MAJOR ARTICLE

Sensitivity, Specificity, and Safety of a Novel ESAT6-CFP10 Skin Test for Tuberculosis Infection in China: 2 Randomized, Self-Controlled, Parallel-Group Phase 2b Trials

Miao Xu,[1,a] Wei Lu,[2,a] Tao Li,[3] Jingxin Li,[2] Weixin Du,[1] Qi Wu,[4] Qiao Liu,[2] Baodong Yuan,[5] Jinbiao Lu,[1] Xiaoyan Ding,[2] Feng Li,[3] Min Liu,[6] Baowen Chen,[1] Jiang Pu,[7] Rongping Zhang,[6] Xiuhong Xi,[3] Rongguang Zhou,[6] Zaoxian Mei,[4] Ronghui Du,[5] Lifeng Tao,[7] Leonardo Martinez,[8] Shuihua Lu,[3,a] Guozhi Wang,[1,a] and Fengcai Zhu[2]

[1]National Institutes for Food and Drug Control, Beijing, PR China; [2]Center for Disease Control and Prevention of Jiangsu Province, Nanjing, Jiangsu Province, PR China; [3]Shanghai Public Health Clinical Center, Fudan University, Shanghai, PR China; [4]Tianjin Haihe Hospital, Tianjin University, Tianjin, PR China; [5]Wuhan Pulmonary Hospital, Wuhan, Hubei Province, PR China; [6]Center for Disease Control and Prevention of Jurong city, Zhenjiang, Jiangsu Province, PR China; [7]Anhui Zhifei Longcom Biopharmaceutical Co, Ltd, Hefei, Anhui Province, PR China; and [8]Boston University, School of Public Health, Department of Epidemiology, Boston, Massachusetts, USA

Background. Diagnostics to identify tuberculosis infection are limited. We aimed to assess the diagnostic accuracy and safety of ESAT6-CFP10 (EC) skin test for tuberculosis infection in Chinese adults.

Methods. We conducted 2 randomized, parallel-group clinical trials in healthy participants and tuberculosis patients. All participants were tested with the T-SPOT.TB test, then received an EC skin test and tuberculin skin test (TST). The diameter of skin indurations and/or redness at injection sites were measured at different time periods. A bacillus Calmette Guerin (BCG) model was established to assess the diagnosis of tuberculosis infection using an EC skin test.

Results. In total, 777 healthy participants and 96 tuberculosis patients were allocated to receive EC skin test at 1.0 µg/0.1 mL or 0.5 µg/0.1 mL. The area under the curve was 0.95 (95% confidence interval [CI], .91–.97) for the EC skin test at 1.0 µg/0.1 mL at 24–72 hours. Compared with the T-SPOT.TB test, the EC skin test demonstrated similar sensitivity (87.5, 95% CI, 77.8–97.2 vs 86.5, 95% CI, 79.5–93.4) and specificity (98.9, 95% CI, 96.0–99.9 vs 96.1, 95% CI, 93.5–97.8). Among BCG vaccinated participants, the EC skin test had high consistency with the T-SPOT.TB test (96.3, 95% CI, 92.0–100.0). No serious adverse events related to the EC skin test were observed.

Conclusions. The EC skin test demonstrated both high specificity and sensitivity at a dose of 1.0 µg/0.1 mL, comparable to the T-SPOT.TB test. The diagnostic accuracy of the EC skin test was not impacted by BCG vaccination.

Clinical Trials Registration. NCT02389322 and NCT02336542.

Keywords. tuberculosis; ESAT6-CFP10; tuberculosis infection; diagnostics.

Approximately one-quarter of the world's population are infected with *Mycobacterium tuberculosis*, and more than 10 million people develop tuberculosis each year [1, 2]. The lifetime risk of developing tuberculosis for persons with tuberculosis infection is estimated to be 5%–10% [3]. In order to achieve the World Health Organization End TB goal by 2035, dealing with the reservoir of tuberculosis infection is essential as it substantially adds to the global tuberculosis burden [2].

Currently, the availability of direct detection methods for tuberculosis infection is limited. The tuberculin skin test (TST) and interferon-gamma release assays (IGRAs) are the most widely applied diagnostics for tuberculosis infection [4, 5]. However, both TST and IGRAs have limitations. The TST is a simple and low-cost test that uses tuberculin purified protein derivative with high sensitivity (approximately 75%–90%) but poor specificity [6]. Previous bacillus Calmette Guerin (BCG) vaccination or environmental exposure to nontuberculous mycobacteria can result in false-positive TST results [7, 8]. IGRAs, including QuantiFERON-TB Gold In-Tube and T-SPOT.TB tests, are blood-based diagnostics on peptides covering tuberculosis-specific and BCG-deleted antigens with a specificity of 98%–100% [9]. Although IGRAs are less likely to be impacted by prior BCG vaccination or natural exposure [10, 11], the test is expensive and requires a well-established laboratory. Therefore, in countries with routine BCG immunization or with a high burden of tuberculosis, new diagnostic methods with high specificity and low cost are needed.

We conducted 2 randomized diagnostic trials to assess the sensitivity, specificity, and safety of a novel ESAT6-CFP10 (EC) skin test for tuberculosis infection in Chinese adults. Compared with the QuantiFERON-TB Gold In-Tube and T-SPOT.TB tests, the EC skin test is performed by intradermal injection of recombinant ESAT-6 and CFP-10 antigens. We also aimed to establish a diagnostic cutoff point for this test and determine an optimal dose for future studies.

METHODS

Study Design and Participants

We conducted 2 randomized, parallel-group clinical trials that involved healthy participants in China to evaluate the sensitivity, specificity, and safety of a novel EC skin test for tuberculosis infection and to assess the proper dose for future clinical trials.

Trial A was carried out at the Jiangsu Center for Disease Control and Prevention, Jiangsu province. Generally healthy participants were enrolled if they were aged between 18 and 65 years with normal chest X-ray results and no tuberculosis history. Only participants with negative results on the T-SPOT.TB test, TST, and EC skin test were involved in the BCG vaccination model. Participants were randomly assigned to BCG and EC skin test (0.5 μg/0.1 mL), BCG and EC skin test (1.0 μg/0.1 mL), placebo and EC skin test (0.5 μg/0.1 mL), or placebo and EC skin test (1.0 μg/0.1 mL). The EC skin test and TST were performed 12 weeks after BCG or placebo vaccination, and the T-SPOT.TB test was performed before the skin tests.

