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Use of bronchoalveolar lavage enzyme-linked immunospot for diagnosis of smear-negative pulmonary tuberculosis

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SUMMARY

OBJECTIVE: To compare the diagnostic validity of blood enzyme-linked immunospot assay (ELISpot), bronchoalveolar lavage (BAL) ELISpot and the tuberculin skin test (TST) in patients with pulmonary smear-negative tuberculosis (TB) in a country with high TB prevalence.

DESIGN: In a prospective, hospital-based study, 107 patients with suspected TB were tested simultaneously using blood and BAL ELISpot and TST.

RESULTS: Of 102 patients with active pulmonary TB, 36 (35.3%) were diagnosed with TB, while 66/102 (64.7%) had a non-TB diagnosis. The sensitivity and specificity for ELISpot on mononuclear cells from BAL fluid was respectively 94.4% (95%CI 81.9–98.5) and 78.1% (95%CI 66.6–86.5). The specificity of BAL ELISpot was significantly higher than that of blood ELISpot ($P = 0.011$). Compared with blood ELISpot and TST, BAL ELISpot was not significantly influenced by previous history of TB (OR 2.05, $P > 0.05$) or household contact with a patient with active TB (OR 2.41, $P > 0.05$).

CONCLUSION: ELISpot on BAL appears to be a more rapid and sensitive supplementary test than on blood for the diagnosis of active TB patients with a negative sputum smear in a developing country setting with high TB prevalence and access to bronchoscopy and ELISpot assay. However, the test's utility was limited by its moderate specificity.

KEY WORDS: tuberculosis; bronchoalveolar lavage; ELISpot assay; interferon-gamma; tuberculin skin test

TUBERCULOSIS (TB) is the most important cause of mortality and morbidity due to an infectious agent worldwide,[1] with an estimated 8.8 million incident cases of TB and 1.1 million deaths in 2010.[2] Microscopy of acid-fast bacilli (AFB) and culture for *Mycobacterium tuberculosis* are gold standards for the diagnosis of active TB; however, suitable specimens are difficult to obtain in those patients unable to produce sputum, and cultures takes an average of ⩾2 weeks to yield results.[3] In ~20% of patients with pulmonary TB (PTB), *M. tuberculosis* cannot be recovered from cultures of bronchial secretions,[4] and although direct smear microscopy is rapid and inexpensive, its sensitivity is limited.[5] The tuberculin skin test (TST), a classic test for the rapid diagnosis of active tuberculosis or latent tuberculosis infection (LTBI),[6] is unreliable, as it has low sensitivity and specificity for TB infection in clinical practice.[7–10] The identification of a fast and accurate diagnostic tool for sputum smear-negative TB patients is therefore crucial.

Interferon-gamma release assays (IGRAs) are based on interferon-gamma (IFN-γ) secretion by lymphocytes exposed to *M. tuberculosis*-specific antigens encoded by the region of difference 1 (RD1): early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10).[11,12] In the past decade, it has been demonstrated that IGRA may be useful for TB control programmes, particularly in non-endemic areas, due to its high accuracy.[13–15] Although enzyme-linked immunospot assay (ELISpot) on peripheral blood mononuclear cells (PBMC) was unaffected by prior bacille Calmette-Guérin vaccination, it cannot distinguish between active and latent TB.[13–15] A recent study showed that ELISpot testing of bronchoalveolar lavage (BAL) fluid is an important advancement for the rapid distinction of sputum AFB smear-negative TB from LTBI in routine clinical practice in low TB incidence countries.[16,17] However, research on direct comparisons of blood ELISpot, BAL ELISpot and TST in the diagnosis of smear-negative TB is still limited, particularly in high TB prevalence areas.

To address this critical public health problem, we conducted a prospective study in Nanning, Guangxi Province, China, an endemic area for TB, with a high

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prevalence rate of 650 per 100 000 population.[18] The aim of this study was to compare the diagnostic validity of blood ELISpot, BAL ELISpot and TST in patients with pulmonary smear-negative TB residing in a high TB prevalence country.

MATERIALS AND METHODS

Study setting

This prospective study was carried out from January to September 2011 at the Nanning Fourth Hospital (NFH), the largest regional infectious disease hospital in Nanning. The protocol was approved by the Ethics Committee of Guangxi Medical University; written informed consent was obtained from each patient.

