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Serodiagnostic Performance of Resat-6-CFP-10 in the Diagnosis of Pulmonary Tuberculosis in Ethiopia

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Abstract

Background: Tuberculosis (TB) is a major public health problem globally. Lack of rapid and accurate diagnostic tests remain an obstacle for effective control. The aim of this study was to evaluate the potential of rESAT-6-CFP-10 in detecting active pulmonary TB in a high burden setting.

Methodology/Principal findings: Three hundred twenty three pulmonary TB suspects were included consecutively from February 2010 to May 2010. Basic socio-demographic data as well as sputum and serum samples were collected. Of the 323 suspects, 107 were confirmed to be pulmonary TB patients using smear microscopy and culture. IgG-based ELISA was run on 204 (89 culture positive and 115 culture and smear negative) serum samples using rESAT-6-CFP-10 antigen. The sensitivity and specificity were 91.0% (83.3 % - 95.4%) and 41.7% (33.1% - 50.9%), respectively. The sensitivities were 94.6% and 88.5% in smear positive and smear negative pulmonary TB patients, respectively. The sensitivity and specificity among HIV positive pulmonary TB suspects were 87.5% and 34.8%, respectively whereas the sensitivity and specificity among HIV negative pulmonary TB suspects were 93.0% and 42.7%, respectively.

Conclusion: The sensitivity of rESA-6-CFP-10 antigen is high but the corresponding specificity is low and therefore, it can't replace smear microscope in the study area.

Keywords: Pulmonary TB; Diagnosis; Sensitivity; Specificity; Resat-6-CFP-10; ELISA

Introduction

According to World Health Organization (WHO), one-third of the world's population is estimated to be infected with *Mycobacterium tuberculosis* [1]. In 2009, there were 9.4 million new cases with 1.7 million deaths globally; however, the vast majority of Tuberculosis (TB) cases and deaths are from developing countries where resources are limited [1] and Ethiopia is 7th among the 22 high burden countries in the world [2].

In response to the global challenge, in 1991, the World Health Assembly set two global targets: to detect at least 70% of new sputum smear positive pulmonary TB patients and to successfully treat 85% of these cases. Although the treatment success rate has exceeded the global target, case detection rate remained short of the global target and the African Region has the lowest estimated (50%) case detection rate in the world [1].

Management of TB patients depends on early diagnosis and appropriate treatment to reduce transmission, morbidity, mortality and development of drug resistance. Currently, detection of patients with TB requires that patients are aware of their symptoms and have access to health facilities. Once they come in contact with a health facility, the diagnosis of TB is mainly based on clinical and radiological findings with subsequent confirmation using smear microscopy or culture. Sputum microscopy is the routinely available test in low income countries. In this complex continuum, anything could go wrong and patients may remain undetected leading to delayed initiation of treatment with high morbidity and mortality as well as continued transmission.

According to a review, the main problem for delayed diagnosis seems to be related to "a vicious cycle of repeated visits of the same health care level, resulting in non-specific antibiotic treatment and failure to access specialized TB services" [3]. In the absence of point-of-care TB diagnostic services at low level health facilities, it is unlikely to reach the global target for case detection rate especially in the African Region.

To overcome diagnostic challenges in tuberculosis, there is a continued effort in the investigation of rapid diagnostic tests. Serologic tests are attractive since they are rapid, simple and robust and hence they could easily be used at low level health facilities in low income countries. In connection with this, a number of mycobacterial antigens were isolated and evaluated to see their potential in the diagnosis of active tuberculosis. A review of studies done on commercial serologic tests for the diagnosis of pulmonary TB [4] showed variation in accuracy of tests across studies with sensitivities ranging from 10% to 100% and specificities ranging from 47% to 100%. Similarly, a recent head to head evaluation of 19 commercial rapid tests using culture as a reference in pulmonary TB patients revealed a low sensitivity (0.97% to 59.7%) and a variable specificity (53% to 98.7%) [5]. Generally, the immune response to mycobacterial antigens is heterogeneous amongst individuals and therefore, the use of recombinant fusion antigens increases sensitivity [6,7] compared to a single antigen.

