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Evaluation of a modified interferon gamma release assay for the diagnosis of latent tuberculosis infection in adult and pediatric populations that enables delayed processing

Shu-Hua Wang, MD, MPH & TM^{1,2}, Dwight A Powell, MD^{1,3}, Haikady N Nagaraja, PhD⁴, Jessica D Morris, BS², Larry S Schlesinger, MD^{1,2}, and Joanne Turner, PhD^{1,2}

¹The College of Medicine, The Ohio State University, Columbus, Ohio

²Center for Microbial Interface Biology, The Ohio State University, Columbus, Ohio

³Department of Pediatrics, Nationwide Children's Hospital, Columbus, Ohio

⁴Department of Statistics, The Ohio State University, Columbus, Ohio

Abstract

The objective of the study was to evaluate the specificity of a modified interferon gamma release assay procedure that allows storage of blood samples for up to 32 hours before processing. A total of 116 subjects were enrolled for the study. Two blood samples were collected from each volunteer; one specimen was processed within 8 hours and analyzed using the T-SPOT.*TB* test; the second specimen was stored overnight and processed 23 to 32 hours later after addition of the T-Cell *Xtend*TM reagent and then analyzed using the T-SPOT.*TB* test. A total of 108 paired T-SPOT.*TB* and T-SPOT.*TB* plus T-Cell *Xtend*TM tests were analyzed on specimens from 97 adults and 11 children. The median age of the subjects was 28 years old with 68.5% female and 78.7% White. The overall agreement between the two tests was 98.2% (106/108). Specificity of the T-SPOT.*TB* test was 99.1% (107/108) and for T-SPOT.*TB* plus T-Cell *Xtend* was 97.2%. The two tests were comparable in results. Increasing storage time of the collected blood specimen prior to processing provides flexibility for clinicians and laboratories. Additional studies in larger and diverse patient populations including immunocompromised, pediatric, patients with active TB disease or latent tuberculosis infection are needed.

Introduction

Approximately one-third of the world's population, including more than 11 million people in the United States, have latent tuberculosis infection (LTBI).[1;2] For an immunocompetent individual the estimated risk for developing active TB disease from reactivation of LTBI is 5 to 10% per life time, and this increases to 10% per year for some immunocompromised individuals.[3] The Centers for Disease Control and Prevention (CDC) recommends targeted screening of high risk *Mycobacterium tuberculosis* infected persons for LTBI or progression to active TB disease.[4] The Tuberculin skin test (TST) has been the traditional screening method for LTBI but the newer interferon gamma release assays (IGRAs) offer several advantages.[5] IGRAs measure interferon gamma released from previously sensitized memory T cells that are specific for *Mycobacterium tuberculosis* antigens resulting in higher specificity than the TST. The IGRAs also decrease the number

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IRB approval obtained from The Ohio State University and Nationwide Children's Hospital. Consent obtained on all study participants.

of false positives due to Bacille Calmette-Guérin (BCG) vaccination or nontuberculous mycobacteria exposure and eliminate the difficulty of TST placement, the variability of TST reading, and the inconvenience of returning for TST reading.[6] Currently, three Food and Drug Administration (FDA) approved commercial IGRAs are available. Two utilize a whole blood enzyme-linked immunosorbent assay - QuantiFERON®-TB Gold (QFT) and QuantiFERON®-TB Gold-In-Tube (Cellestis Ltd., Carnegie, Australia);[7] and one uses an enzyme-linked immunosorbent spot assay (ELISpot) – T-SPOT®.TB (Oxford Immunotec Ltd, Abingdon, UK).[8]

The T-SPOT.TB test requires same day processing. Therefore, clinicians are limited to ordering the test early in the day to allow sufficient time for samples to be delivered to the laboratory for processing. This also limits the laboratory's ability to batch multiple specimens in order to efficiently utilize a technician's time, equipment, and reagents. The objective of this study was to compare the performance of the standard T-SPOT.TB assay procedure with a new protocol involving the addition of T-Cell *Xtend*™ reagent that allows for blood specimens to be stored at room temperature for up to 32 hours prior to any processing or incubation. To our knowledge, this is the first study to evaluate the use of the T-Cell *Xtend*™ reagent in a developed country.

