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High Accuracy of Recombinant Fusion Protein ESAT6-CFP10 Skin Test for the Detection of Tuberculosis Infection: A Phase III, Multi-centered, Double-blind, Hospital-based, Randomized Controlled Trial

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Running title: Phase III trial of EC skin test for TB infection

Highlights

- ECST is a phase III clinical trial.We validated its accuracy for TB infection.
- We identified immune state was associated with the diagnostic accuracy of ECST.
- This study enrich the clinical evidence in related ESAT6/CFP10-based skin tests.

Abstract

Background. The recombinant fusion protein ESAT6-CPF10 (EC) was developed as a novel skin-test reagent to detect Mycobacterium tuberculosis infection. Its diagnostic utility has not been sufficiently verified.

Methods. A multi-centered, double-blind, randomized controlled trial was conducted from December 17, 2015, to March 2, 2018. Participants involved in this study included those with active tuberculosis, suspected pulmonary tuberculosis, or non-tuberculosis pulmonary disease. Each participant received three tests simultaneously, T-SPOT.TB, TST, and ECST, and adverse events were reported.

Results. Diagnostic accuracy was analyzed using data from 1085 protocol-compliant participants. The sensitivities of the ECST, TST, and

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T-SPOT.TB were 91.2% (95%CI, 89.0% - 93.2%), 91.4% (95%CI, 89.1% - 93.3%), and 92.1% (95%CI, 89.9% - 93.9%) respectively. The specificities of the ECST (69.7%, 95%CI, 64.5% - 74.5%) and T-SPOT.TB (76.1%, 95%CI, 71.2% - 80.5%) were significantly higher that of the TST (54.4%, 95%CI, 48.9% - 59.7%). The agreements between ECST and TST (kappa = 0.632) and between ECST and T-SPOT.TB (kappa = 0.780) were substantial. No severe adverse event was reported.

Conclusion. The diagnostic performance of the ECST was close to the T-SPOT.TB assay in the detection of tuberculosis infection and indicated good potential for clinical application in common scenarios.

Introduction

Tuberculosis (TB) remains a leading killer among all infectious diseases globally. The most recent Global Tuberculosis Report from the World Health Organization (WHO) estimated that in 2019 there were about 9.97 million newly developed cases and nearly 2 billion people carrying latent TB infection (LTBI) (World Health Organization, 2021). Rapid detection of TB infection (including active TB and LTBI) is crucial in the eradication of tuberculosis. However, clinical manifestations are absent in latent cases and not easy to detect in most early stages of active TB in which there is a low probability of positive detection by bacteriological examination. Therefore, indirect tests are used to support clinical diagnosis of TB disease. The WHO endorses two commercially available techniques for TB infection: Tuberculin Skin Test (TST) and Interferon-gamma Release Assays (IGRAs) (World Health Organization, 2018). The TST is a traditional test, easy to operate, low cost, but with low specificity in Bacillus Calmette Guerin (BCG)-vaccinated populations or individuals infected with non-tuberculosis mycobacteria (NTM). The IGRAs are less likely to be affected by BCG vaccination or NTM infection, but are costly and require laboratory services.

The recombinant fusion protein ESAT6-CPF10 (EC, Anhui Longcom Biologic Pharmacy Co. Ltd. China.) was developed as a novel skin test reagent to overcome deficiencies of both the TST and IGRA tests. The EC skin test (ECST) utilizes TB-specific antigens (early secreted antigenic target 6-kDa protein and culture

filtrate protein 10) that are not likely to have crossover reaction with BCG or NTM and it retains the user-friendly skin test procedure. The ECST was reported to be safe and effective in phase I and phase II trials(Li et al., 2016; Li et al., 2016; Xu et al., 2022). The phase III trial was designed to confirm the diagnostic utility of ECST through three studies: two hospital-based studies to verify the diagnostic value for adult and childhood TB, and one for LTBI in healthy community individuals. Herein, we report the comparative diagnostic efficacy and safety of the EC skin test in the detection of adult TB.