Study participants

Suspected TB patients attending the NFH during the study period who had negative sputum AFB smear results on three consecutive examinations or were unable to produce sputum were prospectively enrolled as study subjects. The final PTB diagnosis was made on positive bacteriological culture of sputum or BAL fluid, or by the presence of caseating granuloma in the biopsy specimen after 6 months' follow-up.

ELISpot

The T-SPOT.*TB* assay (Oxford Immunotec, Abingdon, UK) was performed according to the instructions for blood included in the assay kit. BAL was performed with 200–300 ml of normal saline from an affected lung segment. Mononuclear cells were obtained by passing the BAL fluid through a stainless steel sieve with a mesh aperture of 0.5 mm (SUS310, Anping Zhongyu WMF, Anping, China). Four wells of a 96-well microtitre plate pre-coated with monoclonal antibodies against IFN-γ were seeded with 2.5×10^5 mononuclear cells from BAL fluid (BALMCs). The plates were incubated at 37°C for 16–20 h with 5% carbon dioxide. Fifty microlitres of AIM-V, ESAT-6, CFP-10 and phytohemagglutinin were added to four wells of nil control, panel A, panel B and positive control, respectively. Culture of the plates, washing, counterstaining and visualisation were performed as previously described.[16] Spot-forming cells (SFCs) were scored using an automated ELISpot plate reader (AID GmbH; Strassberg, Germany). Test wells were considered positive if the test wells contained a mean of at least five SFCs more than the mean of the negative control wells, and if this number was at least twice the number of spots in the negative control wells.[19] Results were considered undetermined if the spot amounts in the positive control were <20 or if spot amounts in the negative control were >10. A trained technician blinded to the TST results and the diagnosis of the patients tested the ELISpot and recorded the test results independently.

Tuberculin skin test

After blood samples had been collected for ELISpot, a TST was performed using the Mantoux method with 5 tuberculin units of purified protein derivative (RT23, Chengdu Institute of Biological Products, Chengdu, China). Any indurations were measured in millimeters 48–72 h later using the ball-point method and interpreted by hospital health care workers. We used 5 mm induration as a positive cut-off value for TST according to the Chinese guidelines for PTB diagnosis.[20]

Statistical methods

Analyses were performed using SPSS for Windows version 17.0 (Statistical Package for the Social Sciences Inc, Chicago, IL, USA) and GraphPad Prism for Windows version 4.0 (GraphPad Software Inc, La Jolla, CA, USA). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio of a positive test result, and likelihood ratio of a negative test result for the diagnosis of active TB disease were calculated for blood ELISpot, BAL ELISpot and TST. Non-parametric testing (Mann-Whitney) was used for continuous variables and the χ^2 test was used to compare the positive proportions. Kappa coefficients were used to assess concordance between test results from the TST and T-SPOT.*TB* assay ($\kappa > 0.75$, excellent agreement; $\kappa < 0.4$, poor agreement; $\kappa \geqslant 0.4$ and $\kappa < 0.75$, fair to good agreement).[1] Multivariate logistic regression was used to analyse diagnostic odds ratios (ORs) and the following risk factors: previous TB history and household contact with an active TB patient for test results of blood ELISpot, BAL ELISpot and TST. $P < 0.05$ was considered significant.

RESULTS

Sample characteristics

Five patients were excluded due to technical errors on ELISpot testing; a final 102 patients suspected of active PTB with negative AFB sputum smear results or who were unable to produce sputum were enrolled in the study (Figure 1). Thirty-six of these were confirmed to have active PTB, of whom 19 (52.8%) were diagnosed using mycobacterial culture from sputum or BAL fluid: 8 (22.2%) were diagnosed using AFB microscopy of BAL fluid, and 16 (44.4%) using transbronchial biopsies through histology of lung tissue. Although some suspects transferred from another hospital had already received anti-tuberculosis treatment, the duration of treatment for all patients with active TB was <14 days. Sixty-six patients received a negative TB diagnosis, including bacterial pneumonia ($n = 41$), sarcoidosis ($n = 10$), bronchiectasis ($n = 8$), bronchogenic carcinoma ($n = 4$) and rheumatoid arthritis ($n = 3$). None of these patients developed TB over a 6-month follow-up period. None of the participants had human immunodeficiency virus (HIV) infection,

