



Rapid diagnosis of pulmonary tuberculosis by combined molecular and immunological methods

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Nearly all patients with active pulmonary TB can be rapidly identified when two diagnostic methods are combined <http://ow.ly/vinu30j5Yk7>

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ABSTRACT Diagnosing pulmonary tuberculosis (TB) may be delayed until culture results become available.

We ascertained the accuracy of a stepwise diagnostic algorithm for the rapid diagnosis of pulmonary TB by GeneXpert from sputum and/or bronchoalveolar lavage (BAL) followed by a *Mycobacterium tuberculosis*-specific BAL ELISPOT assay in patients with a suspected diagnosis of pulmonary TB at a clinical referral centre in Germany.

Among 166 patients with a presumptive diagnosis of pulmonary TB, 81 cases were confirmed by *M. tuberculosis* culture from sputum and/or BAL. In 66 out of 81 (81.5%) cases, patients initially had *M. tuberculosis* detected by GeneXpert from sputum; in addition, six out of 81 (7.4%) cases were diagnosed by GeneXpert on BAL fluid (together 72 out of 81 (88.9%) patients). Out of the remaining nine patients with negative GeneXpert results from sputum and BAL, BAL ELISPOT identified eight patients with culture-confirmed TB correctly (median time to culture positivity 26 days). At a cut-off of >4000 early secretory antigenic target-6- or culture filtrate protein-10-specific interferon- γ -producing lymphocytes per 1 000 000 lymphocytes, the specificity of the BAL ELISPOT for active TB was 97%.

In low TB incidence countries, nearly all patients with active pulmonary TB can be identified within the first few days of clinical presentation using a stepwise strategy with GeneXpert and BAL ELISPOT.

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Introduction

According to the World Health Organization, 10.4 million new cases of tuberculosis (TB) occurred worldwide in 2016 [1]. Early diagnosis of pulmonary TB is crucial to avoid transmission of *Mycobacterium tuberculosis*. *M. tuberculosis* culture remains the gold standard for TB diagnosis, but it takes 2–5 weeks on average until culture results become available [2, 3]. The detection of acid-fast bacilli (AFB) by sputum smear microscopy is a widely used, inexpensive screening test in patients with a presumptive diagnosis of pulmonary TB. However, <50% of all notified cases are sputum smear-positive [4]. Furthermore, microscopy does not distinguish *M. tuberculosis* from other mycobacterial species. Nucleic acid amplification techniques such as GeneXpert (Xpert MTB/RIF; Cepheid, Sunnyvale, CA, USA) have a short time to result of <2 h, and are highly sensitive and specific for the detection of *M. tuberculosis* DNA on AFB smear-positive samples. However, the sensitivity of GeneXpert for the diagnosis of pulmonary TB decreases to <70% for smear-negative cases [5]. Thus, ~15% of adult patients with pulmonary TB remain undetected when initially investigated by GeneXpert.

Following contact with antigen-presenting cells, *M. tuberculosis*-specific T-cells clonally expand in regional lymph nodes and migrate to the site of the infection where they differentiate into effector T-cells [6–8]. After loss of special phenotypical T-cell surface markers, these cell populations are probably unable to re-enter the bloodstream and become enriched at the site of disease [9, 10]. In contrast to detection of *M. tuberculosis*-specific effector T-cells in peripheral blood, detection of *M. tuberculosis*-specific effector T-cells by the interferon (IFN)- γ release assay at the site of the infection has a high diagnostic accuracy for active TB even when AFB and *M. tuberculosis* DNA are not detectable from local samples such as bronchoalveolar lavage (BAL) [11–15], pleural fluid [16, 17], pericardial fluid [18], cerebrospinal fluid [19] or ascites [20]. ELISPOT test results are available within 24 h.

In order to ascertain the diagnostic accuracy of the stepwise testing of sputum or BAL by GeneXpert followed by *M. tuberculosis*-specific BAL ELISPOT for the rapid diagnosis of active pulmonary TB, we prospectively enrolled patients with a presumptive diagnosis of pulmonary TB at a clinical TB referral centre in Germany.