Trial B was carried out at the Shanghai Public Health Clinical Center, Tianjin Haihe Hospital, and Wuhan Tuberculosis Prevention and Control Institute. Tuberculosis patients were enrolled according to China's national diagnostic criteria for pulmonary tuberculosis, which was described previously in the phase 2a clinical trial article [12]. In addition, a group of nontuberculosis patients with other pulmonary diseases but no active tuberculosis were recruited (all pulmonary comorbidities are included in the Supplementary Materials). Enrolled patients were randomly assigned to the EC skin test at 0.5 μg/0.1 mL or at 1.0 μg/0.1 mL. Blood samples of patients in each group were collected for T-SPOT.TB test, and then the EC skin test and TST were performed. Tuberculosis patients were classified into bacteriological positive or negative subgroups on the basis of sputum smear microscopy tests or sputum culture (Supplementary Materials). Tuberculosis treatment history was collected for each tuberculosis patient through medical records. A full list of inclusion and exclusion criteria is provided in the Supplementary Materials.

We obtained approvals from institutional review boards of Jiangsu Provincial Center of Disease Control and Prevention and Shanghai Public Health Clinical Center. Written informed consent was obtained from all participants. Trials were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

Randomization and Masking

Healthy participants were enrolled and randomly assigned in a ratio of 1:1 to be screened with the EC skin test at 0.5 μg/0.1 mL or 1.0 μg/0.1 mL, along with the T-SPOT.TB test and TST. We randomly assigned healthy participants with 3 negative test results into 4 groups to receive BCG vaccination or placebo and EC skin tests. Tuberculosis and nontuberculosis patients were randomly allocated in a ratio of 1:1 to receive the EC skin test at 0.5 μg/0.1 mL or 1.0 μg/0.1 mL.

BCG vaccination and placebo were identical in appearance. EC skin tests administered at different doses were also identical in appearance. A randomization code number on each dose was the only identifier. Investigators and participants were masked to treatment allocations; therefore, both of these trials were double-blinded. Individuals involved in generation of the randomization list were not allowed to participate in any other trial activities. We did not record BCG vaccination history from healthy participants due to recall bias. We observed and recorded participants' BCG scars.

Procedures

The EC antigen is a recombinant reagent of the ESAT-6 and CFP-10 tests, developed by Zhifei Longcom Biologic Pharmacy Company, China.

Blood samples for the T-SPOT.TB test were drawn in participants before administrating the EC skin test or TST. Participants then received the EC skin test on the volar surface of one forearm and TST on the other forearm as a self-control. All participants were observed for at least 30 minutes for acute adverse reactions after receiving each test. Vital signs were measured before and after receiving the skin tests. After leaving the clinic, participants were instructed to record adverse reactions during the following 72 hours. Digital photographs of injection sites were taken and the largest transverse diameter as well as the longitudinal diameter of skin indurations and/or redness at injection sites were measured by investigators at 24, 48, and 72 hours. Since participants received the EC skin test and TST on the same day, all systematic reactions following tests were recorded as related to the EC skin test.

The TST was manufactured by Beijing Gaoke Life and Technology, China, and administrated following the national standard guideline [13]. A positive TST result was defined as an induration reaction ≥5mm [12].

The T-SPOT.TB test was carried out using a commercial kit manufactured by Oxford Immunotec, Ltd, UK. Spots were counted with a magnifying glass and expressed as the number of spots per million peripheral blood mononuclear cells [14]. Two independent observers confirmed spot counts and

determined the results as positive, negative, or indeterminate without awareness of treatment allocation.

Statistical Analysis

Sample size calculations were conducted using PASS software (version 8; Supplementary Materials). The average diameter of the skin reactions (either redness or induration) for the EC skin test at different time points was calculated. We performed a binary classification, where the outcome variable had 2 possible values: negative or positive. The raw prediction of consistency from the model is defined as "(true positives + true negatives)/sample size." The areas under the curve (AUCs) of different EC skin test dose groups were estimated using receiver operating characteristic (ROC) curves to show the capability of distinguishing tuberculosis patients from healthy participants. The optimal diagnostic indicator was determined according to the highest estimated AUC. Cutoff values were chosen by comparing ROC curves at distinct thresholds. We calculated sensitivity, specificity, overall diagnostic accuracy, and positive and negative predictive values of the EC skin test, TST, and T-SPOT.TB test [15]. Safety end points were adverse reactions and serious adverse events after injections in each group. All systemic and local adverse events were observed and recorded. Local skin reactions such as rash, pain, and itch as well as adverse events such as anaphylactic shock, local tissue ulceration caused by a strong positive reaction, local necrosis and liquefaction, systemic allergic rash, systemic urticaria, and allergic purpura were observed at every visit.

The primary analysis was conducted in the entire population, including all participants who received the EC skin test with at least 1 observation available. The Cochran-Mantel-Haenszel χ^2 test or paired χ^2 test was used to compare categorical or paired categorical variables between groups. The t test or paired t test was used to analyze continuous data, when appropriate. We considered P values $\leq$ .05 as statistically significant. All statistical analyses were performed using SAS 9.3 software (SAS Institute, Inc, Cary, NC).

RESULTS

Trial Population

We enrolled 1044 healthy participants, and 1035 (99%) were consented for screening in March 2015. A total of 777 (75.1%) participants were randomly assigned to the EC dose groups of 0.5 µg/0.1 mL (n = 381) or 1.0 µg/0.1 mL (n = 396). Among them, 116 were positive and 661 were negative with the T-SPOT.TB test, 97 were positive and 680 were negative with the EC skin test, and 420 were positive and 357 were negative with the TST. A total of 223 (28.7%) participants had negative results on the EC skin test, T-SPOT.TB test, and TST and were assigned to the BCG vaccination group (Figure 1). There were 169 healthy participants with 3 negative test results who

were randomly allocated into 4 groups (n = 41, 42, 42, and 44, respectively). Participants were allocated to receive BCG or placebo vaccination and the EC skin test at 0.5 µg/0.1 mL or 1.0 µg/0.1 mL 12 weeks after the vaccination along with the TST and T-SPOT.TB test. In November 2014, we enrolled 96 tuberculosis patients and 95 nontuberculosis patients with pulmonary diseases and equally assigned them to receive the EC skin test at 0.5 µg/0.1 mL or 1.0 µg/0.1 mL, respectively (Figure 1). Demographic characteristics of recruited participants were comparable between the different groups (Table 1).