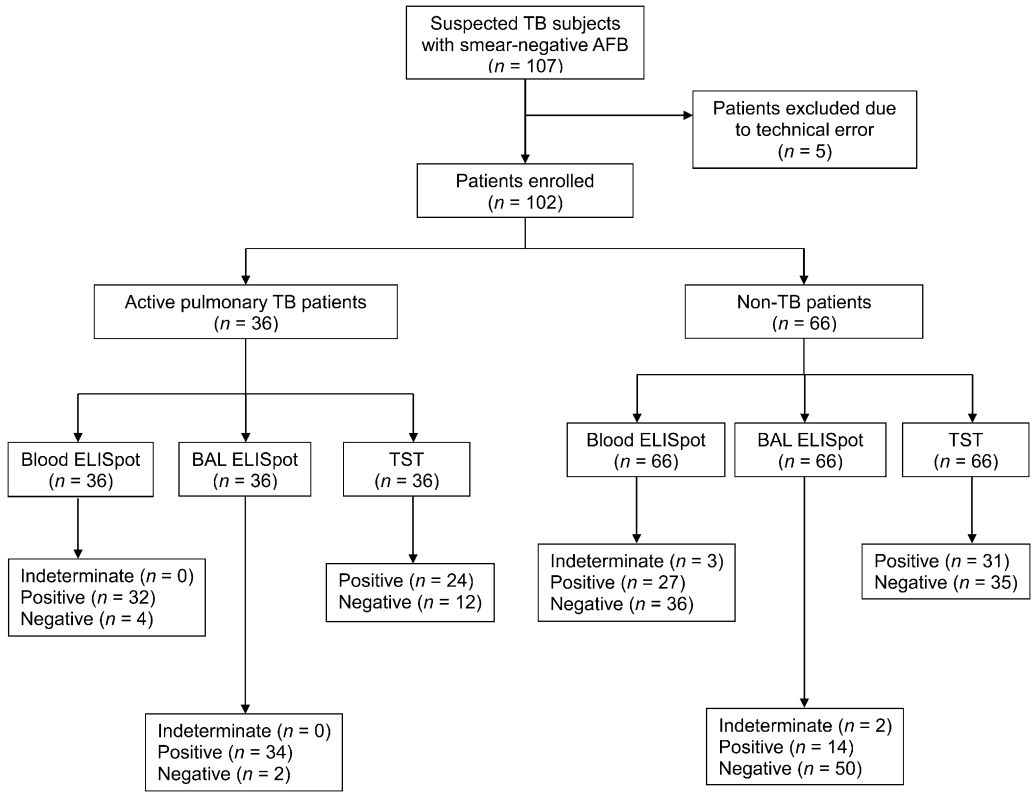

Figure 1 Study flow diagram. TB = tuberculosis; AFB = acid-fast bacilli; ELISpot = enzyme-linked immunospot assay; BAL = bronchoalveolar lavage; TST = tuberculin skin test.

liver or kidney failure. The baseline values of the study population are shown in Table 1.

ELISpot results

All participants were tested using ELISpot simultaneously on PBMCs and BALMCs. Of the 36 subjects with active PTB, ELISpot on PBMCs and BALMCs were positive in respectively 32 and 34. Of the 66 non-TB subjects, ELISpot on PBMCs and BALMCs were positive in respectively 27 and 14. In patients with non-TB disease, 3 (4.5%) had indeterminate blood ELISpot results and 2 (3.0%) had indeterminate BAL ELISpot results.

The sensitivity and specificity for ELISpot on PBMCs were respectively 88.9% (95% confidence interval [CI] 74.7–95.6) and 57.1% (95%CI 44.9–68.6); sensitivity and specificity for ELISpot on BALMCs were respectively 94.4% (95%CI 81.9–98.5) and 78.1% (95%CI 66.6–86.5).