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A recent major advance is the isolation of ESAT-6 (6 kDa Early Secretory Antigen Target) and CFP-10 (10 kDa Culture Filtrate Protein) which are among several immunogenic, early secreted, culture filtrate proteins obtained from *M.tuberculosis* complex [6]. They are encoded within Region of Difference 1 (RD1) which is absent from BCG strains and most non-tuberculous mycobacteria [8]. These antigens have been evaluated in the diagnosis of latent TB and two tests, QuantiFERON-TB Gold and T-SPOT.TB, are commercially available which are important for the diagnosis of latent TB infection.

The potential of ESAT-6 and CFP-10 for the diagnosis of active TB has been evaluated by a few studies. For example, a study in Denmark evaluated the performance of these antigens using whole blood to measure cytokine level (IFN-γ) and found a sensitivity of 85% and a specificity of 60% [9]. Similarly, the performance of rESAT-6-CFP-10 in the serodiagnosis of active TB was evaluated in China and the investigators reported a promising result [10]. In the current study, we report the potential of rESAT-6-CFP-10 in the diagnosis of active TB in a high burden setting.

Methods

Study area

The study was conducted primarily in the Afar Regional State, northeast Ethiopia. Moreover, three health facilities in Dessie Town were included in this study. Dessie Town, with a population of 151,000 [11] is located 400 km Northeast of Addis Ababa in Amhara Regional State. Four hospitals (3 of which are privately-owned) and a number of privately owned clinics are found in the town; patients from Afar Region visit these health facilities frequently.

The Afar Region is one of the 9 administrative regions of Ethiopia with estimated area of 100,000 square km. Semera, capital of the Region, is about 600 km northeast of Addis Ababa on the main road to Djibouti. Administratively, the Region is divided into 5 zones and 30 districts ("Woredas"). According to the 2007 census report, the population size of the Region was about 1.4 million, 87% of which living in rural areas [11]. The Region is arid and semiarid inhabited mainly by pastoralists whose source of livelihood is based on livestock. It is an endemic area to TB; in 2006, the notification rate was 103 per 100,000 populations [12].

The health service coverage in Afar Region is low (40%) and the majority of health facilities are found on the main roads [13]. There is only one functional hospital in the Region. Besides, health facilities are mainly staffed with mid and low-level health workers. The population, whose livelihood is based on pastoralism, constantly moves from place to place in search of pasture and water for their cattle and this could further compromise health service utilization.

Study population

TB management in Ethiopia is currently based on passive case detection in which patients consult health care providers by their own initiative. Subsequently, TB suspects are managed or referred to the next higher level depending on the availability of TB diagnostic services. All pulmonary TB suspects who came to the following health facilities from February till June 2010 were included consecutively: Dubti Hospital and Awash Health Center in Afar Region and Selam Hospital, Bati Hospital and Amir Higher Clinic in Dessie Town.

Inclusion and exclusion criteria

Study participants were selected from the study population according to the following inclusion and exclusion criteria. Patients who were 18 years or older who reported to selected health facilities because of cough lasting 2 weeks or more [14] were included in our study. Critically ill patients requiring urgent intervention and TB patients who already started treatment were excluded.

Socio-demographic data collection

Using structured questionnaire, patients were interviewed on basic socio-demographic characteristics and their symptoms. Participants were examined for BCG scars.

Sputum specimen collection

Three sputum samples were collected from each patient according to the national guideline for the diagnosis of tuberculosis [14]. Smear microscopy was done three times at each health facility's laboratory. The remainder of the sputum from the specimens were transferred to Aklilu Lemma Institute of Pathobiology, Addis Ababa University laboratory. Sputum were pooled, processed and cultured according to WHO guideline [15] using Lowenstein-Jensen medium.

Blood sample collection

About 4 ml blood was collected from each participant. Subsequently, serum was separated and stored at -20 °C until ELISA was done. HIV testing was done according to the national algorithm [16] and those patients with HIV infection were referred to antiretroviral therapy clinics for further intervention.