Methods

Study participants

We performed a prospective analysis of all patients (age ≥ 17 years) admitted to the Medical Clinic of the Research Center Borstel, Germany, between November 2011 and June 2016, presenting with symptoms and/or chest radiography suggestive of pulmonary TB. Patients who had received treatment for TB within the 2 years prior to admission and patients with extrapulmonary TB were excluded from the study. As the initial diagnosis, sputum smear microscopy and/or GeneXpert was performed on up to three sputum samples. In the absence of detectable AFB in smears and/or negative *M. tuberculosis*-specific nucleic acid tests on sputum, bronchoscopy with BAL was performed according to national guidelines [21]. Pooled BAL was divided into two aliquots: one for ELISPOT, and one that was sent for BAL microscopy, GeneXpert and culture to the National Reference Center for Mycobacteria in Borstel. The study flowchart is shown in figure 1.

The study was approved by the Ethics Committee of the University of Lübeck (14-031A). Reporting follows the STARD criteria [22].

GeneXpert

GeneXpert was performed on sputum and BAL according to the manufacturer's guidelines at the National Reference Center for Mycobacteria [23].

M. tuberculosis-specific ELISPOT

M. tuberculosis-specific ELISPOT was performed on peripheral blood mononuclear cells (PBMCs) and BAL cells (BALCs) as previously described [12]. Results of *M. tuberculosis*-specific ELISPOT were considered positive if more than five spot-forming cells (SFCs) were observed in the early secretory antigenic target (ESAT)-6 or the culture filtrate protein (CFP)-10 well, after subtracting the number of SFCs in the negative control well, and if the total number of SFCs in the ESAT-6 or CFP-10 well was at least twice the number of SFCs in the negative control well. The results were considered negative if they did not meet the definition for a positive result and if the number of SFCs in the positive control (anti-CD3, clone X35, 10 ng·mL⁻¹; Beckman Coulter, Krefeld, Germany) was >20 SFCs after subtracting the number of SFCs in the negative control well and if the positive control had at least twice the number of SFCs of the negative control well. Indeterminate results were defined as not meeting the criteria for a positive or a negative test result. Due to the limited number of cells in BAL, some ELISPOT tests were performed with less than 250 000 cells per well; these results were normalised for 250 000 cells per well.

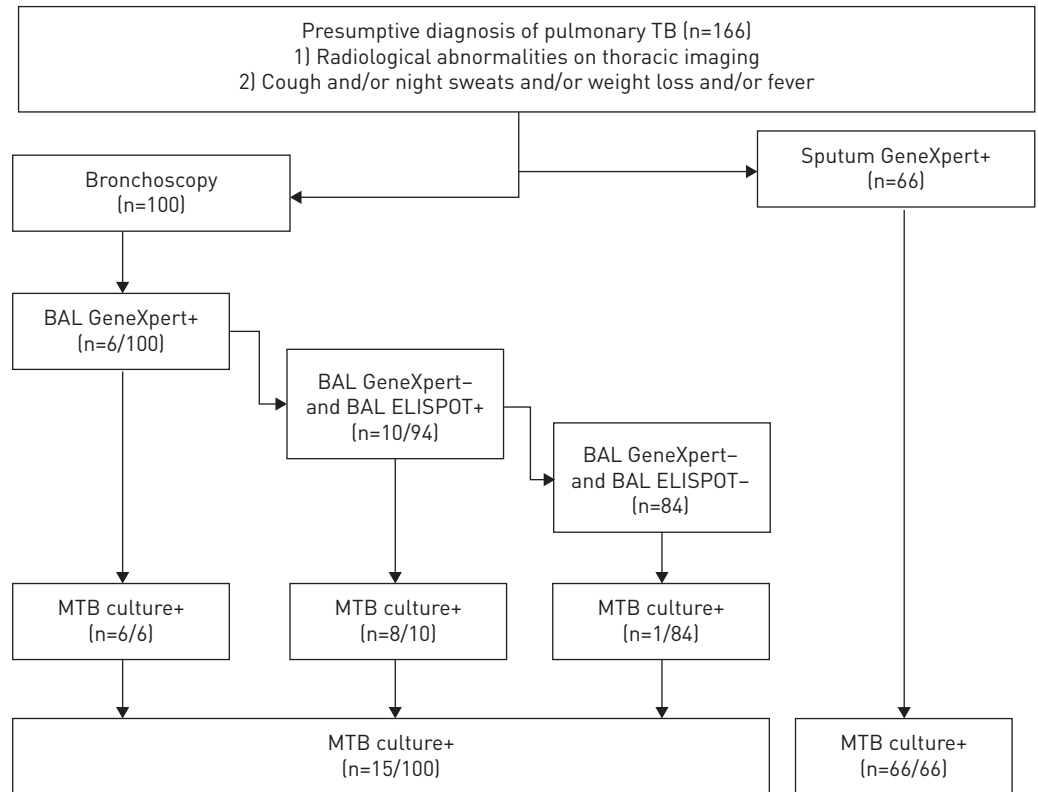


FIGURE 1 Study flowchart. TB: tuberculosis; BAL: bronchoalveolar lavage; MTB: *Mycobacterium tuberculosis*.