Material and methods

Study setting and study subjects

The study was approved by The Ohio State University (OSU) Institutional Review Board (IRB), Western IRB, and Nationwide Children's Hospital IRB. Subjects were recruited from OSU and Nationwide Children's Hospital. Written informed consent was obtained from the participants, parent, or guardian. Using a standard questionnaire, demographic and medical histories were obtained from each participant. Data collected included age, ethnicity, history of TB disease or BCG vaccination, and medical illness including diabetes, human immunodeficiency virus (HIV) infection, use of steroids, anti-tumor necrosis factor blockers, or other immunosuppressive agents.

Blood collection and processing

Blood samples were collected in lithium heparin tubes. For adults and children 10 years and older, 12 mL of venous blood was collected in total. For children 2 to 9 years old and children younger than 2 years, 6 mL and 4 mL of venous blood were collected, respectively. The blood samples were split and one specimen was processed within 8 hours and analyzed using the T-SPOT.TB test; the second specimen was stored at room temperature (18–25°C) for 23 to 32 hours. Prior to processing, 25µL of T-Cell *Xtend*™ reagent was added per mL of blood. The tube was inverted two to three times and incubated at room temperature for an additional 20±5 minutes. The overnight specimens were grouped into 3 processing time blocks of 23–26 hours, 26–29 hours, and 29–32 hours for data analysis.

All T-SPOT.TB tests were performed at the OSU Center for Microbial Interface Biology. To minimize bias, the same laboratory technician performed both tests for the same subjects on two separate days, both test results were read independently and the laboratory technicians were blinded to the subject's clinical and demographic information.

Cells were processed and assays were performed in accordance with the manufacturer's instructions for the T-SPOT.TB test.[9] The results were statistically analyzed using a cut-off of 6 spots or more as indicating a positive test as per FDA approved receiver operating characteristics (ROC) curve.[10] In accordance with protocol, participants were notified if their control arm results were positive. If the final test result revealed a spot count value of <10 spots, a second trained laboratory personnel reviewed the plate to provide additional

verification of the number of spots. Study subject samples with > 10 spots in the Nil control, < 20 spots in the positive control, or high background interference were excluded from the study.

Statistical Analysis

The population analyzed consisted of subjects who had paired T-SPOT.*TB* (control arm) and T-SPOT.*TB* plus T-cell *Xtend* (test arm) assay results. Percent agreement, defined as the number of pairs of final test results with similar control and test arm results divided by the number of tests, was calculated. The Kappa statistic was used to formally measure the overall agreement between the paired test results. A standard error for Kappa as well as the 95% confidence limits, and corresponding exact p-value were calculated. Statistical significance was achieved if the one-sided p-value for Kappa was ≤ 0.05 . Statistical analyses were performed on SAS Version 9.1 and SAS JMP 8.0 software (SAS Institute, Cary, NC).

Results

A total of 116 volunteers, 101 adults and 15 pediatric, were recruited and consented to the study from September to November 2008. Eight participants (8/116; 7.0%) were excluded from the study for physical damage to membrane on the ELISpot plate, insufficient PBMCs or no blood sample collected, processing delay beyond 8 hours for the control arm (for two pediatric specimens), spot counting for both arms was performed on the same day, and high Nil control spot count in the test arm. A total of 108 paired T-SPOT.*TB* results for < 8 hours storage (control arm) and 23–32 hours storage (test arm) from 97 adults and 11 children were included in the final analysis. The participant characteristics are listed in Table I. Median age of the study population was 28 with 68.5% female and 78.7% White. Thirteen of the 108 subjects had a significant current medical condition: six with diabetes, four with asthma, one with a history of bone marrow or organ transplant, one currently on steroid therapy and three with other immunosuppressive treatment or conditions. No participants with a current history of HIV infection or active TB disease were enrolled, but two participants had a history of BCG vaccination.