Enzyme-linked immunosorbent assay

Antigens were donated by Statens Serum Institut, Copenhagen, Denmark. The antigen coating concentration of 4 µg/ml and optimum serum dilution of 1:75 were established at Norwegian Institute of Public Health. ELISA was done at Aklilu Lemma Institute of Pathobiology laboratory. Nunc Maxisorp ELISA plates (flat bottom, Nunc Maxisorp, Roskilde, Denmark) were coated with rESAT-6-CFP10 at 4 µg/ml (0.4 µg antigen per well) in fresh 10 mM PBS (pH 7.4). The plates were then incubated for two days at 4°C before use. After washing 5 times with PBS containing 0.05% Tween20™, 100 µl of PBS containing 2% bovine serum albumin (BSA) was added to each well as a blocking solution and then incubated at 37°C for 1 hour. After washing as described above, 100 µl of patient serum diluted at 1:75 in PBS containing Tween20™ 0.05% and 2% BSA was added in each well and plates were incubated at 37°C for 1 hour. Plates were washed as above and 100 µl of anti-human IgG-alkaline phosphatase (A3187, sigma) diluted at 1:5000 in PBS containing Tween20™ 0.05% and 2% BSA was added in each well. The plates were incubated at 37°C for 1 hour. ELISA was developed using alkaline phosphatase-substrate (S0942, sigma) dissolved in 10% dietanolamine buffer (pH=9.8). Two tablets of alkaline phosphatase-substrate were dissolved per 10 ml dietanolamine buffer and 100 µl of this solution was added per well. Signals were recorded at 405 nm at 30 minutes. Samples were run as duplicates and mean value of the two Optical Density (OD) readings were taken for analysis.

Positive and negative control sera were used in every plate. The negative control sera were from apparently healthy individuals with no chest x-ray abnormality. Besides, they were tuberculin skin test and interferon gamma release assay negative. On the other hand, positive control sera were from culture confirmed pulmonary TB patients with high OD values for the antigen.

Definitions of outcome variables

Sensitivity: proportion of individuals with TB who gave a positive test result using rESAT-6-CFP-10 antigen

Specificity: proportion of individuals without TB who gave a negative test result using rESAT-6-CFP-10 antigen

Likelihood ratio of a positive test: How much more likely is a positive test using rESAT-6-CFP-10 antigen to be found in a person with the condition than in a person without it?

Likelihood ratio of a negative test: How much more likely is a negative test using rESAT-6-CFP-10 antigen to be found in a person without the condition than in a person with it?

Statistical analysis

Data were analyzed using SPSS for Windows version 16. Since the distribution of the OD values were skewed, non-parametric tests were used. The receiver operating characteristic (ROC) curve for the OD values of the antigen was plotted using STATA version 11 and the area under the curve along with its 95% confidence interval (CI) was calculated. In addition, the optimal cut off value was chosen when the Youden's index (sensitivity+specificity-1) was maximum. Serum samples were identified as positive for the specific antibody response when the OD value is greater or equal to the cut-off value. Sensitivity, specificity, and likelihood ratios along with their 95% CI were calculated. In all cases, level of significance was set at $p < 0.05$ and all tests were two-tailed.

Ethics statement

The study has been ethically approved by Norwegian Ethics Committee (Regionale komiteer for medisinsk og helsefaglig forskningsetikk) (2009/284a) and Ethiopian Ministry of Science and Technology (RDHE/66-91/2010). Patients who fulfilled the inclusion criteria were invited to participate in the study and written consent was obtained.

Results

Socio-demographic characteristics

The socio-demographic characteristic of study participants has been summarized (Table 1). About 75% of the study participants were below 45 years and the median age was 30 years (IQR 25-45). The male to female ratio was 1.4 to 1.

Presenting symptoms

Participants were interviewed about the different symptoms they were suffering from. Cough was reported in all patients and fever was the second most frequently reported symptom (93.2%). Moreover, patients reported night sweating (75.2%), loss of appetite (74.3%), weight loss (72.1%), fatigue (65.6%) and haemoptysis (21%).

On bivariate analysis, a higher proportion of pulmonary TB patients reported fever, weight loss, fatigue and loss of appetite compared to non-TB patients. However, none of them were significantly associated with pulmonary TB.

Performance of rESAT-6-CFP-10

Out the 323 pulmonary TB suspects, 101 were culture positive pulmonary TB patients. Forty-one (40.6%) of the culture positive pulmonary TB patients were also smear positive. Moreover, six culture negative patients were found to be smear positive. Taking culture as a reference, 89 serum samples from culture positives and 115 serum samples from culture and smear negatives were randomly selected (Figure 1) and ELISA was run on these samples to detect the presence of IgG in the sera. Table 2 shows the summary statistics.