The median numbers of ESAT-6 and CFP-10 SFCs on PBMCs in active PTB patients (43 and 63/250 000 PBMCs) were significantly higher than among non-TB subjects (11 and 8/250 000 PBMCs, $P < 0.001$). The median numbers of ESAT-6 and CFP-10 SFCs on BALMCs in active PTB (104 and 104/250 000 BALMCs) were significantly higher than among non-TB subjects (5 and 5/250 000 BALMCs, $P < 0.001$, Figure 2).

Tuberculin skin test results

The TST was performed on all subjects. Among the 36 patients with active PTB, 24 were TST-positive. In

Table 1 Demographic and clinical characteristics of the study population

Baseline characteristic	Pulmonary TB (n = 36) n (%)	Non-TB (n = 66) n (%)	P value
Male	21 (58.3)	32 (48.5)	0.341
Smoker	15 (41.7)	31 (47.0)	0.354
Age, years, mean ± SD	46.9 ± 21.7	46.2 ± 19.9	0.865
TST diameter, mm, mean ± SD	9.14 ± 7.08	4.76 ± 3.09	<0.001
BCG vaccination	12 (33.3)	25 (47.9)	0.648
Previous TB history	10 (27.8)	15 (27.3)	0.571
Household contact with an active TB patient	7 (19.4)	11 (16.7)	0.725

TB = tuberculosis; SD = standard deviation; TST = tuberculin skin test; BCG = bacille Calmette-Guérin.

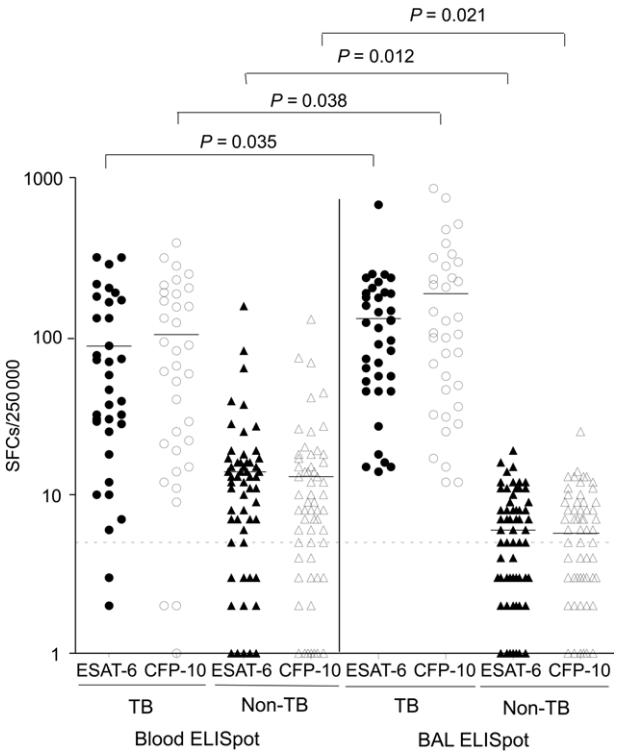

Figure 2 ESAT-6 and CFP-10 SFCs in blood ELISpot and BAL ELISpot per 25 000 cells in patients with active TB and those with non-TB disease. Dashed line = cut-off value of 5 SFCs/250 000 cells. SFC = spot-forming cells; ESAT-6 = early secreted antigenic target 6; CFP-10 = culture filtrate protein 10; TB = tuberculosis; ELISpot = enzyme-linked immunospot assay; BAL = bronchoalveolar lavage.

the 66 patients with non-TB diseases, 31 were TST-positive. The sensitivity and specificity of the TST were respectively 66.7% (95%CI 50.3–79.8) and 53.0% (95%CI 41.2–64.6; Table 2).

Comparison of methods for blood ELISpot, BAL ELISpot and tuberculin skin test

The diagnostic OR of a positive BAL ELISpot result being associated with active TB was 60.71 (95%CI 12.96–284.43, $P < 0.001$) compared with 10.67 for blood ELISpot (95%CI 3.37–33.79, $P < 0.001$) and 2.26 for the TST (95%CI 0.97–5.26, $P = 0.059$; Table 2). The area under the receiver operating characteristic curve (AUC) for blood ELISpot on ESAT-6 was 0.829 compared with 0.922 for BAL ELISpot ($P < 0.001$). The AUC for blood ELISpot on CFP-10 was 0.836, compared with 0.934 for BAL ELISpot ($P < 0.001$). The AUC for the TST was 0.689.