Defining the lymphocyte population by flow cytometry analysis

To adjust for the different percentages of lymphocytes in blood and lavage, 500 000 PBMCs or BALCs were acquired on a FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany). The respective percentage of lymphocytes out of the population of PBMCs or BALCs was used to extrapolate SFCs per 250 000 cells per well to 1 000 000 lymphocytes in blood or BAL specimens [12]. Recruitment of *M. tuberculosis*-specific lymphocytes to the lungs was calculated by dividing the number of SFCs per 1 000 000 lymphocytes in BALCs by the number of SFCs per 1 000 000 lymphocytes in peripheral blood. As previously described, if the denominator had zero SFCs, a value of 0.1 was assigned [12, 24].

Statistical analysis

Statistical analysis was performed using Stata version 14 (StataCorp, College Station, TX, USA) and CATmaker version 1.1 (Center for Evidence-Based Medicine, Oxford, UK). The Mann-Whitney U-test was used to compare groups of continuous variables. The cut-offs were selected using receiver operating characteristic (ROC) curve analysis. The significance level was set at $\alpha=0.05$.

Results

Between November 1, 2011 and June 30, 2016, 412 patients with a presumptive or confirmed diagnosis of TB were admitted to the Medical Clinic of the Research Center Borstel. Of these, 196 patients already had a confirmed diagnosis of pulmonary TB. Another 50 patients were diagnosed with extrapulmonary TB. These patients were excluded. Of the remaining 166 individuals, who were admitted with presumptive pulmonary TB, 66 patients had a microbiological confirmation of the initial diagnosis made by a positive GeneXpert result on sputum. The remaining 100 individuals, in whom the diagnosis of TB could not be confirmed by sputum smear microscopy and sputum GeneXpert, but where pulmonary TB was still part of the differential diagnosis, underwent bronchoscopy with BAL.

The median (interquartile range (IQR)) age of these 100 patients was 52.0 (35.5–63.0) years and 61 (61%) were male.

One patient with culture-confirmed TB and one patient without TB were tested HIV-positive.

15 out of 100 patients had culture-confirmed TB. Six patients with culture-confirmed TB (40%) had a rapid diagnosis of TB by a positive BAL GeneXpert result (of whom two also had detectable AFB on BAL microscopy; four did not have detectable AFB in BAL). Thus, there were nine out of 100 (9%) patients with negative test results on sputum and BAL by microscopy and GeneXpert that had culture-confirmed diagnosis of TB with a median (IQR) time to culture positivity of 26 (16.5–34.5) days. Patient characteristics are shown in table 1.

Among the 100 patients who underwent bronchoscopy, four were diagnosed with nontuberculous mycobacterial (NTM) pulmonary infections. Two patients had evidence of AFB in BAL and GeneXpert did not detect *M. tuberculosis*; the mycobacterial species identified from culture in these patients were *Mycobacterium avium* and *Mycobacterium simiae*. Two individuals with negative results on BAL microscopy and GeneXpert had a positive culture from BAL for *Mycobacterium xenopi* and *Mycobacterium kansasii*. Samples from the remaining 81 patients did not show mycobacterial growth. Patient characteristics and details on the final diagnosis of the patients who underwent bronchoscopy but were ultimately diagnosed as not having pulmonary TB are shown in supplementary table S1.