Methods

Study design and participants

In China, a multi-centered, double-blind, randomized controlled trial was conducted in ten tertiary hospitals from December 2015 to March 2018. Adult participants (18 - 65 years) were consecutively recruited from inpatient services and included active TB, suspected pulmonary TB, or non-TB pulmonary disease. Each eligible participant received IGRA (T-SPOT.TB. Oxford, UK.), TST, and ECST simultaneously, and the results were compared by statistical analysis. The primary outcome of this study was a comparison of the diagnostic accuracy of ECST, TST, and T-SPOT.TB assays for active TB. The secondary outcomes included the consistency between the three assays, the diagnostic yields in different subgroups, and the safety of ECST.

This trial was conducted in compliance with the Declaration of Helsinki and principles of Good Clinical Practice, and all study protocols were reviewed and approved by the ethics committee of Shanghai Public Health Clinical Center. All participants signed the informed consent form. The trial is registered with ClinicalTrials.gov, number NCT02623556.

Case definitions and inclusion/exclusion criteria

In the phase III trial, active TB patients were diagnosed in concordance with the diagnostic standard by the National Health Commission of the People's Republic of China (WS 288–2008), which includes "confirmed TB" as defined by smear microscopy, *Mycobacteria. tuberculosis* (MTB) culture, or positive histopathological examination of a biological specimen, AND "clinically diagnosed TB", defined as those who did not fulfill the criteria for bacteriologically confirmed TB but were diagnosed as having active TB by an experienced clinician who decided to give a full course of TB treatment based on assessing clinical suspicions of TB (fever or cough for more than two weeks, weight loss, or failure to gain weight in the previous three months, or those who had household TB contact within the preceding 24 months, or had either a positive tuberculin skin test or a positive PCR assay, or suggestive imaging evidence). Suspected pulmonary TB cases were defined as those who aroused a suspicion of pulmonary TB but for whom a clinical diagnosis and decisions were not made. Non-TB pulmonary disease included any pulmonary disease that was

diagnosed as not TB by experienced clinicians. Independent investigators conducted follow-up telephone calls to obtain a final diagnosis for those participants who had not received a definitive diagnosis by the end of the trial.

At the time of trial design, domestic standards did not classify cases by "bacteriologically confirmed TB" and "clinically diagnosed TB" as the WHO did. In final analysis, TB cases was redefined in concordance with the revised definitions by the WHO (2013) (World Health Organization, 2013).

Participants were included in the study if they met the following criteria: (1) 18-65 years old; (2) informed consent form obtained; (3) willing to comply with the requirements of the clinical trial.

Exclusion criteria for potential active TB and suspected TB participants included: (1) complicated with the following serious diseases: progressive tumoral disease; acute exacerbation of chronic obstructive pulmonary disease; acute or progressive liver disease or nephropathy; decompensated congestive heart failure; mental disorder; (2) participated in trials of any other new drugs within 3 months; (3) a history of allergy to two or more drugs; (4) pregnant or breastfeeding women; (5) other conditions that could introduce significant bias to the evaluation of the test. Exclusion criteria for non-TB pulmonary disease participants included (2), (3), (4) of those listed above.

Sample size

The sample size was calculated by PASS software (version 11. NCSS, LLC. Kaysville, Utah, USA.). According to previous studies, the sensitivity of T-SPOT.TB assays in tuberculosis patients was estimated to be 85%, and TST sensitivity in tuberculosis patients was estimated to be 80% (Pai et al., 2014; World Health Organization, 2011; Huebner et al., 1993; Losi et al., 2007). Assuming that the positive rate of the EC skin test is not inferior to that of the T-SPOT.TB or TST, we set the critical value of the non-inferiority margin as 10%, the type I error (α) as 0.025, the degree of assurance (1-β) as 0.8, and the ratio of TB to not TB as 2:1. Taking into consideration the requirement of domestic laws for new drug registration that the drop-out rate should not exceed 20%, we determined that a total sample size of 1080 should meet our goals. We expected to enroll about 600 confirmed active TB, 300 non-TB pulmonary disease, and 180 suspected TB in recruiting stage. In final diagnosis, there would be approximately 720 TB patients and 360 non-TB patients.