EC Skin Test Cut-Point Analysis

For T-SPOT.TB⁻/TST, healthy participants and tuberculosis patients were used to assess the diagnostic performance of the EC skin test. The reaction of redness or induration at the injection site after receipt of the EC skin test at 0.5 µg/0.1 mL or 1.0 µg/0.1 mL was observed in 6 participants. In tuberculosis patients, the median diameter of redness or induration at the injection site at 24, 48, and 72 hours following administration of the EC skin test was 22.5, 35.8, and 25.1 mm in the 0.5 µg/0.1 mL group and 23.6, 34.0, and 23.3 mm in the 1.0 µg/0.1 mL group. AUCs were higher at all measured time points for the EC skin test at a dose of 1.0 µg/0.1 mL compared with 0.5 µg/0.1 mL (Figure 2). We suggest that 1.0 µg/0.1 mL is the optimal dose based on these findings; however, this difference was not statistically distinct; therefore, both doses may be acceptable. The highest AUC was 0.95 (95% confidence interval [CI], .91–.97) for the EC skin test at 1.0 µg/0.1 mL at 24–72 hours using redness or induration as indicators. The highest diagnostic values were achieved at a cutoff value of >3.5 mm (Supplementary Materials). The final positive determination criterion of the EC skin test was 1.0 µg/0.1 mL at 24–72 hours using an induration cutoff of ≥5 mm redness or induration for practical implementation purposes and was used in all subsequent analyses.

Sensitivity and Specificity of the EC Skin Test

Among tuberculosis patients, the EC skin test at a dose of 1.0 µg/0.1 mL had a sensitivity of 87.5 (95% CI, 77.8–97.2), while the sensitivity of the T-SPOT.TB test was 86.5 (95% CI, 79.5–93.4). The sensitivity of the TST at 5 mm and 10 mm cutoffs was 86.5 (95% CI, 79.5–93.4) and 82.3 (95% CI, 74.5–90.1; Table 2). High sensitivity was observed when the population was restricted to bacteriologically confirmed tuberculosis (89.7, 95% CI, 77.9–100.0) or tuberculosis cases without bacteriological evidence (84.2, 95% CI, 66.2–100.0). The sensitivity among new and previously treated tuberculosis patients was 90.3 (95% CI, 79.3–100.0) and 82.4 (95% CI, 62.1–100.0), respectively. In TST healthy participants, the EC skin test at 1.0 µg/0.1 mL showed a specificity of 98.4 (95% CI, 95.4–99.7). Among T-SPOT.TB⁻ and T-SPOT.TB⁻/TST healthy participants, specificity was 95.5 (95% CI, 92.7–97.4) and 98.9 (95% CI, 96.0–99.9; Table 2). The EC skin test showed diagnostic performance similar to that
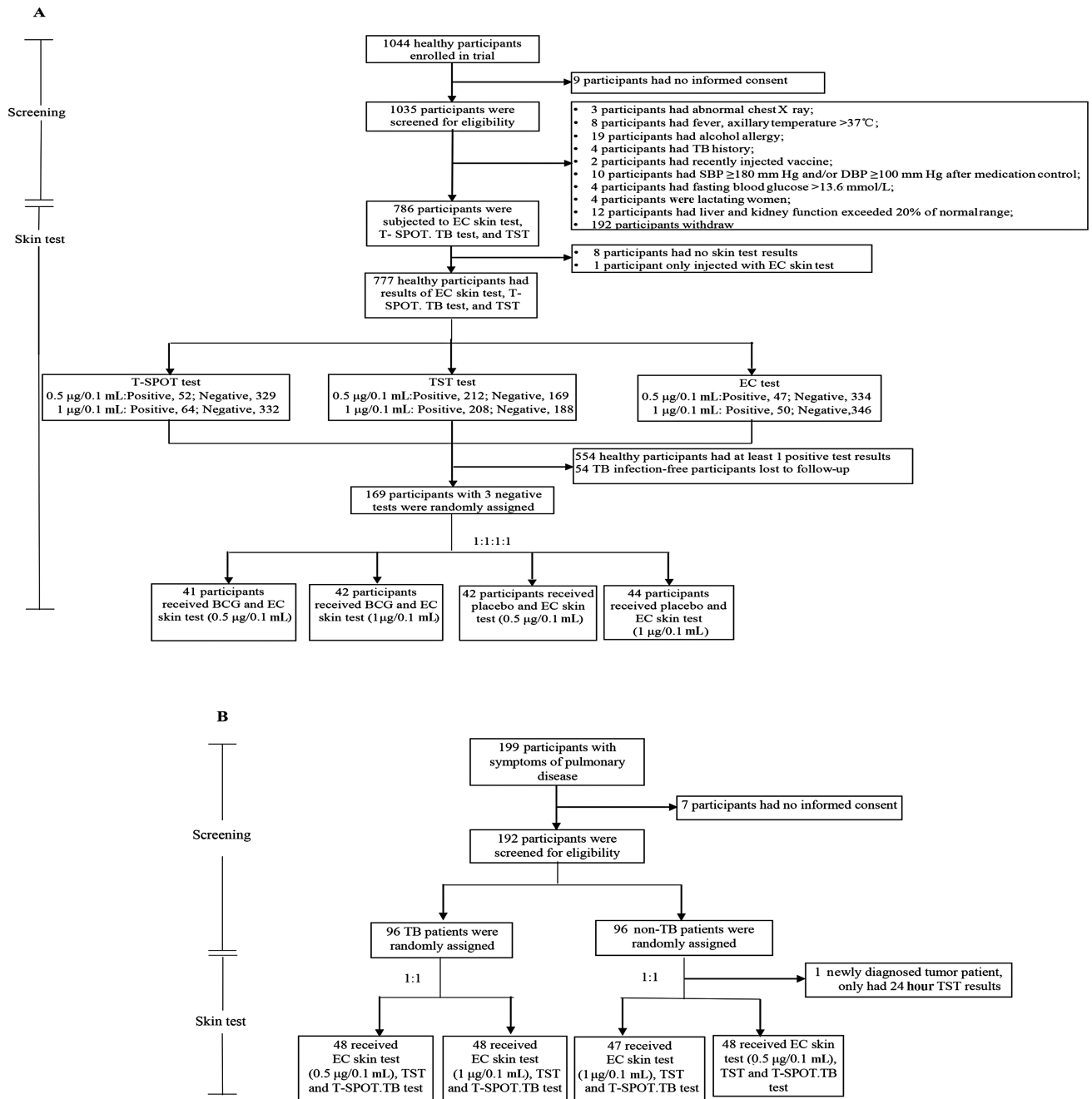
Figure 1. Study profile of the pooled participants from healthy participants (*A*) and patients participants (*B*). Abbreviations: BCG, bacillus Calmette-Guerin; DBP, diastolic blood pressure; EC, ESAT6-CFP10; SBP, systolic blood pressure; TB, tuberculosis; TST, tuberculin skin test.