Possible association of some selected variables with the OD values of pulmonary TB suspects has been evaluated using Mann-Whitney and Kruskal-Wallis tests. The result is summarized in Table 3. There was no statistically significant association of OD values with sex, age, ethnicity, consumption of raw milk, contact with TB patients, being pastoralist and HIV infection. BCG vaccination was found to be significantly associated with higher median OD values compared to the non-vaccinated group (Mann-Whitney, p=0.004).

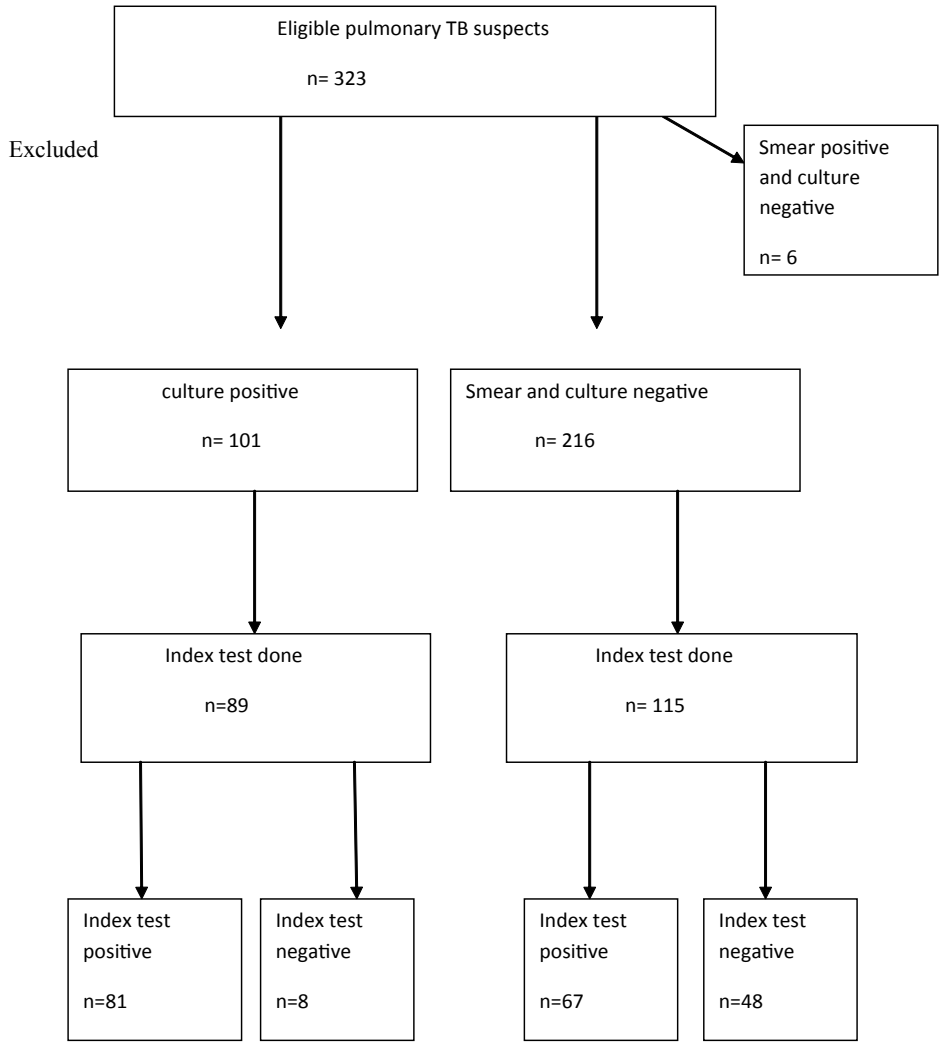
Variable	Count (N=323)	Percent (%)
Age		
18-24	79	24.5
25-44	163	50.4
>44	81	25.1
Sex		
Male	188	58.2
Female	135	41.8
Marital status		
Single	107	33.1
Married	192	59.5
Widowed	10	3.1
Divorced	14	4.3
Residence		
Urban	183	56.7
Rural	140	43.3
Religion		
Muslim	236	73.1
Christian	87	26.9
Ethnicity		
Afar	145	44.9
Amhara	126	39.0
Others	52	16.1
Education		
None	205	63.5
Primary (1-6)	77	23.8
Post-primary (>6)	41	12.7
Occupation		
Pastoralist	147	45.5
Non-pastoralist	176	54.5

Table 1: Socio-demographic characteristics of pulmonary TB suspects, Ethiopia.