Concordance between tests in active pulmonary tuberculosis and non-tuberculosis patients

The blood ELISpot and BAL ELISpot assays showed good agreement in all participants ($\kappa = 0.69$), and in both those with active PTB ($\kappa = 0.64$) and those with non-TB disease ($\kappa = 0.53$). The concordance between blood ELISpot assay and the TST was good overall ($\kappa = 0.40$); however, concordance was poor in patients with active PTB ($\kappa = 0.25$). Similarly, the concordance was poor between the BAL ELISpot assay and the TST ($\kappa = 0.30$ overall, $\kappa = 0.05$ for active PTB).

Risk factors for test results of blood ELISpot, BAL ELISpot and tuberculin skin test

In multivariate logistic regression analysis, previous history of TB was significantly related to a positive blood ELISpot result (OR 3.10, $P = 0.046$) and TST (OR 6.51, $P = 0.002$), but not to the BAL ELISpot (OR 2.05, $P = 0.141$). Household contact with an active TB patient was significantly related to a positive blood ELISpot result (OR 5.15, $P = 0.023$) and TST (OR 3.55, $P = 0.046$), but not to the BAL ELISpot (OR 2.41, $P = 0.112$; Table 3).

DISCUSSION

In this study, we assessed the usefulness of ELISpot testing of BAL in the diagnosis of smear-negative PTB in clinical routine in China, a developing country with high TB incidence. The sensitivity and specificity of the BAL ELISpot (respectively 94.4% and 78.1%) is comparable to those previously reported in low-incidence countries,[17] which suggests that BAL ELISpot would have a high diagnostic value in clinical practice in high TB prevalence countries.

Table 2 Diagnostic validity of active TB for blood ELISpot, BAL ELISpot and TST*

	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)	LR+ (95%CI)	LR− (95%CI)	Diagnostic OR (95%CI)
Blood ELISpot	88.9 (74.7–95.6)	57.1 (44.9–68.6)	54.2 (41.7–66.3)	90.0 (77.0–96.0)	2.07 (1.53–2.82)	0.19 (0.075–0.50)	10.67 (3.37–33.79)
BAL ELISpot	94.4 (81.9–98.5)	78.1 (66.6–86.5)	70.8 (56.8–81.8)	96.2 (87.0–98.9)	4.32 (2.70–6.91)	0.071 (0.018–0.28)	60.71 (12.96–284.43)
TST	66.7 (50.3–79.8)	53.0 (41.2–64.6)	43.6 (31.3–56.7)	74.5 (60.5–84.8)	1.42 (1.00–2.00)	0.63 (0.38–1.05)	2.26 (0.97–5.26)

*Differences in sensitivity and specificity between blood ELISpot and BAL ELISpot respectively $P = 0.394$ and $P = 0.011$.
Differences in sensitivity and specificity between blood ELISpot and TST respectively $P = 0.023$ and $P = 0.64$.
Differences in sensitivity and specificity between BAL ELISpot and TST respectively $P = 0.003$ and $P = 0.003$.
TB = tuberculosis; ELISpot = enzyme-linked immunospot assay; BAL = bronchoalveolar lavage; TST = tuberculin skin test; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value; LR+ = positive likelihood ratio; LR− = negative likelihood ratio; OR = odds ratio.

Table 3 Risk factors for blood ELISpot, BAL ELISpot and TST test in all participants

	Blood ELISpot-positive			BAL ELISpot-positive			TST-positive		
Variable	*n/N* (%)	OR (95%CI)	*P* value	*n/N* (%)	OR (95%CI)	*P* value	*n/N* (%)	OR (95%CI)	*P* value
Previous TB history		3.10 (1.02–9.44)	0.046		2.05 (0.79–5.34)	0.141		6.51 (2.01–21.13)	0.002
No	40/75 (53.3)			33/76 (42.9)			34/77 (44.2)		
Yes	19/24 (79.2)			15/24 (62.5)			21/25 (84.0)		
Household contact with an active TB patient		5.15 (1.28–28.73)	0.023		2.41 (0.81–7.14)	0.112		3.55 (1.02–12.29)	0.046
No	45/82 (54.9)			36/82 (43.9)			42/84 (50.0)		
Yes	15/17 (88.2)			12/18 (66.7)			14/18 (77.8)		

ELISpot = enzyme-linked immunospot assay; BAL = bronchoalveolar lavage; TST = tuberculin skin test; OR = odds ratio; CI = confidence interval; TB = tuberculosis.