of the T-SPOT.TB test in active tuberculosis (Supplementary Materials).

Diagnostic Agreement of the EC Skin Test, TST, and T-SPOT.TB Test

Diagnostic agreement between the EC skin test at 1.0 μg/0.1 mL and the T-SPOT.TB was 85.4% (95% CI, 75.1–95.8) in tuberculosis patients and 88.9% (95% CI, 85.8–92.0) in general healthy participants. Diagnostic agreement between the EC skin test at 1.0 μg/0.1 mL and

the TST (5 mm) was 81.3% (95% CI, 69.8–92.7) in tuberculosis patients and 58.6% (95% CI, 53.7–63.5) in healthy participants. Diagnostic agreement between the EC skin test at 1.0 μg/0.1 mL and the TST (in 10 mm cutoff) was 77.1% (95% CI, 64.8–89.4) in tuberculosis patients and 70.5% (95% CI, 65.9–75.0) in healthy participants. Diagnostic agreement of the EC skin test at 1.0 μg/0.1 mL and the TST at either cutoff ranged from 77.1% to 81.3% for tuberculosis patients (Table 3).

Table 1. Demographic Characteristics of the Recruited Tuberculosis Patients, Nontuberculosis patients, and Healthy Participants

Demographic Characteristic	General Healthy Participants		Tuberculin Skin Test–Negative Participants		T-SPOT.TB–Negative Participants		Tuberculosis Patients		Nontuberculosis Patients With Other Pulmonary Diseases	
	0.5 µg/0.1 mL	1.0 µg/0.1 mL	0.5 µg/0.1 mL	1.0 µg/0.1 mL	0.5 µg/0.1 mL	1.0 µg/0.1 mL	0.5 µg/0.1 mL	1.0 µg/0.1 mL	0.5 µg/0.1 mL	1.0 µg/0.1 mL
N	381	396	169	188	329	332	48	48	48	47
Sex										
Male	189 (49.6)	191 (48.2)	67 (39.6)	81 (43.1)	158 (48.0)	148 (44.6)	29 (60.4)	38 (79.2)	24 (50.0)	32 (68.1)
Female	192 (50.4)	205 (51.8)	102 (60.4)	107 (56.9)	171 (52.0)	184 (55.4)	19 (39.6)	10 (20.8)	24 (50.0)	15 (31.9)
Mean age, years (SD)	45.8 ± 9.4	45.6 ± 9.0	46.3 ± 10.0	45.8 ± 9.6	45.3 ± 9.4	45.1 ± 8.9	37.2 ± 14.2	38.8 ± 14.2	47.5 ± 13.9	51.0 ± 12.7
Mean height (SD), cm	163.9 ± 7.3	162.6 ± 7.4	162.8 ± 7.5	162.0 ± 7.6	164.0 ± 7.4	162.5 ± 7.3	167.2 ± 8.2	169.5 ± 7.2	164.5 ± 78	1672 ± 8.2
Mean weight (SD), kg	66.8 ± 10.5	65.0 ± 9.9	64.0 ± 10.6	62.7 ± 9.2	66.7 ± 10.6	64.4 ± 9.5	55.9 ± 11.3	57.3 ± 10.8	61.2 ± 10.2	65.5 ± 11.6
Ethnic group										
Han	379 (99.5)	389 (98.2)	167 (98.8)	184 (97.9)	328 (99.7)	327 (98.5)	46 (95.8)	48 (100.0)	47 (97.9)	47 (100.0)
Minority	2 (0.5)	7 (1.8)	2 (1.2)	4 (2.1)	1 (0.3)	5 (1.5)	2 (4.2)	0 (0.0)	1 (2.1)	0 (0.0)
Chest X ray										
Normal	381 (100.0)	396 (100.0)	169 (100.0)	188 (100.0)	329 (100.0)	332 (100.0)	1 (2.1)	1 (2.1)	1 (2.1)	3 (6.4)
Abnormal	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	47 (97.9)	47 (97.9)	47 (97.9)	44 (93.6)
Bacillus Calmette Guerin scar										
No	209 (54.9)	223 (56.3)	110 (65.1)	111 (59.0)	177 (53.8)	187 (56.3)	-	-	-	-
Yes	172 (45.1)	173 (43.7)	59 (34.9)	77 (41.0)	152 (46.2)	145 (43.7)	-	-	-	-
Treatment history										
New patients	-	-	-	-	-	-	33 (68.8)	31 (64.6)	-	-
Previously treated patients	-	-	-	-	-	-	15 (31.3)	17 (35.4)	-	-
Bacteriological examination										
Positive	-	-	-	-	-	-	26 (54.2)	29 (60.4)	-	-
Negative	-	-	-	-	-	-	22 (45.8)	19 (39.6)	-	-

Abbreviation: SD, standard deviation.

