Determination of levels of specific IgA to the HspX recombinant antigen of *Mycobacterium tuberculosis* for the diagnosis of pleural tuberculosis*

Pesquisa de IgA contra o antígeno recombinante HspX de *Mycobacterium tuberculosis* no diagnóstico de tuberculose pleural

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Abstract

Objective: To evaluate the accuracy of determining specific IgA to HspX recombinant antigen in pleural fluid and serum samples for the diagnosis of pleural tuberculosis in patients with pleural effusion. **Methods:** This was a cross-sectional study. Serum and pleural fluid samples of patients with pleural effusion and suspected of having pleural tuberculosis were tested with indirect ELISA in order to determine the optical density of specific IgA to HspX. **Results:** We evaluated serum and pleural fluid samples from 132 patients: 97 diagnosed with pleural tuberculosis (study group) and 35 diagnosed with pleural effusion due to other causes (control group). The determination of IgA in pleural fluid satisfactorily discriminated between pleural tuberculosis patients and control patients. The sensitivity of the test in pleural fluid and in serum was 69% and 30%, respectively, whereas the specificity was 83% and 84%, respectively. **Conclusions:** Our data suggest that this test can be used in the diagnosis of pleural tuberculosis. Further studies, involving larger patient samples and different epidemiological scenarios, are warranted.

Keywords: Pleural effusion; Tuberculosis/diagnosis; Enzyme-linked immunosorbent assay.

Resumo

Objetivo: Avaliar a acurácia da dosagem de IgA contra o antígeno recombinante HspX no líquido pleural e no soro de pacientes com derrame pleural para o diagnóstico de tuberculose pleural. **Métodos:** Estudo transversal de teste diagnóstico. Amostras de líquido pleural e de soro de pacientes com derrame pleural e suspeita de tuberculose pleural foram avaliadas para a determinação da densidade óptica de IgA contra HspX utilizando ELISA indireto. **Resultados:** Foram avaliadas amostras de líquido pleural e de soro de 132 pacientes: 97 com tuberculose pleural (grupo de estudo) e 35 com derrame pleural por outras causas (grupo controle). A dosagem de IgA em líquido pleural foi capaz de discriminar os pacientes com tuberculose pleural dos controles. A sensibilidade do teste em líquido pleural e em soro foi, respectivamente, de 69% e 30%, enquanto a especificidade foi de 83% e 84%, respectivamente. **Conclusões:** Os dados sugerem o potencial da utilização deste teste no diagnóstico de tuberculose pleural. Estudos com amostras maiores e em diferentes cenários epidemiológicos são necessários.

Descritores: Derrame pleural; Tuberculose/diagnóstico; ELISA.

* Study carried out in the Laboratory of Immunopathology of Infectious Diseases, Federal University of Goiás Institute of Tropical Pathology and Public Health, Goiânia, Brazil.
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Financial support: This study received financial support from the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq, National Council for Scientific and Technological Development) and the *Fundação de Amparo à Pesquisa do Estado de Goiás* (FAPEG, Goiás Research Foundation).
Submitted: 18 January 2011. Accepted, after review: 21 March 2011.

Introduction

Brazil is one of the 22 countries that are responsible for approximately 80% of the cases of tuberculosis (TB) worldwide, and, within Latin America, it is the country with the largest number of TB cases.[1] Pulmonary TB accounts for approximately 85% of all TB cases, whereas extrapulmonary TB accounts for approximately 14%. Among the cases of extrapulmonary TB in non-HIV-infected individuals, the most common form is pleural TB.[1,2]

The diagnostic method that has the highest yield in patients with pleural TB is histology combined with culture of a pleural sample obtained by means of biopsy. However, the procedure to obtain a pleural sample is invasive, and pleural sample culture requires at least three weeks.[3-5] Therefore, researchers have sought to identify tests for the diagnosis of pleural TB that are more rapid, as well as being noninvasive or only minimally invasive.

Recent studies have demonstrated that the presence of HspX recombinant antigen—Rv2031c—in serum can be related to latent TB and to active pulmonary TB.[6,7] However, TGF-□, one of the many cytokines induced by *Mycobacterium tuberculosis*, is increased in patients with pleural TB, as well as being involved in the regulation of the cellular immune response and in the induction of IgA production.[8,9]

On the basis of what has been presented above, we conducted a study to determine the accuracy of quantifying, with indirect ELISA, specific IgA to HspX recombinant antigen in pleural fluid (PF) and serum samples as a means of diagnosing pleural TB in patients with pleural effusion.