Randomization and masking

Using a central stratified block randomization method, a third-party statistician generated a random code table. Each random code corresponded to a set of experimental skin test reagents, including an EC and a purified protein derivative (PPD, Beijing Xiang Rui Biological Products Co., Ltd., China), randomly allocated to the left and right arms. The unblind form was restored blind to participants and investigators and copied in emergency letters. After enrollment,

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each participant was assigned a random code according to their participant series numbers.

Study procedures

At enrollment, the participants' sex, age, weight, height, pulmonary symptoms, infection site, previous treatment of tuberculosis infection, and HIV status were recorded. Blood samples for the T-SPOT.TB tests were collected before administrating ECST or TST. Participants then received the ECST intradermally on the volar surface of one forearm and TST on the other forearm according to the coding of the reagent. The subcutaneous induration or redness of the injection site was examined at 24 h, 48 h, and 72 h after injection. Two experienced nurses measured the skin test response independently. If the difference was larger than 0.2 mm, the results were rechecked. All participants were observed for at least 30 minutes for adverse reactions after receiving each test. Vital signs were measured before and after receiving the skin tests. Participants were instructed to record adverse reactions during the following 72 hours. All the local skin reactions, systemic symptoms, signs, and abnormal laboratory examination results (>± 20% of the reference range) were recorded as adverse events. The causality of adverse events was assessed as certainly related, probably related, possibly related, possibly unrelated, and unrelated to the injection. The grade of adverse events was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) v5.0.

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A positivity of ECST was determined as a diameter of redness or induration ≥ 5 mm at 48 hours after injection, which was consistent with phase II study of ECST (Miao Xu et al., 2022). For the TST, a positive result was defined as a diameter of induration ≥ 5 mm 48 hours after injection. The T-SPOT.TB test was read manually according to the manufacturer's instructions. If the result was indeterminate, a certificated third-party clinical laboratory reread the board or retested the sample.

Statistical analysis

IBM SPSS statistics (version 22.0) was used for statistical analysis. The demographic characteristics of all subjects were described by mean ± standard deviation, numerical value, median, minimum and maximum values. The diagnostic values of EC skin test, T-SPOT.TB, TST were compared by sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Kappa values were used to compare the agreement between assays. A difference with $P < 0.05$ was considered to be statistically significant. In this study, the safety of skin test reagents was analyzed in the full-analysis set and diagnostic efficacies were analyzed in the per-protocol set.

Results

Study population

From December 17, 2015, to March 2, 2018, 1169 participants were

screened. Of them, 1090 eligible participants were enrolled and then received

ECST, TST, and T-SPOT.TB test. The safety cohort included reports from all 1090

participants. Five participants dropped out during the subsequent visits (lost to

follow-up), and therefore there were 1085 participants in the accuracy cohort

(742 TB and 343 not-TB) (Figure 1). The average age of the accuracy cohort was

40.8 ± 14.3 years, and 65.3% were male. 67.2% of them had urban dwellings

according to their medical insurance records. 12.4% of them reported a TB

history. In the TB group, 90.70% (673/742) had pulmonary TB (including some

who also had extra-pulmonary TB), and 9.30% (69/742) only had

extra-pulmonary TB; 50.7% (376/742) were culture-positive, 48.7% (361/742)

were culture-negative, and 0.7% did not have MTB culture results. There were

significant differences in age, sex, BMI, and TB history between the TB and the

Not TB groups. (Table 1).