A The estimated area under the curve of the diagnosis of EC skin-test at different time by redness or induration
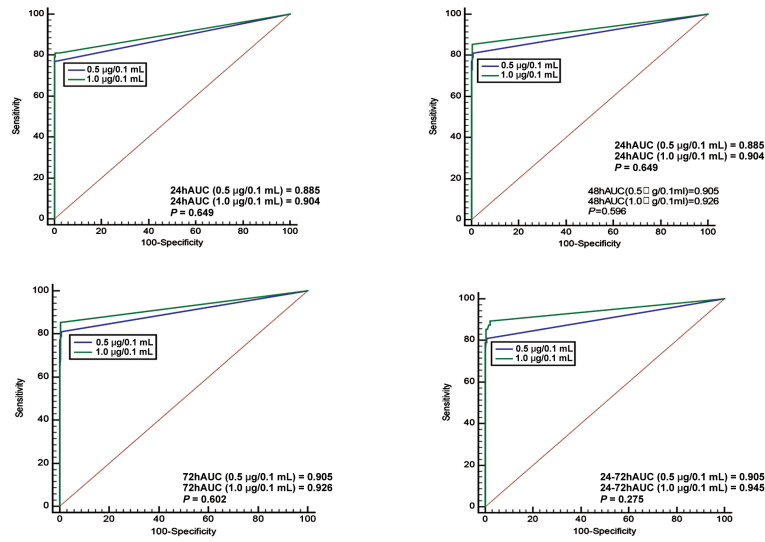
B The estimated area under the curve of the diagnosis of EC skin-test at different time by redness
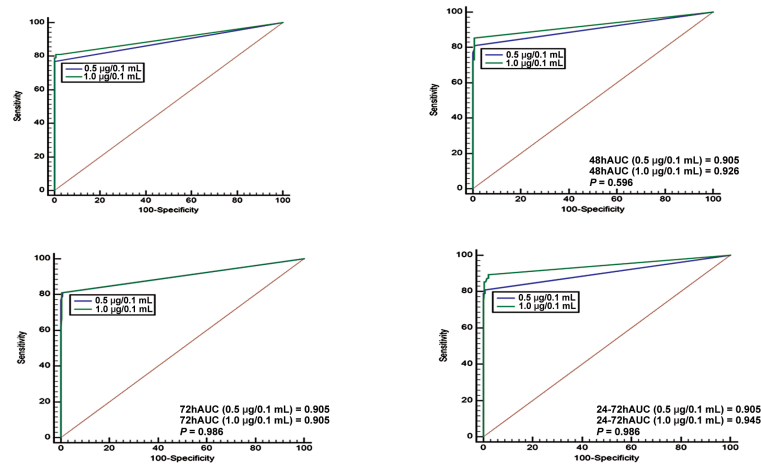
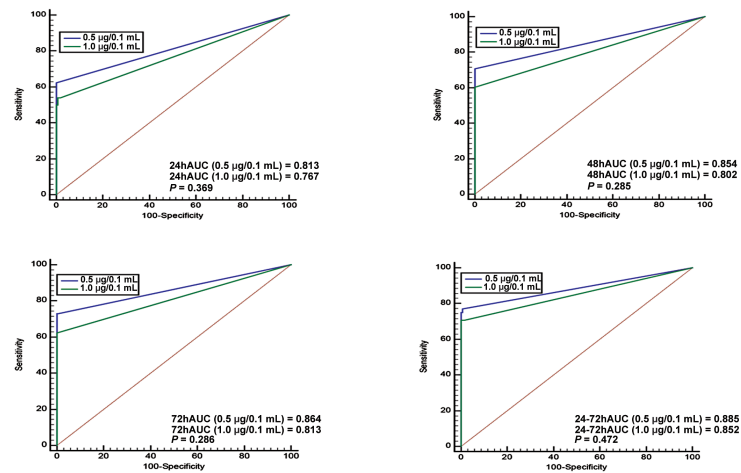**C** The estimated area under the curve of the diagnosis of EC skin-test at different time by induration

Figure 2. Estimated AUC for diagnosis with the ESAT6-CFP10 skin test with different indicators at different doses and time points. Abbreviations: AUC, area under the curve; EC, ESAT6-CFP10.

Table 2. Diagnostic Performance of ESAT6-CFP10 Skin Test, T-SPOT.TB Test, and Tuberculin Skin Test

Test Performance	EC Skin Test, 0.5 µg/0.1 mL n/N	Estimate (95% CI)	EC Skin Test, 1.0 µg/0.1 mL n/N	Estimate (95% CI)	T-SPOT.TB Test n/N	Estimate (95% CI)	TST (≥5 mm) n/N	Estimate (95% CI)	TST (≥10 mm) n/N	Estimate (95% CI)
Sensitivity, tuberculosis infection										
Tuberculosis patients	39/48	81.3 (69.8–92.7)	42/48	87.5 (77.8–97.2)	83/96	86.5 (79.5–93.4)	83/96	86.5 (79.5–93.4)	79/96	82.3 (74.5–90.1)
Bacteriologically confirmed tuberculosis	24/26	92.3 (81.3–100)	26/29	89.7 (77.9–100)	49/55	89.1 (80.6–97.6)	48/55	87.3 (78.2–96.4)	45/55	81.8 (71.3–92.3)
Not bacteriologically confirmed tuberculosis	15/22	68.2 (47.0–89.3)	16/19	84.2 (66.2–100)	34/41	82.9 (70.9–95.0)	35/41	85.4 (74.1–96.7)	34/41	82.9 (70.9–95.0)
New tuberculosis patients	25/33	75.8 (60.3–91.2)	28/31	90.3 (79.3–100)	57/64	89.1 (81.2–96.9)	54/64	84.4 (75.2–93.5)	52/64	81.3 (71.4–91.1)
Previously treated patients	14/15	93.3 (79.0–100)	14/17	82.4 (62.1–100)	26/32	81.3 (67.0–95.5)	29/32	90.6 (79.9–100)	27/32	84.4 (71.1–97.7)
Specificity, tuberculosis infection										
TST negative participants	166/169	98.2 (94.9–99.6)	185/188	98.4 (95.4–99.7)	343/357	96.1 (93.5–97.8)	-	-	-	-
T-SPOT.TB negative participants	315/329	95.7 (93.0–97.7)	317/332	95.5 (92.7–97.4)	-	-	343/661	51.9 (48.0–55.8)	464/661	70.2 (66.5–73.7)
TST and T-SPOT.TB negative	165/166	99.4 (96.7–100)	175/177	98.9 (96.0–99.9)	-	-	-	-	-	-

Abbreviations: CI, confidence interval; EC, ESAT6-CFP10; TST, tuberculin skin test.