PBMC and BALC ELISPOT

Among 100 patients with a presumptive diagnosis of pulmonary TB, one had an indeterminate test result in PBMCs. In one out of 100 (1%) there was failure of the positive control in the PBMC ELISPOT. In five out of 100 (5%) the BALC ELISPOT was indeterminate: two had failure of the negative control and three had failure of the positive control. Using the manufacturer's definition of a positive test result, among the patients with valid test results, PBMC and BALC ELISPOT were positive in 62 out of 99 (62.6%) and 40 out of 95 (42.1%) individuals, respectively. Active TB could be excluded in 34 out of 62 (54.8%) individuals with a positive PBMC ELISPOT and 25 out of 40 (62.5%) individuals with a positive BALC ELISPOT. Out of 15 patients with culture-confirmed pulmonary TB, PBMC ELISPOT was positive in 14 out of 15 (93.3%) and negative in one out of 15 (6.7%). BALC ELISPOT was positive in 15 out of 15 (100%).

For patients with culture-confirmed pulmonary TB, PBMC ELISPOT had a sensitivity of 93% (95% CI 81–100%) and a specificity of 50% (95% CI 38–62%), while BALC ELISPOT had a sensitivity of 100% and a specificity of 77% (95% CI 67–87%) when the manufacturer's definitions of positive and negative test results on PBMCs were used.

Table 2 shows all individual test results for the nine culture-confirmed pulmonary TB patients with negative AFB and negative GeneXpert results in BAL. The respective numbers of positive or negative paired PBMC and BALC ELISPOT results in patients with culture-confirmed pulmonary TB and patients without TB are given in supplementary figure S1.

Concentration of antigen-specific SFCs at the site of infection

In patients with culture-confirmed pulmonary TB, the median (IQR) SFC count was 25 (6.3–210) per 250 000 PBMCs for ESAT-6 and 53.8 (22.5–177) per 250 000 PBMCs for CFP-10. BALC ELISPOT median (IQR) was elevated to 295 (52.5–489) SFCs per 250 000 BALCs for ESAT-6 and 160 (61–453) SFCs per

TABLE 1 Individual test results and patient characteristics of the nine patients with culture-confirmed tuberculosis with undetectable acid-fast bacilli (AFB) in bronchoalveolar lavage (BAL) and negative GeneXpert results

Patient	Age years	Sex	Country of origin	MTB culture	AFB BAL	GeneXpert BAL	PBMC ELISPOT	BALC ELISPOT	TPP days
1	30	Male	Somalia	+	–	–	+	+	17 [#]
2	31	Male	Ghana	+	–	–	+	+	31 [#]
3	69	Male	Germany	+	–	ITR	+	+	16 [¶]
4	62	Female	Afghanistan	+	–	–	+	+	38 [¶]
5	27	Female	Germany	+	–	–	+	+	21 [¶]
6	43	Male	Germany	+	–	–	+	+	16 [#]
7	19	Female	Germany	+	–	–	+	+	28 [¶]
8	48	Male	Russia	+	–	–	+	+	26 [¶]
9	33	Male	Eritrea	+	–	–	+	+	39 [#]

MTB: *Mycobacterium tuberculosis*; PBMC: peripheral blood mononuclear cell; BALC: BAL cell; TPP: time to culture positivity of the first culture growing MTB; ITR: indeterminate test result. [#]: sputum; [¶]: BAL.

TABLE 2 Individual ELISPOT results and recruitment factors for the nine patients with culture-confirmed tuberculosis with negative acid-fast bacilli microscopy and negative GeneXpert results in bronchoalveolar lavage (BAL)

Patient [#]	SFCs per 250 000 cells				SFCs per 1 000 000 lymphocytes				Recruitment factor [¶]	
	PBMCs		BALCs		PBMCs		BALCs		ESAT-6	CFP-10
	ESAT-6	CFP-10	ESAT-6	CFP-10	ESAT-6	CFP-10	ESAT-6	CFP-10		
1	473	301	407	279	3300	2100	15654	10731	4.7	5.1
2	210	195	526	351	1761	1635	6370	4251	3.6	2.6
3	291	64	450	621	1229	270	4780	6599	3.9	24.4
4	6	6	546	479	44	44	12248	10734	279.2	244.7
5	2.5	10	251	160	12	47	6058	3858	514.9	81.9
6	25	23	295	349	122	110	18820	21930	145.3	199.8
7	15	33	176	159	73	161	5949	5359	81.3	33.3
8	20	29	52.5	50	113	162	4595	4376	40.8	27
9	5	47	6	61	24	229	107	1085	4.4	4.7

SFC: spot-forming cell; PBMC: peripheral blood mononuclear cell; BALC: BAL cell; ESAT: early secretory antigenic target; CFP: culture filtrate protein. [#]: see table 1 for patient characteristics; [¶]: SFCs in BALCs/SFCs in PBMCs multiplied by the lymphocyte factor (% of lymphocytes in PBMCs/% lymphocytes in BALCs).