Diagnostic performance of ECST, TST, and T-SPOT.TB assay for active TB

The sensitivities of the ECST, TST, and T-SPOT.TB were 91.2% (95%CI, 89.0%

- 93.2%), 91.4% (95%CI, 89.1% - 93.3%), and 92.1% (95%CI, 89.9% - 93.9%)

respectively, and ECST was non-inferior to both the TST and the T-SPOT.TB assay.

The specificities of ECST (69.7%, 95%CI, 64.5% - 74.5%) and T-SPOT.TB (76.1%,

95%CI, 71.2% - 80.5%) were significantly higher than the specificity of the TST

(54.4%, 95%CI, 48.9% - 59.7%). The specificity of the ECST was non-inferior to

that of the T-SPOT.TB assay. The area under the curve (AUC) for the ECST was

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0.81 (95%CI, 0.77 - 0.84), 0.75 for the TST (95%CI, 0.72 - 0.79), and 0.84 (95%CI, 0.81 - 0.87) for the T-SPOT.TB assay. (Table 2) There was substantial diagnostic agreement between ECST and TST (kappa = 0.632), and between ECST and T-SPOT.TB (kappa = 0.780)

Factors associated with sensitivity and specificity of ECST

Stratified by median age (40 years), the sensitivity and specificity of ECST were both higher in the younger group (94.4 vs. 87.4% and 81.0 vs. 63.5%, P < 0.05). The sensitivity was higher in MTB culture-positive participants (94.4% vs. 88.1%, P < 0.05) and the specificity was higher in those without TB history (70.6% vs. 52.9%, P > 0.05) (Figure 2, Supplemental data). Sex, BMI, injection site (left or right arm), dwelling place (urban or rural), allergic history (any confirmed or suspected), and lymphocyte count have not significantly associated with ECST positivity in either the TB or not TB group.

Safety of the ECST

Some 118 participants (10.83%) reported 140 local adverse events related to the EC skin test, and 112 participants (10.32%) reported 122 local adverse events related to the TST. The most common local adverse events were itching and pain. A total of 55 participants (5%) reported 66 systemic adverse events, of which fatigue, headache, and fever were relatively common (Table 3). No severe adverse events or death were reported in this trial.

Discussion

The recombinant fusion protein ESAT6-CFP10 was designed as a point-of-care diagnostic tool for detecting TB infection (including active TB and LTBI), and its sensitivity and specificity were intended to be similar to the results of IGRAs. The phase II trial showed that the sensitivity and specificity of the ECST were 87.5% and 98.9% for active TB at an optimized dose of 1.0 μg/0.1 ml at 24 - 72 hours, and no serious adverse events occurred (Li F et al., 2016; Miao Xu et al., 2022). In this hospital-based study we compared ECST with two other globally recognized assays (TST and T-SPOT.TB) for diagnostic accuracy and safety using active TB as a surrogate reference (because there is currently no globally accepted gold standard for the diagnosis of LTBI). With a larger population size, we confirmed the non-inferiority of the ECST to the other assays and validated its safety.

The recombinant fusion protein ESAT6-CFP10 was designed as a point-of-care diagnostic tool for detecting TB infection (including active TB and LTBI), and its sensitivity and specificity for TB disease were intended to be similar to the results of IGRAs. Probably because of fewer false positive results due to BCG vaccination, there is currently no globally accepted gold standard support the clinical diagnosis of LTBI, studies comparing difference between similar assays may use active TB as a surrogate reference (Li F et al., 2016; Miao Xu et al., 2022). The phase II trial showed that the sensitivity and specificity of the ECST were 87.5% and 98.9% for clinical diagnosis of active TB at an optimized dose of 1.0

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μg/0.1 ml at 24 - 72 hours, and no serious adverse events occurred. In this hospital-based study we compared ECST with two other globally recognized assays (TST and T-SPOT.TB) for diagnostic accuracy and safety with a larger population size. We confirmed the non-inferiority of the ECST to the other assays and validated its safety.