Methods

This was a cross-sectional study involving a convenience sample composed of individuals consecutively treated in the Department of Special Procedures of the Federal University of Rio de Janeiro Thoracic Diseases Institute, located in the city of Rio de Janeiro, Brazil. Individuals were eligible for inclusion in the study if they had been under clinical or radiological suspicion of having pleural TB and were referred to the Department for thoracentesis and pleural biopsy between September of 2002 and July of 2007.

We sequentially included individuals who met the following inclusion criteria: being ≥ 12 years of age; presenting with free pleural fluid or pleural effusion, as demonstrated on lateral decubitus chest X-rays; and presenting with a Karnofsky performance status ≥ 60%. We excluded patients presenting with clinical or biochemical evidence of kidney, heart, or liver failure, those with a history of anticoagulant use or acetylsalicylic acid use in the 10 days preceding the procedure, those in whom the diagnosis was not confirmed, and those who did not complete all of the study procedures.

All of the patients were submitted to the following: standardized interview; physical examination; anteroposterior and lateral decubitus chest X-rays; thoracentesis; pleural biopsy with a Cope needle; and sputum induction. Sputum was induced as previously described.[10] Pleural effusion was classified as exudative or nonexudative, in accordance with the criteria proposed by Light et al.[11] Pleural effusion was considered lymphocytic if lymphocyte levels were higher than 80%. A 5-mL sample of PF, two pleural samples, and the induced sputum were stained by the Ziehl-Neelsen method. The samples were cultured on Löwenstein-Jensen and Sabouraud media in accordance with the standard protocols.[12] Another three pleural samples were stained with H&E, and the PF was stained by the Papanicolaou method. All of the specimens with positive culture for mycobacteria were tested by biochemical methods in order to distinguish between *M. tuberculosis* and other mycobacteria that cause TB. Approximately 500 µL of PF and 500 µL of blood were collected and immediately frozen at −20°C for subsequent ELISA.

The individuals who were diagnosed with pleural TB met one or more of the following criteria: *M. tuberculosis* growth in the PF, pleural sample, sputum sample, or any combination of the three; presence of granuloma with or without caseous necrosis in the pleural tissue; and presence of exudative lymphocytic pleural effusion with negative cytology for malignant cells, and complete resolution of the clinical and radiological profiles occurring after treatment with rifampin, isoniazid, and pyrazinamide.

We selected 132 serum and PF samples of patients with pleural effusion: 97 from patients with pleural TB and 35 from patients with diseases

other than TB (28 patients with metastatic cancer, 1 patient with congestive heart failure, 2 patients with liver failure, 1 patient with systemic lupus erythematosus, and 3 patients with parapneumonic effusion). In order to detect IgA antibodies against HspX recombinant antigen in PF and serum samples, we used ELISA, as previously described, except for some changes after standardization.[13] For ELISA, the antigen was diluted in carbonate/bicarbonate buffer (pH = 9.6), at a final concentration of 2.5 µg/mL (50 µL/well), and incubated at 4°C for 18 h in the ELISA plates. Subsequently, we added 50 mL of 0.05 M carbonate/bicarbonate buffer with 1% powdered skim milk. This was followed by incubation at 37°C for another 2 h. Serum and PF samples were diluted in PBS and 0.1% skim milk at dilutions of 1/100 and 1/10, respectively, and incubated at 37°C for 2 h (serum samples) or for 24 h (PF samples). The plates were washed six times with PBS and 0.05% Tween 20. The solutions containing the peroxidase-conjugated anti-human IgA were diluted in PBS and 0.05% skim milk, in a final dilution of 1/2,000, distributed into each well (50 µL), and incubated at 37°C for 1 h. After that period, the plates were again washed with PBS and 0.05% Tween 20. The substrate buffer (a solution of 5 mg of orthophenylenediamine, 20 µL of oxygen peroxide, and 5 mL of citrate/phosphate buffer; pH = 5.2) was drawn with a pipette and incubated for 15 min. Subsequently, 50 µL of 4 N sulfuric acid solution were added to each well. The ELISA results, obtained with an ELISA reader (Thermo Lab Systems, Franklin, MA, USA), are expressed as optical density values at a wavelength of 492 nm. The procedures were performed in the Laboratory of Immunopathology of Infectious Diseases of the Federal University of Goiás Institute of Tropical Pathology and Public Health, located in the city of Goiânia, Brazil.

The data were analyzed with the programs GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2003. In order to determine the optical density cut-off value that would maximize the sensitivity and specificity of the tests in PF and serum, we used the ROC curve, and the best cut-off value was defined as that closest to the upper left corner of the curve (sensitivity and specificity of 100%). The medians were compared by the Mann-Whitney test.[14] The level of significance was set at 5%.

