

Evaluation of Commercial Enzyme-Linked Immunosorbent Assay Kits for Detection of Tuberculosis in Argentinean Population

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Pathozyme-Myco G (Myco G), M, A, and TB complex plus (Omega Diagnostics Ltd., Alloa, Scotland) were evaluated for the serological diagnosis of pulmonary tuberculosis (TB) in an Argentinean population. Sera from 58 patients with pulmonary TB, 24 subjects with pulmonary mycobacteriosis or mycoses (pulmonary MM group), and 45 subjects with other underlying disorders (control group) were analyzed. The sensitivities of the tests ranged from 29% (Myco M) to 82% (Myco G) in smear-positive patients (17 subjects) and from 29% (TB complex plus) to 49% (Myco G) in smear-negative patients (41 subjects). The specificities of the assays varied from 93% (Myco M) to 100% (Myco G and TB complex plus) in controls and from 62% (Myco A) to 96% (TB complex plus) in the pulmonary MM group. Overall, for the diagnosis of smear-negative patients, Myco G had the best characteristics, with a sensitivity of 49% and specificities of 100% for controls and 75% for the pulmonary MM group; after its combination with TB complex plus, its sensitivity improved to 59%. Nevertheless, despite its relatively poor capacity to discriminate between pulmonary TB and pulmonary MM, Myco G, alone or in combination with TB complex plus, would be a useful diagnostic tool for patients with suspected pulmonary TB living in areas where the relative prevalence of pulmonary MM was low.

Several enzyme-linked immunosorbent assays (ELISAs) have been tried to achieve the rapid, early, and easy diagnosis of pulmonary tuberculosis (TB) (4, 5). However, a large variability in their accuracy has been reported depending on the antigen employed, the immunoglobulin (Ig) class measured, the strain variation, and the deviation in the antibody response among different ethnic groups (4). The evaluation of different serological kits at the same time with the same group of sera has been rarely carried out (4, 6, 17). This study was conducted to evaluate the potential of four ELISA kits: Pathozyme-TB complex plus (TB complex plus), Pathozyme-Myco G (Myco G), Myco M, and Myco A (Omega Diagnostics Ltd., Alloa, Scotland) for pulmonary TB diagnosis. The first kit detects IgG antibodies against recombinant forms of two antigens from the *Mycobacterium tuberculosis* complex: r38 kDa (PstS-1, PhoS, antigen 5, antigen 78 [1]), expressed in and purified from *Escherichia coli* (20), and r16 kDa, a member of the alpha-crystalline family of low-molecular-weight heat shock proteins (23). The details regarding the method for producing r16 kDa antigen are considered proprietary by the manufacturer and are unknown to the authors. Both antigens have been considered species-specific antigens of the *M. tuberculosis* complex (14, 23). The other tests, which utilize the r38 kDa antigen and the lipoarabinomannan (LAM; a common lipoglycan component of the mycobacterial cell wall [7]) purified from *M. tuberculosis* (13), detect human IgG, IgM, or IgA, respectively, in

response to infection with mycobacterial species. Sera and sputum samples were obtained from ambulatory patients with suspected but not proven pulmonary disease who were being investigated for TB at the Instituto Nacional de Enfermedades Respiratorias “Emilio Coni.” Sera of those patients whose sputum cultures were positive (58 patients; median age, 37 years; age range, 16 to 62 years; percentage of males, 64%) were selected for the study, with 17 being smear positive and 41 being smear negative. The times from admission to diagnosis and to treatment were recorded. Sera from 24 patients (median age, 30 years; age range, 20 to 55 years; percentage of males, 58%) with pulmonary diseases usually considered in the differential diagnosis of pulmonary TB (pulmonary mycobacteriosis or mycoses [pulmonary MM]) were also included, as follows: (i) pulmonary diseases produced by nontuberculous mycobacteria (NTM) (*Mycobacterium avium* complex, nine subjects; *Mycobacterium chelonae*, two subjects; *Mycobacterium fortuitum*, one subject), (ii) pulmonary mycoses (paracoccidioidomycosis, seven subjects; aspergillosis, three subjects; histoplasmosis, two subjects). The control population also consisted of 45 patients (median age, 40 years; age range, 20 to 58 years; percentage of males, 62%) without clinical evidence of TB and who were being treated for underlying disorders other than mycobacteriosis or mycoses (respiratory diseases other than TB, including pneumonia, 12 subjects; lung cancer, 5 subjects; asthma, 3 subjects; nonrespiratory diseases including diabetes and cardiovascular disorders, 10 and 15 subjects, respectively). Furthermore, all controls with respiratory symptoms were assessed by X-ray; those with productive cough were bacteriologically evaluated. An additional control group was established with four cases of leprosy (median age, 40 years; age range, 22 to 47 years; percentage of males, 75%). All