The specificity of the T-SPOT.*TB* study results for the control arm was 99.1% (107/108; 95% CI 94.9–100%). The estimated specificity for the modified procedure using T-Cell *Xtend*TM and overnight stored blood was 97.2% (105/108; 95% CI 92.1–99.4%). The overall agreement between the two test arms was 98.2% (106/108) (see Table II). The Kappa statistic for the overall agreement between the paired T-SPOT.*TB* results for the two study arms was 0.493 with a 95% confidence interval of (−0.1071, 1.00) and p-value of 0.0278. The two discordant samples were both negative in the control arm but positive in the test arm. One subject had 3 spots in the control arm and 12 spots in the test arm, making this a positive test arm result (subject #15). The other subject, however, was only borderline in the test arm. The technician had read the test arm sample as having 5 spots, but the validating reviewer observed 6 spots, making this a positive test arm result (subject #70, Table III). Three additional subjects had a negative control arm result with borderline test arm results with 5 spot counts (subjects #47, 65, 94, III3). Only one subject had a positive T-SPOT.*TB* result for the control arm, which was also positive for the test arm.

A lower number of viable PBMCs was observed in the test arm. This did not reach statistical significance and the assay procedure controlled for differences by the addition of 2.5×10^5 total cells to each well (in a final volume of 200 μ l). The average viable PBMCs for the control and test arms were 155.5 and 140.1 respectively, and a 95% CI for the average difference was (1.9, 28.91). For the Nil control arm, the average number of spots was 0.18 and for the test arm it was 0.44, with a 95% CI of (0.02, 0.51) for the average difference. Only 1/116 (0.9%) of samples showed a nil control spot count outside the valid range (>10

spots), and only 2/108 (1.9%) of samples had a positive test but negative control result. No difference in the positive control responses was noted. The number of spots for the PHA positive control for Tspot test were too numerous to count on Day One for all 108 subjects and 106 subjects on Day 2. They were 37 and 48 for the remaining two subjects on Day 2. The overnight stored samples, 71% (10/14) Leucosep cell separation method led to higher RBC contamination when compared to 1.1% (1/94) Ficoll separation. However, the three overnight storage time blocks of 23–26, 26–29, and 29–32 hours showed agreements of 15/16 (93.8%), 69/70 (98.6%) and 22/22 (100%), respectively. In this study 11/11 (100%) pediatric samples were concordant between the two arms of the study amongst which 3 samples were obtained from children < 5 years old. One sample from a child of 4 years old yielded an invalid result due to a high nil spot count.

Discussion

This study evaluated the agreement between the standard T-SPOT.*TB* assay protocol and a modified protocol which enables blood samples to be stored for an extended period of time, and which would allow the clinician to mail specimens directly to the reference laboratory without any intermediary processing or incubation. The T-Cell *Xtend*TM proprietary reagent is an antibody which cross-links red blood cells to granulocytes, thereby decreasing the number of unwanted granulocytes that accumulate in the PBMC fraction during cell separation in samples that have had prolonged storage. This should theoretically result in recovery of IFN- γ responses comparable to samples stored for up to 8 hours.[11] Our results demonstrate an agreement of 98.2% (106/108; 95%CI 93.5–99.5%) between the currently approved T-SPOT.*TB* assay procedure and the modified protocol providing evidence for the validity of the use of the T-Cell *Xtend*TM reagent in this protocol. The kappa value of 0.493 represents a good concordance, but is lower than expected. This is due to the fact that almost all of the test results were of only one type (negative) (Table II). Furthermore, we observed no significant differences between a processing time delay between 23 and 32 hours indicating a degree of flexibility with the overnight blood storage time.