The study project was approved by the Research Ethics Committee of the Clementino Fraga Filho University Hospital/Federal University of Rio de Janeiro Thoracic Diseases Institute. All participants gave written informed consent.

Results

We evaluated 175 individuals with pleural effusion and suspected of having pleural TB. Of those, 43 were excluded from the study: 13 because of sample hemolysis; and 30 because the final diagnosis was unknown. Therefore, we selected 132 patients. Of those, 97 were diagnosed with pleural TB, and 35 were diagnosed with diseases other than TB (metastatic cancer, congestive heart failure, liver failure, systemic lupus erythematosus, and parapneumonic effusion). The 35 patients who were diagnosed with diseases other than TB constituted the control group. We evaluated serum and PF samples collected from the 132 patients. Table 1 shows the profiles of the individuals included in the present study. Figure 1 shows the optical density values obtained by indirect ELISA in serum and PF samples. As can be seen in Figure 1A, the serum levels of IgA were higher in the patients with pleural TB than in the control group patients (0.244 ± 0.377 vs. 0.137 ± 0.174). However, the difference was not statistically significant ($p > 0.05$). Nevertheless, as can be seen in Figure 1B, IgA levels were significantly higher in the PF of patients with pleural TB than in that of patients in the control group (0.481 ± 0.300 vs. 0.203 ± 0.204; $p < 0.05$).

Table 2 shows the cut-off values (obtained by means of the ROC curve) for the evaluation of the test in serum and in PF. For the diagnosis of pleural TB in the study sample, the sensitivity, specificity, and positive predictive value of the test were better in PF samples than in serum samples.

Discussion

The determination of the levels of specific IgA to HspX recombinant antigen in PF by means of ELISA satisfactorily discriminated between patients with pleural effusion due to TB and those with pleural effusion due to other

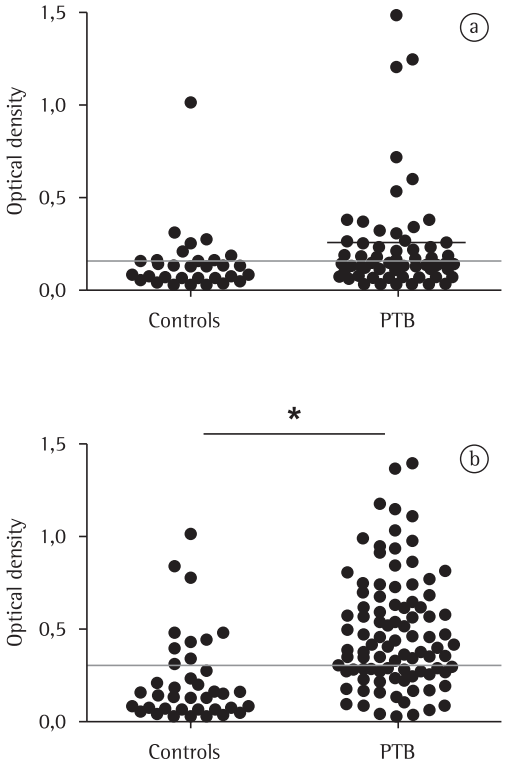

Figure 1 - Optical density values for the levels of specific IgA to HspX recombinant antigen in serum samples (in a) and pleural fluid samples (in b) of pleural tuberculosis (PTB) patients and control patients. The horizontal line in each graph represents the cut-off value. *p < 0.05.

diseases. In addition, the sensitivity of the test was 69%, which was greater than is that of culture for *M. tuberculosis*—the conventional test for the diagnosis of TB in PF—the reported sensitivity of which ranged from 10% to 20% in different case series.[3,15]