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subjects had *Mycobacterium bovis* BCG vaccination history and were human immunodeficiency virus-negative. The purified protein derivative (PPD) status of the patients was unknown. In many countries, such as Argentina, the PPD test for the diagnosis of adult pulmonary TB is considered difficult to interpret and, therefore, suspected TB patients are not usually evaluated by tuberculin skin test in routine clinical practice (10, 16).

All kits were tested simultaneously according to the manufacturer's instructions. In brief, for TB complex plus, a total of 100 μ l of diluted (1/50) serum was distributed in microtiter wells and incubated for 60 min at 37°C. Unbound serum was removed by washing with a buffer solution. The wells were subsequently incubated with 100 μ l of peroxidase-labeled anti-human conjugate at 37°C for 30 min. After another wash cycle, 100 μ l of peroxidase substrate tetramethylbenzidine containing hydrogen peroxide was added to the wells and the colorimetric reaction was prolonged for 15 min in the dark at 37°C until 100 μ l of stop reagent was added. The absorbance values at 450 nm were recorded. Three standards (with 2, 4, and 16 serounits/ml) were provided to generate a semilog reference curve. Because the sera were diluted 1/50, the units extrapolated from the curve were multiplied by 50 to obtain serounits for result interpretation. According to the manufacturer's instructions, a result was considered positive when the level of antibodies in a sample was higher than 200 serounits/ml. The procedures for Myco G, A, and M were identical to those described for TB complex plus except that the sera were diluted 1/100 and all incubations were at room temperature. For Myco G and A, three standards (with 2, 4, and 16 serounits/ml) were provided but, because sera were diluted 1/100, the units extrapolated from the reference curve were multiplied by 100 to obtain serounits for interpretation of results. ELISA-positive sera were those having more than 400 and 300 serounits/ml for Myco G and A, respectively. For Myco M, low-positive and a highly positive control sera were provided. The absorbance value for the low-positive control was considered the cutoff point. The specimens were labeled with sequential numbers and processed in the blind. Samples from controls and patients were tested together in an interspersed fashion. Each sample was tested twice on different days. The assay reproducibility was examined by determining the coefficient of variation (CV%). Since the between-assay CV% were ± 6.8 , ± 5.9 , ± 7.1 , and ± 6.1 % for Myco G, Myco M, Myco A, and TB complex plus, respectively, the kits were considered acceptable for routine clinical assay in terms of reproducibility (21).

The results obtained with tuberculous patients are summarized in Table 1. The assays yielded a significantly higher sensitivity when applied to smear-positive pulmonary TB patients (chi square test, $P < 0.05$) than to smear-negative pulmonary TB patients, except for the Myco M kit. A generally higher sensitivity for the smear-positive group was also observed when Myco G, A, and M were evaluated in a survey performed in New Zealand (17). However, no significant differences were found, possibly due to the lower number of pulmonary TB patients that were included in that study. Whether a higher rate of seropositivity for the smear-positive group simply reflects a higher bacillary loads resulting in a greater exposure to antigen and thus a more vigorous antibody response or a qualitatively different immunological response to *M. tuberculosis* is