Sensitivities between the three assays were quite similar. The major difference between assays lies in the specificities. In this study, the specificity of ECST was significantly higher than TST (69.7% VS. 54.4%, P<0.05) because it yielded fewer false positives. This advantage may facilitate the differential diagnosis of active tuberculosis. The overall diagnostic accuracy of ECST was quite similar to that of the T-SPOT.TB assay, mainly because they are designed based on the same immune profile.

The definition of TB at trial design was different from that of the WHO. The main difference between the two standards is the perception of PCR-based rapid diagnostic tests and histopathological examination. For the generalizability of the research results, we redefined and classified the participants in this study.

We did not calculate the diagnostic accuracy against bacteriological confirmed TB. During the research implementation stage, some WHO-endorsed PCR tests, such as GeneXpert MTB/RIF assay, were not routinely performed on participants. This may introduce bias in calculation.

Using a threshold of 5 mm for redness or induration at 48 hours, the ECST was as sensitive as the TST (5 mm), and in BCG vaccinated participants, the specificity of the ECST was higher than that of the TST. This finding was similar to reports from evaluation of other ESAT6/CPF10-based skin tests, including Diaskintest® and C-tb skin test (Hoff ST et al., 2016; Starshinova A et al., 2018; Krutikov et al., 2022), suggesting a universal threshold of 5 mm for screening TB in high-risk individuals. As was found with the TST, the diameter of induration in the ECST could be used as a semi-quantitative guide for predicting active TB (Lewinsohn et al., 2017). However, since the ECST is responsive to latent infection, it is more likely to be used as a TB infection rule-out test rather than a decision-making test for active TB.

The finding that the diagnostic accuracy of ECST was better in younger groups (Figure 2) was similar to studies of IGRAs and TST (Pan et al., 2015; Almeida et al., 2020). This may be because younger people are more immunocompetent than the elderly, and consequently are less likely to present negative results. Younger people are also less likely to get exposed to active-TB patients which would result in a lower rate of LTBI-caused ECST-positive results. These considerations indicate that a negative ECST result in elderly patients with suspected TB should be interpreted with particular care.

Notably, compared with TST, the sensitivity of ECST seems less affected by lymphocyte count in HIV-negative population (Gallant et al., 2010;

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Kowalewicz-Kulbat et al., 2018). We believe that the ECST should be evaluated in HIV-positive populations in future studies because they often have low lymphocyte counts and are most likely to benefit from this feature of the ECST.

The specificity of each assay in this study may be affected by TB history despite the lack of statistical significance (partly associated with a small sample size of participants with TB history) (Figure 2). The immune memory after TB infection can yield false-positive assay results even though the infection is inactive. It should be noted that the test was designed based on immunological response to TB infection and theoretically cannot differentiate between active TB and LTBI. The "false positive" was unlikely technically false positive but more likely to be immune reaction to latent infection or just immune memory. It can detect an immune reaction induced some time ago (months or years) by a contact with Mtb, independently of the persistence of living mycobacteria and may remain positive for years after cure of TB or disappearance of the mycobacteria (Mark U, et al.,2009). We can reasonably speculate that in people with LTBI, the ECST will also have a relatively high positive rate compared to people who have completely resolved the infection to a state of irreversible dormancy or sterility.

In low TB-incidence settings, the rate of positivity of TST, ECST and T-SPOT in healthy people without exposure to TB (non-contacts) is different. The rate for ECST and T-SPOT is close to the known rate of positivity in the Chinese

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population (Supplement Tables) (Gao L et al., 2015; Xin H et al., 2019) and worldwide (Cohen A et al., 2019), whereas the rate of positivity of TST is increased due to the high proportion of subjects vaccinated with BCG.

The local adverse events from ECST were quite similar to those from TST. The adverse reactions were mainly mild or moderate, and the overall incidence was low, confirming the clinical acceptability of the ECST. Systemic adverse events could not be distinguished from ECST and TST because the tests were simultaneously implemented in each arm.