Table 3. Agreement of the Diagnostic Results of ESAT6-CFP10 Skin Test at 1.0 µg/0.1 mL Compared With the Tuberculin Skin Test and T-SPOT.TB Test

Population	EC Skin Test	T-SPOT.TB Test Positive n	Negative n	Consistency, % (95% CI)	TST (≥5 mm) Positive n	Negative n	Consistency, % (95% CI)	TST (≥10 mm) Positive n	Negative n	Consistency, % (95% CI)
Tuberculosis patients	Positive	37	5	85.4 (75.1–95.8)	37	5	81.3 (69.8–92.7)	35	7	77.1 (64.8–89.4)
	Negative	2	4		4	2		4	2	
Bacteriologically confirmed tuberculosis	Positive	22	4	79.3 (63.6–95.0)	23	3	82.8 (68.1–97.4)	21	5	75.9 (59.3–92.4)
	Negative	2	1		2	1		2	1	
Not bacteriologically confirmed tuberculosis	Positive	15	1	94.7 (83.7–100.0)	14	2	78.9 (58.8–99.1)	14	2	78.9 (58.8–99.1)
	Negative	0	3		2	1		2	1	
Nontuberculosis patients	Positive	13	8	76.6 (64.0–89.2)	18	3	68.1 (54.3–81.9)	17	4	72.3 (59.1–85.6)
	Negative	3	23		12	14		9	17	
General healthy participants	Positive	35	15	88.9 (85.8–92.0)	47	3	58.6 (53.7–63.5)	40	10	70.5 (65.9–75.0)
	Negative	29	317		161	185		107	239	
TST-negative participants[a]	Positive	1	2	93.6 (90.1–97.1)	-	-	-	-	-	-
	Negative	10	175		-	-		-	-	
T-SPOT.TB-negative participants[a]	Positive	-	-	-	13	2	56.6 (51.3–62.0)	10	5	71.1 (66.2–76.0)
	Negative	-	-		142	175		91	226	

We performed a binary classification in which the outcome variable had 2 possible values: negative or positive. The raw prediction of consistency from the model is defined as "(true positives + true negatives)/sample size."

Abbreviations: CI, confidence interval; EC, ESAT6-CFP10; TST, tuberculin skin test.

[a]We calculated specificity by comparing participants with TST- and T-SPOT.TB-negative results. Consequently, participants with TST and T-SPOT.TB positive results were not included in this table.

Diagnostic agreement between the EC skin test at 0.5 μg/0.1 mL and the T-SPOT.TB was 81.3% (95% CI, 69.8–92.7) in tuberculosis patients and 91.3% (95% CI, 88.5–94.2) in general healthy participants. However, in healthy participants 12 weeks after BCG vaccination, the EC skin test showed a significant superiority over the TST; the TST had high false positivity in BCG recipients. Consistency between the EC skin test and the T-SPOT.TB test was 96.3% (95% CI, 92.0–100.0) among healthy participants with negative results on all 3 tests. Diagnostic agreement with the TST was much lower (39.5%, 95% CI, 29.0–50.1 and 52.3%, 95% CI, 41.6–63.1) with the T-SPOT.TB test. In the BCG vaccination group, the EC skin test showed high consistency (97.4%, 95% CI, 92.2–100.0) with the T-SPOT.TB test but low consistency with the TST (16.7%, 95% CI, 4.9–28.4; Supplementary Materials).

Safety of the EC Skin Test

In total, 333 (27.8%) adverse events were related to the EC skin test, while 198 (16.5%) were related to the TST ($P < .001$). For both the EC skin test and the TST, the most common adverse reaction was pruritus at the injection site followed by pain at the injection site (Figure 3). The overall incidence of adverse reactions between different EC skin test dose groups was comparable with 26.8% and 28.7% for 0.5 μg/0.1 mL and 1.0 μg/0.1 mL doses (Supplementary Materials). Pruritus at the injection site occurred more frequently in the 1.0 μg/0.1 mL group compared with the 0.5 μg/0.1 mL group (13.5% vs 8.4%; $P = .005$). Most adverse reactions were mild and self-limiting, arising during the first 24 hours after injection and lasting less than 48 hours. Only 1 serious adverse event was reported. A fatal myocardial infarction episode occurred in a nontuberculosis patient with lung disease 1 day after receiving the 0.5 μg/0.1 mL EC skin test. Considering his medical history at enrollment, this event was determined to be not related to study procedures.