	Summary statistics	† Culture negatives	Culture positives	p-value
n		115	89	
Age (years)	Median (10^{th}-90^{th} percentile)	35(20-60)	30(19-55)	0.11
OD values	5^{th} percentile	0.14	0.18	
	10^{th} percentile	0.15	0.21	
	25^{th} percentile	0.17	0.24	
	Median	0.23	0.35	<0.001
	75^{th} percentile	0.35	0.88	
	90^{th} percentile	0.71	2.34	
	95^{th} percentile	0.91	3.27	

† both culture and smear negatives
n= sample size for ELISA

Table 2: Summary statistics of OD values for rESAT-6-CFP-10 in culture positive and culture negative patients in Ethiopia



A flow diagragm showing sampling procedure and how the reference test and the index test was applied among TB suspects.
Reference test: Culture
Index test: the ELISA test using ESAT-6-CFP-10 antigen

Figure 1: A flow diagragm showing sampling procedure and how the reference test and the index test was applied among TB suspects. Out of 323 suspects, 6 were smear positive but culture negative patients were excluded. A total of 101 suspects were confirmed as culture positive pulmonary TB patients whereas 216 suspects were both culture and smear negative. The index test was done on 89 culture confirmed pulmonary TB patients and 115 culture and smear negative non-TB patients. The sensitivity and specificity of the index test were 91.0% and 41.7%, respectively. Index test: ELISA using rESAT-6-CFP-10 antigen.

OD values of culture positive and negative patients were compared to see if there is a difference. Culture positive TB patients did have significantly higher OD values compared to non-TB patients (Mann-Whitney, $p<0.001$) (Figure 2). The median OD values of smear positives (n=37) and negatives (n=52) among culture positive pulmonary TB patients (n=89) was compared and there is no statistically significant difference between them (Mann-Whitney, p=0.733).

Non-parametric ROC curve (Figure 3) for the antigen has been constructed as sensitivity versus 1-specificity taking each OD value as a possible cut-off point. The area under the curve was 0.71 (CI 0.64- 0.78) indicating a good overall performance of the antigen. With an optimum cut-off value of 0.21, the sensitivity and specificity were 91.0% (83.3 % - 95.4%) and 41.7% (33.1% - 50.9%), respectively. The positive and negative likelihood ratios are 1.6 and 0.2, respectively. Generally, the further the likelihood ratios are from 1 the stronger the evidence for the presence or absence of disease. In this study, the negative likelihood ratio is farther from 1 implying that a suspect who is negative using this antigen is most likely a true negative patient (Table 4).

After dividing the sample as positive (OD $\geq$ 0.21) and negative (OD < 0.21), the sensitivities of the antigen among smear negative and smear positive pulmonary TB patients were 88.5% and 94.6%, respectively. Similarly, the sensitivity and specificity of the antigen among HIV positives were 87.5% and 34.8%, respectively whereas the sensitivity and specificity among HIV negatives were 93.0% and 42.7%, respectively.

Discussion

Early detection and effective treatment are key strategies to control TB. Early detection could be facilitated if an accurate and rapid diagnostic test is available. We evaluated the potential of a recombinant fusion antigen in the diagnosis of active pulmonary TB among pulmonary TB