Our study was limited by the low risk study population however this population was purposefully chosen to study specificity since lower risk patients would be a population to benefit from not requiring same day processing of samples. It was important to evaluate the potential for false positives. Prolonged blood storage time could lead to nonspecific cell activation of PBMCs leading to IFN- γ release and a high incidence of false positive assays. Thus, low risk individuals who would have tested negative would be most impacted by any adverse conditions that lead to non-specific IFN- γ release, leading to a false negative result that would be easily detected. In comparison, the test status would not directly change in individuals with TB or latent TB, where numerous SFU are routinely observed. In our study the low number of invalid Nil specimens or positive test with negative control findings would argue against nonspecific cell activation. Additionally, extended storage of whole blood could potentially increase the number of PBMCs that are not functional in the ELISpot assay, or may also result in increased cell death of viable PBMCs necessary to measure the IFN- γ response, and both of these factors would affect the test results. Smith et al., showed that delayed processing of ELISpot assays for *M. tuberculosis* lead to a reduction in spot forming units and Lenders et al., also showed a reduction in the conversion/reversion rate when the specimens were processed with the addition of the T-Cell *Xtend*TM reagent however, this difference was not statistically significant.[12, 13] In our study, with the addition of T-Cell *Xtend*TM reagent, there were no significant differences observed in the overall proportion of viable PBMCs or the total PBMC concentrations between blood stored for up to 8 hours and blood stored for 23–32 hours. Additional information regarding the T-Cell *Xtend*TM reagent would have been gained by comparing the Tspot TB test to Tspot plus T-Cell *Xtend*TM on Day one in our study.

The T-SPOT.*TB* study results for the control arm in a low risk population demonstrated a specificity for the current T-SPOT.*TB* test of 99.1% (107/108; 95% CI 94.9–100%). The estimated specificity for the modified procedure using T-Cell *Xtend*[™] and overnight stored blood was 97.2% (105/108; 95% CI 92.1–99.4%); this is not significantly different from the control arm value and demonstrates the feasibility of extended time between blood collection and analysis using the T-cell *Xtend*[™] reagent.

Both Leucosep and standard Ficoll separation methods are approved with the T.SPOT[®].*TB* test. It is unclear why higher RBC contamination was observed with Leucosep method. However all of the Leucosep processed samples yielded valid results. Only one sample was excluded due to poor PBMC yields and this was processed in Ficoll. No samples were excluded due to a low positive control response.

As per IRB protocol, the one participant with the positive control arm was notified of their T-SPOT.*TB* test result. The subject voluntarily informed the study investigator that they had emigrated from a TB endemic country with a known history of positive TST. It is important to note that this subject tested positive in both the control arm and the test arm of the study. The subjects with positive or borderline results in the test arm only, were not notified of their T-SPOT.*TB* plus *Xtend*[™] assay results as per IRB protocol. Thus, we do not have any prior TST results or follow-up assessments on these subjects with positive Day 2 only specimens and are unable to determine their TB risk factors. Of the four individuals with negative control arm results and borderline test arm results, three had 5 spots (which is negative according to the FDA approved ROC curve based cut-off for the assay) and one had 6 spots. It is our experience that most positive T-SPOT.*TB* test results for active TB disease and LTBI have a much higher number of spots than the cut-off threshold.

To minimize any variation due to spot counting, a second reader verified the spot count in wells containing < 10 spots. The technician and the verifying reader disagreed on only one sample where the final test result was discordant (subject #70, Table III). The result changed from 5 spots (negative) to 6 spots (positive) although both results are in the borderline region for the assay (5 – 7 spots). This, in our opinion, stresses the importance of the need to take particular care in the reading of low spot counts near the borderline cut-off threshold and justifies the provision of a borderline region. We also recommend that clinicians receive the actual spot count in addition to positive, negative, or borderline test results.

The updated CDC guidelines state that IGRAs may be used in any situation in place of TST for screening of LTBI, however, TST is preferred in children < five years of age.[14] While our sample size is small the data suggest that the modified procedure is applicable to pediatric samples. Two pediatric participants were excluded due to delayed processing of > 8 hours in the initial specimen. The distance from Children's Hospital to the laboratory was approximately 5 miles with transportation provided by a local courier service. This highlights the difficulty in getting specimens to the laboratory in a timely fashion and illustrates the potential for the extended blood storage procedure.