TABLE 1. Results of four serological kits with different groups of patients

Diagnostic group	No. tested	% of seropositive individuals (no. of positive results)			
		Myco G	Myco M	Myco A	TB complex plus
Smear-positive TB	17	82.3 (14)	29.4 (5)	76.5 (13)	58.8 (10)
Smear-negative TB	41	48.8 (20) ^a	31.7 (13)	34.1 (14) ^a	29.3 (12) ^a
Mycobacteriosis or mycoses	24	25.0 (6) ^b	20.8 (5)	37.5 (9) ^b	4.2 (1)
Mycobacteriosis	12	25.0 (3) ^b	25.0 (3)	16.7 (2)	8.3 (1)
Mycoses	12	25.0 (3) ^b	16.7 (2)	58.3 (7) ^b	0 (0)
Leprosy	4	50.0 (2) ^b	100.0 (4) ^b	100.0 (4) ^b	75.0 (3) ^b
Others	45	0 (0)	6.7 (3)	2.2 (1)	0 (0)

^a Chi square test for significance of difference of positivity percentages (in comparison with smear-positive TB) yielded P values < 0.05 .

^b Fisher's exact test for significance of differences of positivity percentages (in comparison with the "others" group) yielded P values < 0.05 .

not clear (11). TB complex plus showed a low degree of sensitivity (29%) for the smear-negative group included in this study. Pathozyme-TB complex (a previous version that contained only r38 kDa) showed contrasting results when it was used to evaluate sera from smear-negative patients in surveys performed on populations from different ethnic origins (17, 24). Genetic restriction of the immune response to mycobacteria (9) and/or differential characteristics among mycobacterial strains prevalent in distinct regions of the world, inducing different host responses, could account for these differences.

As the antigens used in the kits were not specific for *M. tuberculosis* and are present in *M. bovis* as well (8, 23), it is possible that BCG vaccination could influence the antibody levels of the population, possibly affecting the rate of specificity. Nevertheless, in spite of the fact that all of the 45 control subjects with diseases other than pulmonary MM and leprosy have BCG vaccination history, the specificities of the tests were good, ranging from 93 (Myco M) to 100% (Myco G and TB complex plus) (Table 1). However, Myco G, A, and M were not useful in discriminating TB from pulmonary MM (specificities ranging from 62 to 79%) and, in addition, showed positive reaction (50 to 100%) to sera from leprosy patients (Table 1). These cross-reactivities were not distinguished in the previous evaluation performed in New Zealand (17). On the other hand, while TB complex plus was found to be highly specific for distinguishing NTM and mycoses from pulmonary TB (specificity: 96% for pulmonary MM), it also showed cross-reactivity with sera from leprosy patients. It is interesting that, unlike tests based in protein antigens (TB complex plus), those kits including LAM as antigen gave a high percentage of false positives when evaluated for patients with mycoses (Table 1). Our findings are supported by an earlier study of Sada et al. (19), which reported a high level of anti-LAM antibodies in patients with histoplasmosis.

Patients who have smear-positive pulmonary disease currently receive a standardized anti-TB treatment. It is known that, in some proportion of these cases, the positive smears are due to NTM, so the value of a serological test for smear-positive pulmonary patients must be limited to its ability to distinguish TB from NTM. This seems to be the case for TB complex plus, which has proved to be a very specific test.

TABLE 2. Results of combination of serological tests

Combination	% Positivity among:				
	Active pulmonary TB patients		Controls		
	Smear ⁺ (n = 17)	Smear ⁻ (n = 41)	NTM or mycoses ^a (n = 24)	Leprosy (n = 4)	Others (n = 45)
Myco G + Myco M	82.3	63.4	37.5	100.0	6.7
Myco G + Myco A	94.1	58.5	41.7	100.0	2.2
Myco G + TB complex plus	88.2	58.5	25.0	75.0	0
Myco M + Myco A	82.3	48.8	45.8	100.0	8.9
Myco M + TB complex plus	70.6	51.2	25.0	100.0	6.7
Myco A + TB complex plus	82.3	48.8	37.5	100.0	2.2
Myco G + Myco M + Myco A	94.1	68.3	54.2	100.0	8.9
Myco G + Myco M + TB complex plus	88.2	70.7	37.5	100.0	6.7
Myco G + Myco A + TB complex plus	93.1	65.8	41.7	100.0	2.2
Myco A + Myco M + TB complex plus	88.2	61.0	45.8	100.0	8.9
Myco G + Myco M + Myco A + TB complex plus	94.1	75.6	54.2	100.0	8.9

^a Patients with pulmonary diseases produced by NTM or mycoses.