Owing to the high incidence and high risk of infection, priority should be given to the treatment of active TB cases, which will lead to a reduction in the risk of transmission in developing countries.[21] In clinical practice, suspected active TB patients with negative sputum AFB smear results or those unable to produce sputum are common. Those patients are hard to diagnose due to the lack of rapid and accurate laboratory techniques. In developing countries, the majority of these cases have been treated only on the basis of clinical and chest radiographic findings.[22] At NFH, all suspected TB patients with negative smear results were offered bronchoscopy with BAL for mycobacterial culture if the procedure was safe and appropriate; ELISpot on BALMCs was thus available for TB diagnosis.

We found that significantly more SFCs were produced by ESAT-6 and CFP-10 on BALMCs in active PTB than among non-TB subjects ($P < 0.001$). Among patients with active TB, more SFCs were produced on BALMCs than the cut-off value of 5. In contrast, in patients with non-TB disease, a median of five SFCs was produced by PBMCs. This confirms that ELISpot performed on mononuclear cells using BAL fluid can distinguish active TB patients from those with non-active TB more accurately than blood ELISpot.

BAL ELISpot showed higher sensitivity and specificity than blood ELISpot, which is similar to the findings of a study conducted in a low-incidence country.[17] The diagnostic OR of a positive BAL ELISpot result being associated with active TB was much higher than with blood ELISpot and TST, which suggests that the diagnostic value of BAL ELISpot is superior for the rapid diagnosis of patients with sputum AFB smear-negative TB.

One of the major limitations of ELISpot performed on PBMCs is that it is unlikely to distinguish active TB from LTBI. There is a high LTBI rate among the general population in China (about 44.5%),[23] and most people may be infected by *M. tuberculosis* even if they have no definite history of TB exposure, which inevitably reduces the specificity of blood ELISpot. However, a moderate specificity of 78.1% was observed for BAL ELISpot in this study, although it was lower than that reported by Dheda et al. (94%) in South Africa.[24] Interestingly, we found that household contact with an active TB patient was significantly related to a positive blood ELISpot result (OR 5.15, $P = 0.023$) and TST (OR 3.55, $P = 0.046$), but not to a positive BAL ELISpot (OR 2.41, $P = 0.112$). Although the blood and BAL ELISpot assays showed good agreement in patients with non-TB disease ($\kappa = 0.53$), 13 subjects were positive on blood ELISpot but negative on BAL ELISpot. Of these 13, 7 (53.8%) had a definite history of household contact with an active TB patient, and were considered to be more likely to be latently infected with *M. tuberculosis*. This suggests that ELISpot responses in mononuclear cells from BAL can distinguish active TB patients from LTBI more precisely than responses with PBMC.

We confirm previous findings[17,18] that patients with a previous history of TB without current reactivation were significantly more likely to have a positive blood ELISpot result (OR 3.10, $P = 0.046$) and TST (OR 6.51, $P = 0.002$), but not a BAL ELISpot (OR 2.05, $P = 0.141$). As no patients with non-TB disease developed TB during follow-up, we can speculate that the previous history of TB may have caused the false-positive results.

This study had several limitations. First, the sample size was small, which may have led to inaccurate sensitivity and specificity for BAL ELISpot. Second, as the study setting was monocentric, the findings are not representative of situations in other areas. Third, as HIV-infected patients were not enrolled, the diagnostic value of BAL ELISpot in those patients cannot be assessed. Finally, it would be highly unrealistic to expect bronchoscopy and ELISpot facilities to be available at all high TB burden settings for routine use in smear-negative patients. Our findings are therefore most relevant to research settings and referral/tertiary centres that offer such services.