DISCUSSION

The antigens used in the traditional TST are not unique to *M. tuberculosis* and can be found in BCG and environmental nontuberculous mycobacteria [16, 17]. Therefore, TST testing often results in high false positivity in BCG vaccinated populations, leading to unnecessary antibiotic treatment and causing risk of drug toxicity [18-20]. The T-SPOT.TB test is considered to have superior specificity compared with the TST [21]. However, due to high costs and laboratory requirements to process the T-SPOT.TB test, the World Health Organization issued a "negative" policy statement with a caution against replacing the TST with the T-SPOT.TB test, especially in low- and middle-income settings [22]. Thus, there is an urgent need for efficient and reliable new tools to improve the ability to diagnose tuberculosis in resource-poor settings. We conducted these trials to assess the efficacy and proper dose of a novel EC skin test compared with the reference standard (TST and T-SPOT.TB test). Our results indicate that the EC skin test at a dose of 1.0 μg/0.1 mL has satisfactory diagnostic accuracy and good agreement with the T-SPOT.TB test. This dose may be optimal for a phase 3 trial, although both doses demonstrated satisfactory results. The EC skin test was unaffected by prior BCG vaccination, which is critical in countries with high levels of BCG vaccination or in areas with high tuberculosis risk.
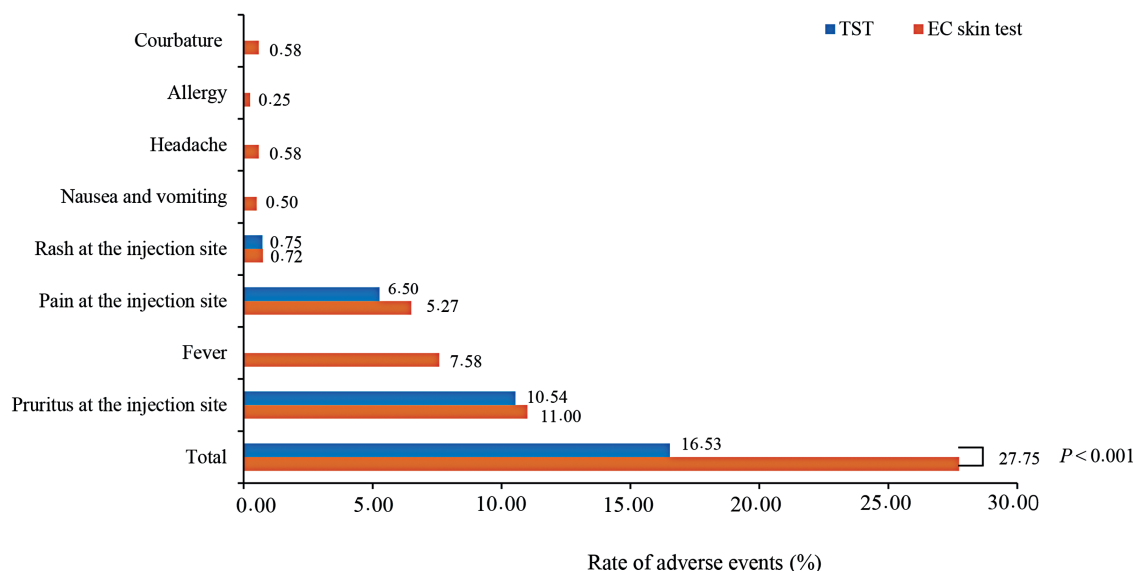

Figure 3. Incidence of adverse reactions at injection sites after receiving the ESAT6-CFP10 skin test and TST. Courbature means muscle aches. Abbreviations: EC, ESAT6-CFP10; TST, tuberculin skin test.

We also noted a moderate diagnostic specificity of the 1.0 μg/0.1 mL EC skin test in nontuberculosis patients with pulmonary diseases. This population was distinguished from tuberculosis patients based on China's currently implemented national diagnostic criteria for pulmonary tuberculosis [23]. However, clinically, it is difficult to determine whether persons had potential tuberculosis infection or not, thus misclassification of nontuberculosis patients with pulmonary diseases may be possible. Nevertheless, the diagnostic consistency of the EC skin test at 1.0 μg/0.1 mL and the T-SPOT.TB test is still considerably high in this cohort, demonstrating a generally congruent specificity and sensitivity. The EC skin test is easy to use programmatically, relatively inexpensive, and convenient for administration in low-income countries.

Generally, the EC skin test was safe in participants from this cohort. Most adverse reactions were mild or moderate, and no related serious adverse reactions were found during follow-up. The incidence of adverse reactions observed in this study was in line with previous phase 1 trials of the EC skin test [24]. Our results suggests that the EC skin test may be a safe and potentially efficient tool for diagnosing tuberculosis infection.

Cost analyses will be important when evaluating novel diagnostic tests for tuberculosis infection. At present, the price of national centralized procurement is 4.68 dollars per person for EC, 50 cents per person for BCG-pure protein derivative (PPD), 2.1 dollars per person for TB-PPD, 31.2 dollars for QuantiFERON-TB Gold (QFT-G) and 46.8 dollars for T-SPOT.TB Tests. The cost of the EC skin test is about one-seventh of the QFT-G and one-tenth of the T-SPOT.TB test. Given the comparable diagnostic value suggested by our study, the EC skin test may be a more efficient method than QFT-G or T-SPOT.TB and more effective than the TST in populations that are BCG-vaccinated. However, diagnostic costs may be distinct between settings.

This study has limitations. First, the lack of a gold standard for diagnosing tuberculosis infection provides difficulties in determining the diagnostic accuracy of both the T-SPOT.TB test and the EC skin test, especially in nontuberculosis patients with pulmonary diseases [25, 26]. Second, the EC skin test was administered only in specific populations. Certain immunocompromised patients, such as persons living with human immunodeficiency virus, autoimmune diseases, silicosis, or cancer, were not included. The diagnostic value of the EC skin test needs to be evaluated in other special populations (eg, those with extrapulmonary tuberculosis, pediatric tuberculosis). In addition, interactions between repeated administration of EC skin test may also need to be evaluated. "Booster" responses from repeat TSTs within 1 year after an initially negative TST have been reported in previous studies [27, 28] and is commonly observed in individuals who have been BCG-vaccinated [29, 30]. In our study, among tuberculosis infection-free and healthy participants, 6 and 2

participants became positive in the 0.5 μg/0.1 mL EC skin test and T-SPOT.TB test group, while 3 and 1 became positive in the 1 μg/0.1 mL EC and T-SPOT.TB test group after receiving vaccination. T-SPOT.TB conversion may suggest a novel exposure or tuberculosis infection. However, repeated skin tests may lead to false-positive results and overestimation of tuberculosis infection [31].

In conclusion, the experimental EC skin test has a good safety profile and high diagnostic accuracy, with the potential to become an efficient tool for the diagnosis of tuberculosis infection. Based on our findings, 1.0 μg/0.1 mL EC was identified as the candidate formulation for a phase 3 trial (ClinicalTrials.gov, NCT02795260, NCT02623556, and NCT03027154 in progress) to further assess the diagnostic accuracy and safety in larger populations.

Supplementary Data

Notes

References

1. World Health Organization. Global tuberculosis report 2019. Geneva, Switzerland: WHO, **2019**.

2. Houben RM, Dodd PJ. The global burden of latent tuberculosis infection: a re-estimation using mathematical modelling. PLoS Med **2016**; 13:e1002152.