Determination of immunoglobulin levels is relatively simple and inexpensive. Therefore, the determination of immunoglobulin levels in serum samples, PF samples, and other samples has long been investigated as a means of diagnosing TB.[16] A sensitive, reproducible, safe, and easy-to-perform technique, ELISA does not require sophisticated, expensive instruments, meaning that it can be easily performed in any laboratory. However, although promising, studies involving purified antigens in serum for the diagnosis of pulmonary TB have shown that the determination of the serum levels of such antigens is still not accurate enough to warrant its incorporation into routine medical practice.[17-20] In a meta-analysis of extrapulmonary TB, conducted in 2007, the sensitivity of immunoenzymatic assays of PF samples ranged from 26% to 59%, despite the fact that the specificity ranged from 81% to 100%.[21] The authors concluded that although the tests were rapid, simple, and noninvasive, there was no evidence to support their use in the diagnosis of extrapulmonary TB.[21] However, none of the tests evaluated in that meta-analysis involved the determination of specific IgA to HspX recombinant antigen in PF. Another meta-analysis, conducted in 2009, evaluated the performance of serological tests for pulmonary TB.[22] In 25 studies, the mean sensitivity was found to be 40% (range, 10-90%), and the mean specificity was 96% (range, 48-100%). These values are similar to those found for the test in serum in the present study, i.e., 30% (95% CI: 16-49) and 84% (95% CI 74-83), respectively. However, in the present study, the determination of specific IgA to HspX recombinant antigen in serum did not discriminate between patients with pleural TB and control patients, suggesting that the humoral immune response (in the serum) is not as significant as is the local response (in the PF).

Regarding the antigen used in the present study (HspX recombinant antigen), a previous, preliminary study demonstrated, by means of immunoblot assay, that 27-kDa proteins and 16-kDa proteins (which is the case of HspX recombinant antigen) are detected in the serum of patients with active TB but not in that of control patients without active TB.[23] Nevertheless, a previous study suggested that the response to HspX recombinant antigen was greater in the serum of individuals with latent *M. tuberculosis* infection than in that of those with active TB.[13] A recent study demonstrated a greater response to HspX recombinant antigen in individuals with latent *M. tuberculosis* infection.[23] However, the authors identified

Table 1 - Characteristics of the patients included in the present study.

Characteristics	Pleural TB group (n = 97)	Control group (n = 35)
Gender (M/F)	69/28	21/14
Age, years[a]	33 (16-70)	61 (17-94)

TB: tuberculosis. [a]Values expressed as median (range).

Table 2 - Accuracy of the immunoenzymatic assay for determination of the levels of specific IgA to HspX recombinant antigen in the serum and pleural fluid of 132 individuals with pleural effusion and suspected of having tuberculosis.

IgA levels	Optical density cut-off value	PPV	NPV	Sensitivity	Specificity
		%	%	% (95% CI)	% (95% CI)
Serum	0.060	84	32	30 (16-49)	84 (74-90)
PF	0.216	87	47	69 (52-83)	83 (74-90)

PPV: positive predictive value; NPV: negative predictive value; and PF: pleural fluid.

HspX recombinant antigen expression in the various stages of TB, from infection with *M. tuberculosis* to active disease.[23]

The various studies mentioned here suggest that, although immunoenzymatic assays can detect latent *M. tuberculosis* infection, as well as active pulmonary or extrapulmonary TB, none have proved sufficiently accurate to warrant their use. The results obtained by determining the levels of specific IgA to HspX recombinant antigen in PF samples in the present study reignite this discussion and stimulate further studies in this line of research. Studies involving larger samples in which the control groups comprise a larger number of cases of pleural effusion caused by other infectious agents—cases that can be clinically and radiologically mistaken for TB—should be conducted, as should studies involving immunocompromised individuals. It is of note that the use of other antigens in combination with HspX recombinant antigen can increase the sensitivity of the test by as much as 20% and constitutes an additional resource that can be employed in future studies.[24]

To our knowledge, the present study was the first study to evaluate the determination of levels of specific IgA to HspX recombinant antigen in PF samples for the diagnosis of pleural TB in Brazil, and the results were encouraging. However, the study has some limitations. The study sample comprised individuals who had pleural effusion and were clinically or radiologically suspected of having pleural TB. In addition, the study was conducted in an area where the prevalence of TB is high. This selection bias influenced the prevalence of TB in the study sample and, consequently, the predictive values of the test. In addition, the present study involved a convenience sample, and the wide confidence interval for the sensitivity and specificity of the test is evidence that our sample, especially the control group, should have been larger. Therefore, similar studies, involving different epidemiological scenarios and larger patient samples, should be conducted. Another limitation of the present study is that, in our hospital, the routine evaluation of patients who present with pleural effusion and are suspected of having TB does not include determination of adenosine deaminase levels, and a comparison between that test and the one performed in the present study could have made our results more generalizable.

In conclusion, the determination, by means of indirect ELISA, of specific IgA to the HspX recombinant antigen of *M. tuberculosis* in PF samples satisfactorily discriminated between patients with pleural effusion due to pleural TB and those with pleural effusion due to other diseases. The good accuracy of the test in the present study strongly suggests that this line of research should be further explored. However, before the test can be incorporated into routine medical practice, further studies are needed. Such studies should involve patient samples that are larger and more diverse, as well as addressing different epidemiological scenarios.

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