The sample size was too small to determine if pre-existing medical conditions played a role in the study results. One of six diabetic patients had a positive result after overnight blood storage. More studies need to be conducted in special patient populations including diabetics, immunocompromised, pregnancy, and pediatrics before conclusions can be drawn regarding pre-existing conditions and the extended blood storage option in such cohorts.

In summary, the use of T-Cell *Xtend*[™] with overnight stored blood provided comparable data to the use of < 8 hours stored blood providing evidence for the feasibility of extending the period of time between blood collection and execution of the T-SPOT.*TB* assay. The specificity of the T-SPOT.*TB* assay and T-SPOT.*TB* assay plus T-Cell *Xtend*[™] was high at

99.1% and 97.2% respectively. Furthermore, the time to assay was flexible, with no significant differences in assay results between 23 and 32 hours of delay prior to processing. Increasing the storage time of the collected blood specimen prior to processing or incubation can provide flexibility for clinicians to order the sample at anytime and provides flexibility for the laboratories to process the specimens in a wider time period, avoiding discarding any samples that may have surpassed the time frame for assay setup. Lastly, larger studies in diverse patient populations including individuals that are immunocompromised, pediatric populations, and patients with active TB disease or a high probability of LTBI are needed.

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SHW drafted and revised the manuscript and assisted in data analysis; SHW and DAP assisted in application of to the Institutional Review Boards; DAP, LSS, and JT performed critical review and editing of the manuscript; HNN performed the data analysis and statistical calculations; JM carried out the T.SPOT®.TB and T.SPOT.TB plus T-Cell Xtend™ assay.

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Table I

Demographic characteristics of the study participants

	Total	Pediatric	Adult
Number of subjects (%)	108	11 (10%)	97 (90%)
Age, years;			
Mean	31.4	8.2	34.1
median (range)	28 1.3 – 68	8 1.3 – 14	29 18 to 68
Gender			
female	74	5	69
male	34	6	28
Racial Group, n (%)			
Asian	5 (4.6)	1 (9.1)	4 (4.1)
Black (non-Hispanic)	12 (11.1)	3 (27.3)	9 (9.3)
Hispanic	3 (2.8)	0	3 (3.1)
Native American Indian	1 (0.1)	0	1 (1.0)
White (non-Hispanic)	85 (78.7)	7 (63.6)	78 (80.4)
Unknown	2 (0.2)	0	2 (2.1)

Comparative T-SPOT®.TB assay outcomes for the control arm (blood stored for <8 hours) and test arm (blood stored for 23–32 hours and processed with the T-Cell Xtend™ reagent).

Table II

		Test arm					
		Positive			Negative		
		≥8 spots	7 spots	6 spots	5 spots	≤4 spots	Total
Control arm	Positive	1	0	0	0	0	1
	Negative	1	0	1	3	102	107
	Total	2	0	1	3	102	108

Table III

Subjects with borderline T.SPOT[®].TB plus *Xtend*[™] (test arm) results.

Study ID# T.SPOT.TB study arm	Nil control	Panel A	Panel B	Assay value *	Assay result	Cell separation method	Comments
#47 Control	2	3	3	1	Negative	Ficoll	
#47 Test	3	8	1	5	Borderline	Ficoll	
#65 Control	0	0	2	2	Negative	Leucosep	
#65 Test	0	0	5	5	Borderline	Leucosep	RBC contamination
#70 Control	1	0	0	-1	Negative	Leucosep	
#70 Test	6	12	6	6	Borderline	Leucosep	High background and RBC contamination
#94 Control	0	0	1	1	Negative	Ficoll	
#94 Test	0	0	5	5	Borderline	Ficoll	

ID#, identification number; Panel A, Early secreting antigen (ESAT)-6; Panel B, cell filtrate protein (CFP)-10; RBC, red blood cell.

* Assay value is obtained by subtracting the Nil control result value from the higher result value of Panel A or Panel B.