3. Gao L, Li X, Liu J, et al; LATENTTB-NSTM Study Team. Incidence of active tuberculosis in individuals with latent tuberculosis infection in rural China: follow-up results of a population-based, multicentre, prospective cohort study. Lancet Infect Dis **2017**; 17:1053-61.

4. Pai M, Denkinger CM, Kik SV, et al. Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. Clin Microbiol Rev **2014**; 27:3-20.

5. Yang H, Kruh-Garcia NA, Dobos KM. Purified protein derivatives of tuberculin—past, present, and future. FEMS Immunol Med Microbiol **2012**; 66:273-80.

6. Lu P, Liu Q, Zhou Y, et al. Predictors of discordant tuberculin skin test and quantiFERON-TB gold in-tube results in Eastern China: a population-based, cohort study. Clin Infect Dis **2021**; 72:2006-15.

7. Trajman A, Steffen R, Menzies D. Interferon-gamma release assays versus tuberculin skin testing for the diagnosis of latent tuberculosis infection: an overview of the evidence. Pulm Med **2013**; 2013:601737.

8. Pai M, Riley LW, Colford Jr JM. Interferon-γ assays in the immunodiagnosis of tuberculosis: a systematic review. Lancet Infect Dis **2004**; 4:761-76.

9. Jung Y, Lyu J, Yoo B, et al. Combined use of a TST and the T-SPOT®. TB assay for latent tuberculosis infection diagnosis before anti-TNF-α treatment. Int J Tuberculosis Lung Dis **2012**; 16:1300-6.

10. Metcalfe JZ, Everett CK, Steingart KR, et al. Interferon-γ release assays for active pulmonary tuberculosis diagnosis in adults in low- and middle-income countries: systematic review and meta-analysis. J Infect Dis **2011**; 204:S1120-9.

11. King TC, Upfal M, Gottlieb A, et al. T-SPOT.TB interferon-γ release assay performance in healthcare worker screening at nineteen U.S. hospitals. Am J Respir Crit Care Med **2015**; 192:367-73.

12. Li F, Xu M, Qin C, et al. Recombinant fusion ESAT6-CFP10 immunogen as a skin test reagent for tuberculosis diagnosis: an open-label, randomized, two-centre phase 2a clinical trial. Clin Microbiol Infect **2016**; 22:889.e9-16.

13. Huebner RE, Schein MF, Bass JB Jr. The tuberculin skin test. Clin Infect Dis **1993**; 17:968-75.

14. Losi M, Bossink A, Codecasa L, et al. Use of a T-cell interferon-γ release assay for the diagnosis of tuberculous pleurisy. Eur Resp J **2007**; 30:1173-9.

15. Akobeng AK. Understanding diagnostic tests 1: sensitivity, specificity and predictive values. Acta Paediatr **2007**; 96:338-41.

16. Brock I, Weldingh K, Lillebaek T, Follmann F, Andersen P. Comparison of tuberculin skin test and new specific blood test in tuberculosis contacts. Am J Resp Critical Care Med **2004**; 170:65-9.

17. Fietta A, Meloni F, Cascina A, et al. Comparison of a whole-blood interferon-gamma assay and tuberculin skin testing in patients with active tuberculosis and individuals at high or low risk of *Mycobacterium tuberculosis* infection. Am J Infect Control **2003**; 31:347-53.

18. Vanhoof J, Landewe S, Van Wijngaerden E, Geusens P. High incidence of hepatotoxicity of isoniazid treatment for tuberculosis chemoprophylaxis in patients with rheumatoid arthritis treated with methotrexate or sulfasalazine and anti-tumour necrosis factor inhibitors. Ann Rheum Dis **2003**; 62:1241-2.

19. Liu Q, Lu P, Leonardo M, et al. Undiagnosed diabetes mellitus and tuberculosis infection: a population-based, observational study from eastern China. Diabetes/Metab Res and Rev **2020**; 36:e3227.

20. Rodger M, Ramsay T, Fergusson D. Diagnostic randomized controlled trials: the final frontier. Trials **2012**; 13:1-7.

21. Moon HW, Hur M. Interferon-gamma release assays for the diagnosis of latent tuberculosis infection: an updated review. Ann Clin Lab Sci **2013**; 43:221-9.

22. World Health Organization. Use of tuberculosis interferon-gamma release assays (IGRAs) in low-and middle-income countries: policy statement. **2011**.

23. Health Commission of China. Diagnostic criteria for pulmonary tuberculosis (WS 288-2017). **2017**.

24. Li F, Xu M, Zhou L, et al. Safety of recombinant fusion protein ESAT6-CFP10 as a skin test reagent for tuberculosis diagnosis: an open-label, randomized, single-center phase I clinical trial. Clin Vaccine Immunol **2016**; 23:767-73.

25. Herrera V, Perry S, Parsonnet J, Banaei N. Clinical application and limitations of interferon-γ release assays for the diagnosis of latent tuberculosis infection. Clin Infect Dis **2011**; 52:1031-7.

26. Kleinert S, Tony HP, Krueger K, et al. Screening for latent tuberculosis infection: performance of tuberculin skin test and interferon-γ release assays under real-life conditions. Ann Rheum Dis **2012**; 71:1791-5.

27. Menzies R, Vissandjee B, Rocher I, St Germain Y. The booster effect in two-step tuberculin testing among young adults in Montreal. Ann Intern Med **1994**; 120:190-8.

28. Hizel K, Maral I, Karakus R, Aktas F. The influence of BCG immunisation on tuberculin reactivity and booster effect in adults in a country with a high prevalence of tuberculosis. Clin Microbiol Infect **2004**; 10:981-4.

29. Menzies D. Interpretation of repeated tuberculin tests. Boosting, conversion, and reversion. Am J Resp Critical Care Med **1998**; 159:15-21.

30. Menzies R, Vissandjee B. Effect of bacille Calmette-Guérin vaccination on tuberculin reactivity. Am Rev Respir Dis **1992**; 145:621-5.

31. Soborg B, Andersen AB, Larsen HK, et al. Detecting a low prevalence of latent tuberculosis among health care workers in Denmark detected by *M. tuberculosis* specific IFN-gamma whole-blood test. Scand J Infect Dis **2007**; 39:554-9.