the first manifestation of HIV infection, indicating that these patients are in the early stages of immune suppression. Unfortunately, the viral loads and CD4 T cell counts from the patients in these studies were not reported (6, 14). A South African study using immunohistochemistry to detect the MPT64 antigen in biopsy tissue of patients with pleural effusion attained sensitivities of 80% (20/25) and 81% (13/16) for HIV-positive and HIV-negative patients, respectively, at a specificity of 100% (2). It can be conjectured that the detection of this antigen at the infection site is more sensitive to a TB diagnosis in HIV patients. For the present findings, similar sensitivity was obtained in the group of TB/HIV$^-$ patients (83.7%; 41/49), which in the TB/HIV$^+$ group decreased to 66.7% (4/6). It is possible that the small number of PL-TB/HIV$^+$ patients in our study may have contributed to the discrepancy between the HIV$^+$ results.

There was no significant difference between the mean reactivity obtained in the PF samples of the patients according to their histopathological versus microbiological results (data not shown). Even so, the combinatory analysis of the immunoassay results showed better sensitivity for the clinical specimens in patients for which the histopathological examination was inconclusive (94.4%; 17/18) and in those with a negative culture (85.2%; 23/27). High sensitivity was also obtained in the PF from patients with negative sputum (81.3%; 52/64). It is known that extrapulmonary TB is a paucibacillary disease, suggesting that the immune response is not dependent on bacterial load. Moreover, it has been reported that the expression of the mycobacterial proteins MPT64 and MT10.3 occurs during the onset of infection and the spread of *M. tuberculosis* (1, 26, 35).

It is, therefore, not far-fetched to conclude that because of the slow proliferation of the bacilli in conjunction with the acute presentation of PL-TB, detection of the disease during the active phase has decreased accuracy as a result of the small bacillary load when applying methods that require the detectable presence of bacilli and/or granulomas at the time the specimen is collected. However, the immune system may already have come into contact with these proteins, secreted early by *M. tuberculosis*, thereby triggering the initiation of antibody production. Therefore, when the bacterial load increases, the secretion of these two antigens could be stopped, as it is known that there is a dynamic variation in the expression and secretion of antigens by *M. tuberculosis* to evade the host immune response (35). Perhaps these events explain the higher sensitivity found in subgroups with lower bacterial loads for certain antigens, such as those used in our study.

According to Taha et al., IgA is secreted during the contact of *M. tuberculosis* and/or its antigens with the mucosal surface, on which an interaction occurs with specific receptors located in the host cells, stimulating the release of cytokines (interleukin-4 [IL-4], IL-6, and IL-10) (38). This provokes an adjuvant effect on the immune response by inducing the exchange of immunoglobulin isotype secretion (IgM to IgA). Moreover, other studies have shown the presence of IgA against mycobacterial antigens regardless of the time of pulmonary TB infection (0 to 24 months), suggesting that this immunoglobulin isotype response is present in both newly acquired infections and in those of a longer duration, indicating that the production of IgA is probably more closely associated with active infection (3). Thus, the fact that PF IgA is associated with reactivity to MT10.3-MPT64 contributes to its utility in the immunodiagnosis of PL-TB.

It is also important that several studies including our own have reported that hemolyzed specimens may have an influence on ELISA performance (4, 14, 36, 43). In the present study, inconclusive ELISA results were obtained in PF samples with a considerable degree of hemolysis, even though no interference was observed in those having mild hemolysis. As a result, only the first type of samples was removed from the analysis.

Another important issue is the possibility of passive diffusion of blood antibodies to the PF (18). Other authors have shown that the use of body fluids from the infection site provides better sensitivity to the ELISA for extrapulmonary TB, indicating the probability of local antibody secretion (17). Early studies reported differences in the localized versus systemic immune responses in tuberculous pleuritis, as PF T cells showed a significant response to purified protein derivative, eliciting selective gamma interferon-positive $CD4^+$ concentrations. Conversely, peripheral blood T cells show a lesser response (10, 29, 31). IgA is the most abundantly produced mammalian immunoglobulin, and although IgA-secreting plasma cells are concentrated along mucous membrane surfaces, it is produced in the pleural space, since the pleura is a serous membrane (28, 41). On the other hand, several authors have pointed to the presence of IgA against the mycobacterial antigens in the serum of TB patients (3, 11, 33, 45). Regrettably, in our study, serum samples of patients were not available for a matching analysis.

Of concern regarding the fusion of two or more antigens is whether the conformational B cell epitopes will be conserved in the hybrid molecule. A similar reactivity was found for most of the PF samples when we compared the single-antigen results and MT10.3-MPT64 fusion results, except for four patients in whom only single antigens were recognized. On the other hand, antigens in the other 11 patients were only detected by the IgA MT10.3-MPT64 ELISA (Fig. 4). This discrepancy in sensitivity could be related to small differences in the exposure of epitopes in the fused protein, since even though sequencing has only rarely shown point changes, they did not appear to have interfered with the amino acid sequence (data not shown).

In conclusion, the sensitivity of the constructed fusion protein MT10.3-MPT64 was found to be similar to that obtained for the single antigens. However, the cumulative results of the PF dilutions showed higher sensitivity without jeopardizing specificity. It might be useful to implement these strategies in the development of a potentially new diagnostic test for extrapulmonary tuberculosis.

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