CONCLUSIONS

The ELISpot assay on BAL fluid showed high sensitivity, PPV and NPV in the diagnosis of active TB. Moreover, the diagnostic validity of BAL ELISpot was

higher than that of blood ELISpot and the TST, and was not affected by previous history of TB and LTBI. BAL ELISpot thus appears to be a more rapid and sensitive supplementary test than blood ELISpot for the diagnosis of active TB patients with negative sputum smear results in high-prevalence developing countries with access to bronchoscopy and ELISpot assay. However, the utility of the test was limited by its moderate specificity.

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RÉSUMÉ

OBJECTIF : Comparer la validité en matière de diagnostic de l'ELISpot sur le sang et sur le lavage bronchoalvéolaire (BAL) et du test cutané tuberculinique (TST) chez les patients atteints d'une tuberculose (TB) à bacilloscopie négative des frottis dans un pays à haute prévalence de TB.

SCHÉMA : Dans une étude prospective basée sur l'hôpital, on a testé simultanément 107 patients suspects de TB par l'ELISpot sur le sang, l'ELISpot sur le BAL et le TST.

RÉSULTATS : Le diagnostic de TB pulmonaire active a été porté chez 36 des 102 patients (35,3%) et un diagnostic autre que la TB chez 66 patients (64,7%). La sensibilité et la spécificité de l'ELISpot sur les cellules mononucléaires du BAL ont été respectivement de 94,4% (IC95% 81,9–98,5) et de 78,1% (IC95% 66,6–86,5). La spécificité de l'ELISpot sur le BAL est significativement plus élevée que celle de l'ELISpot sur le sang ($P = 0,011$). Par comparaison avec l'ELISpot sur le sang et le TST, l'ELISpot sur le BAL n'est pas significativement influencé par les antécédents de TB (OR 2,05 ; $P > 0,05$), ni par un contact dans le ménage avec un patient atteint de TB active (OR 2,41 ; $P > 0,05$).

CONCLUSION : L'ELISpot sur le BAL s'avère un test complémentaire rapide et plus sensible que l'ELISpot sur le sang pour le diagnostic d'une TB active chez les patients dont les résultats des frottis de crachats sont négatifs dans le contexte d'un pays en développement où la prévalence de la TB est élevée et où la bronchoscopie et le test ELISpot sont disponibles. Toutefois, l'utilité de ce test est limitée par sa spécificité, qui est modérée.

RESUMEN

OBJETIVO: Comparar la validez diagnóstica de las pruebas ELISpot en la sangre y el lavado broncoalveolar (BAL) y la prueba cutánea de la tuberculina (TST) en pacientes con tuberculosis (TB) pulmonar y baciloscopia negativa del esputo, en un país con alta prevalencia de TB.

MÉTODO: Se llevó a cabo un estudio hospitalario en el cual participaron 107 pacientes con presunción diagnóstica de TB. Se practicó a los pacientes en forma simultánea la prueba ELISpot en sangre y en el BAL y la TST.

RESULTADOS: Se diagnosticó TB pulmonar en 36 de los 102 pacientes (35,3%) y en los 66 restantes (64,7%) el diagnóstico fue diferente de TB. La prueba ELISpot exhibió una sensibilidad de 94,4% (IC95% 81,9–98,5) y una especificidad de 78,1% (IC95% 66,6–86,5) en las células mononucleares del BAL. La especificidad de esta prueba fue significativamente superior en las muestras de BAL que en las muestras sanguíneas ($P = 0,011$). En comparación con la prueba ELISpot en sangre y la TST, el antecedente de TB no influyó de manera significativa sobre los resultados de la prueba ELISpot del BAL (OR 2,05; $P > 0,05$), tampoco se modificaron con la presencia de contactos domiciliarios con diagnóstico de TB activa (OR 2,41; $P > 0,05$).

CONCLUSIÓN: La prueba ELISpot del BAL aparece como un método complementario rápido y sensible, mejor que la prueba ELISpot en sangre, en el diagnóstico de los pacientes con TB activa y baciloscopia negativa del esputo, en un país en desarrollo con alta prevalencia de la enfermedad y donde se puede practicar la fibroscopia y la prueba ELISpot. La utilidad de la prueba se ve limitada, no obstante por una especificidad moderada.