Variable	Count	Median (IQR)	p-value
Age (n=204)			
18-24	46	0.33 (0.21-0.71)	0.27ª
25-44	103	0.26 (0.19-0.45)	
>44	55	0.27 (0.18-0.45)	
Sex (n=204)			
Male	116	0.28 (0.20-0.48)	0.93
Female	88	0.27 (0.19-0.51)	
Ethnicity (n=204)			
Afar	91	0.26 (0.18-0.58)	0.48ª
Amhara	83	0.29 (0.21-0.45)	
Others	30	0.32 (0.20-0.52)	
Occupation (n=204)			
Pastoralist	99	0.28 (0.18-0.52)	0.75
Non-pastoralist	105	0.28 (0.21-0.47)	
Raw milk consumption (n=204)			
Yes	99	0.27 (0.19-0.55)	0.91
No	105	0.29 (0.20-0.46)	
Contact history (n=204)			
Yes	74	0.27 (0.19-0.54)	0.60
No	130	0.29 (0.20-0.48)	
BCG vaccination (n=204)			
Yes	30	0.44 (0.24-0.81)	0.004
No	174	0.26 (0.19-0.41)	
Smear result (n=89)*			
Positive	37	0.32 (0.24-1.23)	0.73
Negative	52	0.36 (0.24-0.68)	
HIV status (n=201)			
Positive	55	0.29 (0.21-0.46)	0.56
Negative	146	0.27 (0.19-0.51)	

*Smear result of culture positive TB patients
ª Kruskal-Wallis

Table 3: Associations of socio-demographic, cultural and health-related factors with OD values of rESAT-6-CFP-10 in Ethiopia

suspects in an endemic setting. A total of 204 serum samples (89 from culture verified pulmonary TB patients and 115 culture/smear negative non-TB patients) were used to run ELISA. Our ELISA result showed a significantly higher OD values for the antigen among pulmonary TB patients compared to non-TB patients consistent with a report from Ethiopia [17] among TB patients and their TB contacts.

In the present study, we found an overall sensitivity of 91.0% with a corresponding specificity of 41.7%. Generally, the sensitivity for this antigen is better than most of the commercial tests [4,5] as well as the purified in house antigens [7] reported so far. Besides, the low negative likelihood ratio indicates that a suspect who is negative using this antigen is most likely a true negative suspect. However, the specificity of this antigen is lower compared to the specificities reported for most other antigens [4,5,7].

Some studies reported the performance of ESAT-6 and CFP-10 antigens as a separate or fusion antigen. A study done in China [10] reported a sensitivity and specificity of 66.7% and 81.8%, respectively for ESAT-6-CFP-10 antigen. Another study from India reported a sensitivity and specificity of 64.9% and 88.9% for rESAT-6 and 66% and 85.2% for rCFP-10 antigen, respectively [18]. In these studies, they used healthy controls to determine specificity which might have resulted in a higher specificity [7]. Similarly, a study from South Korea [19] reported

	Culture (reference test)		
	Positive	**Negative**	**Total**
Positive	81	67	**148**
Negative	8	48	**56**
Total	**89**	**115**	**204**

ELISA (index test)
Sensitivity (95 % CI) =91.0% (83.3 % - 95.4%)
Specificity (95 % CI) =41.7% (33.1% - 50.9%)
Likelihood ratio positive (95% CI) =1.6 (1.3-1.9)
Likelihood ratio negative (95% CI) =0.2 (0.1-0.4)

Table 4: Performance of rESAT-6-CFP-10 antigen in the diagnosis of pulmonary TB in Ethiopia.

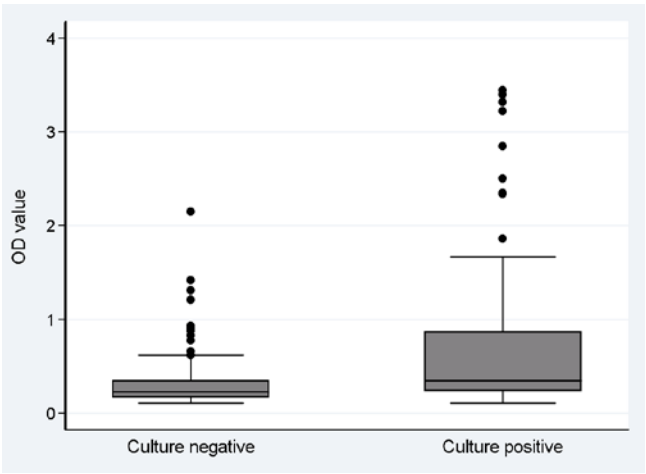


Figure 2: OD values for rESAT-6-CFP-10 antigen among culture positive and culture negative patients in Ethiopia. A box plot comparing the OD values for rESAT-6-CFP-10 antigen among culture positive pulmonary TB patients and culture negative non-TB patients. There is a clear difference between the two groups, culture confirmed pulmonary TB patients having a higher median OD value. The difference was found to be statistically significant (p<0.001).