This study has some limitations. First, this is a hospital-based study. The participants were recruited from confirmed TB, suspected TB, and non-TB pulmonary disease, but no healthy contacts or volunteers were involved. This may have influenced the apparent specificity of ECST because healthy contacts tend to yield false-positive results. As far as we are aware, no community-based evaluation of ESAT6/CFP10 tests has been published, and such an investigation would help in assessing the ECST. Second, the clinical characteristics of the participants in this study were not in concordance with the patients seen in routine practice. The test may be implemented in patients with more complicated disease conditions, such as critically ill patients, patients receiving solid organ transplantation, or hemodialysis. In these cases, the diagnostic accuracy of the ECST may be lower than usual. Third, HIV-positive individuals were not involved in this study because the prevalence of HIV in China is very low.

Lastly, the participants received both ECST and TST, and this procedure was not consistent with routine practice. Potentially, cross-reaction may have affected the results of ECST and TST.

Conclusion

Our data have provided robust evidence that the ECST is a reliable skin test and may replace the TST for TB infection. The sensitivity and specificity were similar to the T-SPOT.TB assay using active TB as a surrogate reference. However, we should note that the ECST is not a decision-making test for active TB. It would be more suitable for the detection of infection in TB contacts or immunocompromised patients who may benefit from preventive interventions. Future studies may focus on its diagnostic performance in different clinical practice scenarios.

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Conflict of interests

The authors declare no conflict of interests. The funder played no role in data collection, analysis, interpretation, or writing this report.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

All authors contributed to the interpretation of the data and reviewed this report. Shuihua Lu, Guozhi Wang and Xuhui Liu contributed to designing the trials, revising study protocols, and critically reviewing and revising the report. Lu Xia, Miao Xu, and Feng Li contributed equally to data collection, analysis, and writing.

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Table1: Clinical characteristics of participants in the TB and Not-TB groups.

	TB (N=742)	Not TB (N=343)	P value
Age, years (mean ± SD)	38.8±14.1	45.1±13.6	<0.001
Male sex, n. (%)	512 (69.0)	196(57.1)	<0.001
BMI, mean (± SD)	20.3±3.1	22.4±3.3	<0.001
Urban population, n. (%)	484 (65.2)	245(71.4)	0.044
Allergic history, n. (%)	47 (6.3)	26 (7.6)	0.437
TB history, n. (%)	118 (15.9)	17 (5.0)	<0.001
Smear positive, n. (%)	230 (31.0)	6 (1.7) *	
Culture positive, n. (%)	376 (50.7)	9 (2.6) *	
Bacteriologically confirmed TB, n. (%) **	459 (61.9)	N/A	

* These cases were identified as *Non-tuberculosis Mycobacterium* infection by MPT 64 assays or sequencing methods. ** This includes positivity by smear, culture, and WHO-approved rapid diagnostics (such as Xpert MTB/RIF) on biological specimen.

Table2: Diagnostic accuracy indices of ECST, TST, and T-SPOT.TB assay for active tuberculosis.

	Result	TB (n.)	Not TB (n.)	Sensitivity	95%CI	Specificity	95%CI	AUC*	95%CI	PPV**	95%CI	NPV**	95%CI
ECST	+	677	104	91.2	89.0-93.2	69.7	64.5-74.5	0.81	0.77-0.84	86.7	84.1-89.0	78.6	73.6-83.1
	-	65	239										
TST	+	678	157	91.4	89.1-93.3	54.4	48.9-59.7	0.75	0.72-0.79	81.2	78.4-83.8	74.5	68.6-79.8
	-	64	186										
T-SPOT.TB	+	683	82	92.1	89.9-93.9	76.1	71.2-80.5	0.84	0.81-0.87	89.3	86.9-91.4	81.6	76.9-85.7
	-	59	261										

*AUC: area under the curve; **PPV: positive predictive value, NPV: negative predictive value.