Among the 12 pulmonary mycobacteriosis patients included in this study, four individuals were smear positive and none of them had a TB complex plus-positive result. Nevertheless, because of the low sensitivity of TB complex plus, negative results might be obtained both in patients with a disease due to NTM and in 41% of smear-positive pulmonary TB patients, which nearly completely destroys the usefulness of this kit in planning the treatment regimen.

To improve the sensitivity of detection for smear-negative patients, different combinations of the tests were analyzed (Table 2). The best combination of two different tests was that of TB complex plus with Myco G, which gave an improved sensitivity of 58.5% for smear-negative patients, with a specificity of 87.7% (64 of 73). However, even when the results of the four kits were combined, 10 out of the 41 smear-negative patients remained negative by all the assays, yielding a maximum sensitivity of 75.6% for the smear-negative population. The possibility that these 10 smear-negative patients had falsely positive (cross-contaminated) cultures was highly unlikely, since all of them had more than one positive culture for *M. tuberculosis*. Either generalized immune suppression, immune complex formation (18, 22), specific inhibition of lymphocyte subsets (12), or insufficient antigenic stimulation could explain the lack of antibody responsiveness in some patients with active TB.

The present study suggests that Myco G is an easy and reliable test. Although this test yielded higher sensitivity when applied to the study of patients with advanced smear-positive TB than when applied to smear-negative patients, it identified 49% of the cases which could not be detected by microscopic examination. However, to appraise its diagnostic value, it is necessary to know its positive and negative predictive values (PV), which depend not only on its sensitivity and specificity but also on the prevalence of the disease in the community. As Myco G's specificity for patients with pulmonary MM was significantly lower than that for patients with other underlying disorders (Table 1), the value of the overall specificity markedly depends on the relative composition of the control population. At a 15% prevalence of pulmonary TB (prevalence of bacteriologically confirmed pulmonary TB in our patients with two consecutive negative sputum acid-fast stains and sugges-

tive clinical and chest radiological findings of TB [15]) and considering the compositions of the controls, for whom the percentages of mycoses and NTM with respect to TB were 5 and 0.5%, respectively (2), the overall specificity of Myco G was 99.8% and the positive and negative PV were 97.4 and 91.8%, respectively. Therefore, this kit might have a potential use as a rapid presumptive diagnostic test for patients with suspected TB long before culture results are available in our clinical population. This kit might be used only for populations where the relative proportion of mycobacteriosis and mycoses to TB is very low, as in Argentina.

Traditionally, in smear-negative cases the treatment may be delayed until culture results are available. So we attempted to gauge the effect that use of Myco G would have had if the results had been known within the first week of admission in terms of reducing the time before treatment initiation. On this basis, the 20 positive Myco G results (49% of the smear-negative patients) would have allowed chemotherapy to start on average 45 days earlier. Although patients who are smear negative clearly expel fewer organisms than those who are smear positive, the delay of treatment and lack of isolation may significantly contribute to the propagation of TB. Measures to accelerate the rapid diagnosis of these patients may contribute to controlling TB (3).

In the present study we showed that Myco G, alone or in combination with TB complex plus, would be a complementary diagnostic tool in patients with suspected TB long before culture results are available. Nevertheless, because of the relatively poor capacity to discriminate between pulmonary TB and pulmonary MM, this kit might be used only for populations where the relative proportion of mycobacteriosis and mycoses to TB is very low. A better understanding of the repertoire and dynamics of antibody responses in patients with *M. tuberculosis* infection and other mycobacterial infections may facilitate the development of more-sensitive and -specific antibody-based methods for the diagnosis of active pulmonary TB.

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