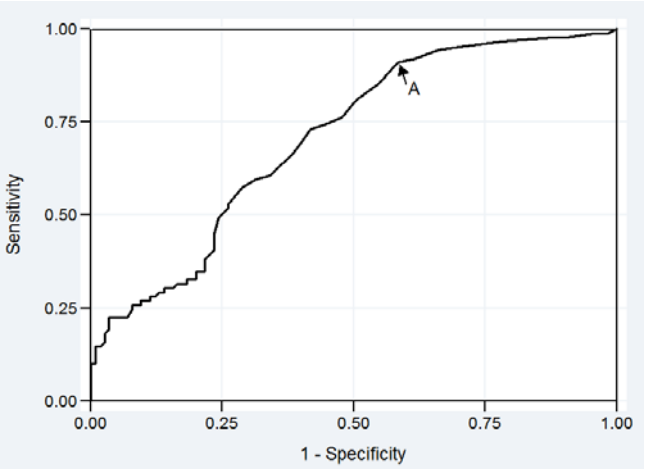


Figure 3: ROC curve of OD values for rESAT-6-CFP-10 antigen among pulmonary TB suspects in Ethiopia. This figure shows the sensitivity and 1-specificity of the antigen taking all possible cut-off values. The area under the curve is 0.71 (CI 0.64- 0.78). A cut-off value, 0.21, was selected when senstivity+specifcity-1 is maximum and the sensitivity and specificity became 91.0% and 41.7%, respecively. A: Sensitivity and 1-specificity values corresponding to OD value of 0.21

a lower sensitivity for rESAT-6 (37%) and rCFP-10 (46%). However, in this study, the cut-off value was determined when the specificity is 100% for each antigen. A lower specificity in our study might be attributable to the high prevalence of TB infection in the study area as well as the use patients rather than healthy controls.

Generally, differences in the performance of the same antigen in different settings have been reported frequently. The difference in the sensitivity and specificity of an antigen across studies might be attributable to differences in the study population, the stage of illness, choice of control groups, choice of cut-off values as well as choice of the reference standard. We used culture as a reference test which is the best test available but its sensitivity is not high enough to accurately classify all suspects as TB and non-TB patients and therefore some TB patients might have been misclassified as non-TB patients affecting the sensitivity and specificity of ELISA.

We found a higher sensitivity among smear positive compared to smear negative but culture positive pulmonary TB patients, a finding in agreement with previous reports [20-25]. Moreover, the sensitivity as well as the specificity of the antigen among HIV negative pulmonary TB patients in the present study were higher compared to the corresponding sensitivity and specificity among HIV positive pulmonary TB patients which is in agreement with a review on commercial rapid tests for the diagnosis of pulmonary TB [4]. HIV is known for immune suppression and it might reduce the humoral immune response to mycobacterial antigens among HIV co-infected patients resulting in lower sensitivity of TB rapid tests.

Interestingly, we found a significant difference in the IgG-specific immune response to rESAT-6-CFP-10 antigen between BCG vaccinated and non-vaccinated patients, those vaccinated having higher median OD values. This is consistent with the findings of the study from China [10]. Generally, the RD-1 which codes ESAT-6 and CFP-10 is absent from BCG strains [26] and therefore, it is not clear from this study why such differences in the IgG-specific immune response between BCG vaccinated and non-vaccinated was observed. One possible explanation could be due to shared epitopes of the antigen and the BCG vaccine as suggested by Wu et al [10]. Further study is needed to clarify this association since it has an implication in countries where BCG is routinely used.

The clinical impact of serodiagnosis using rESAT-6-CFP-10 antigen was not evaluated in this study. The other limitation of this study is that it was not designed to evaluate the use of the serodiagnostic test as a triage test followed by other tests like microscopy.

Conclusions

The sensitivity of this antigen is high. The sensitivity remained high even in smear negative as well as HIV positive pulmonary TB patients; however, the specificity of the antigen is low with high false positive rate and therefore, it can't be used as a diagnostic tool in the study area. Further study is needed to evaluate its value when used as a triage test together with smear microscope and chest x-ray.

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