Table 3: Incidence of adverse events in participants after ECST and TST

AEs*	ECST, n=1090(%)	TST, n=1090(%)
SAEs**	0 (0)	0 (0)
Local AEs	140 events in 118 (10.8)	122 events in 112 (10.3)
AEs occurring in ≥1% in any skin test		
Pruritus at the injection site	106 (9.7)	103 (9.5)
Pain at the injection site	26 (2.3)	15 (1.4)
Systemic AEs	66 events in 55 (5.0)	
AEs occurring in ≥1% in any skin test		
Fatigue	14 (1.3)	
Headache	13 (1.2)	
Fever ≥37.2℃	11 (1.0)	

* AEs: adverse events; ** SAEs: severe adverse events.
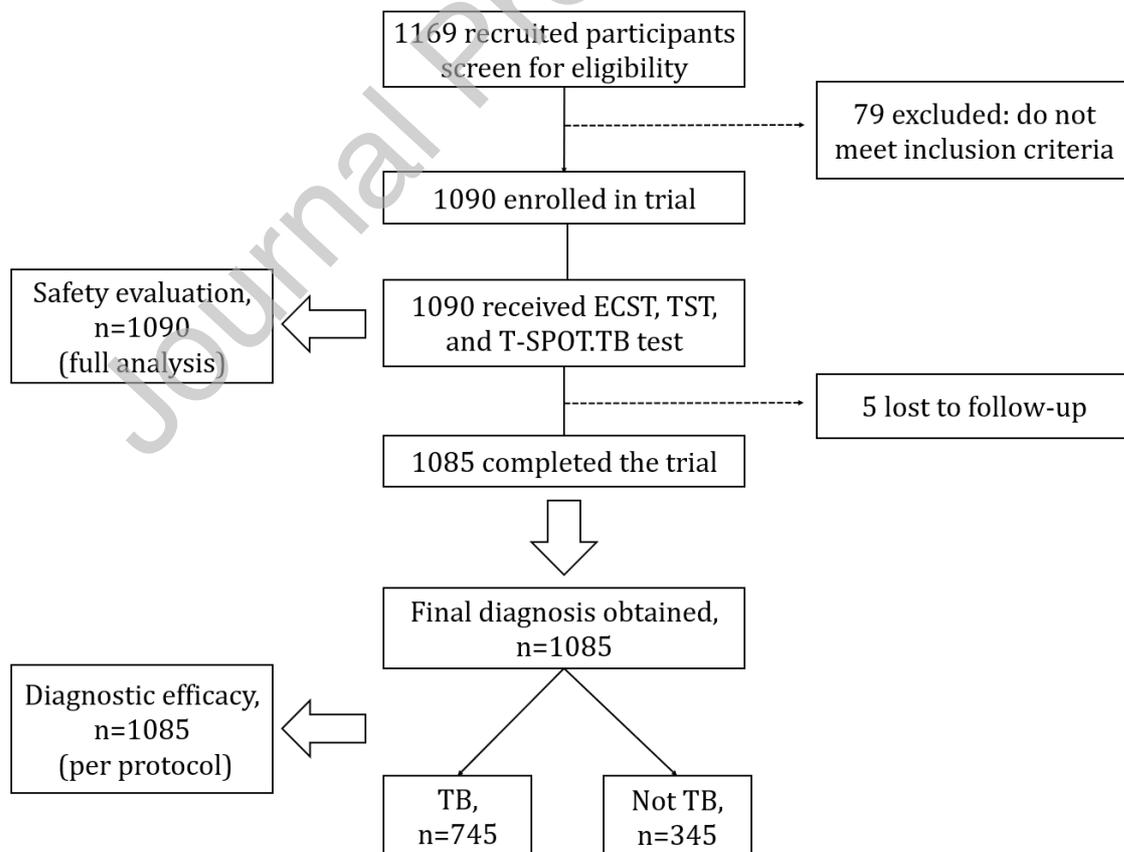
Figure 1: Participant flowchart in this study

Figure 2: Comparative diagnostic efficacy of ECST, TST, and T-SPOT.TB assay for active tuberculosis by age and TB history