250 000 BALCs for CFP-10. In contrast, patients without TB had a median (IQR) of 3.8 (0.1–17.5) SFCs per 250 000 PBMCs for ESAT-6 and 4.3 (0.1–16.9) SFCs per 250 000 PBMCs for CFP-10, and in the BAL the median for both ESAT-6 and CFP-10 was low with 0 SFCs per 250 000 BALCs. Figure 2 and supplementary figure S1 show the number of SFCs in 250 000 PBMCs and BALCs in patients with

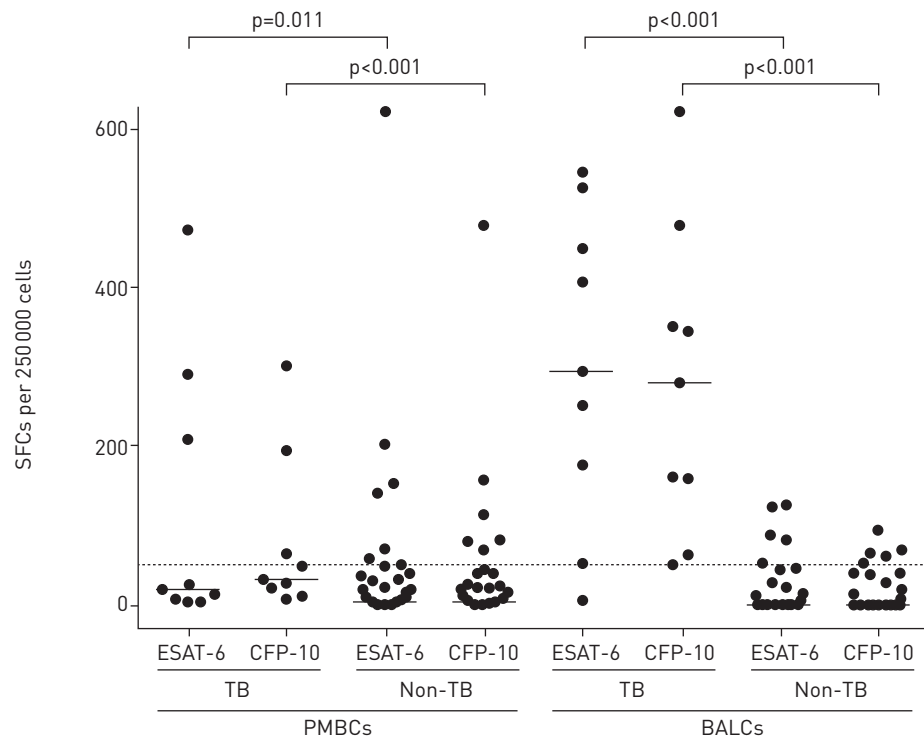


FIGURE 2 Number of spot-forming cells (SFCs) of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10-specific peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage cells (BALCs) per 250 000 cells in individuals where tuberculosis was excluded (non-TB) and in patients with culture-confirmed pulmonary TB with undetectable acid-fast bacilli on microscopy and negative GeneXpert results (n=9). A suggested cut-off >50 SFCs per 250 000 cells is indicated.

culture-confirmed pulmonary TB but undetectable AFB on microscopy and negative GeneXpert results compared with patients in whom TB had been excluded.

Concentration of antigen-specific lymphocytes at the site of infection

To account for the different percentages of lymphocytes between individuals, SFC results in PBMCs and BAL were normalised for 1 000 000 lymphocytes. In patients with culture-confirmed pulmonary TB, the median (IQR) of antigen-specific lymphocytes per 1 000 000 in peripheral blood was 121 (43.9–1228.8) SFCs for ESAT-6 and 270 (109.8–1363.8) SFCs for CFP-10, while in the BAL the median (IQR) was 6370 (4595.2–14 541.7) SFCs for ESAT-6 and 6598.5 (3857.7–13 503.7) SFCs for CFP-10. By comparison, patients with a diagnosis other than TB had a median (IQR) of 25.5 (0.8–99.9) SFCs for ESAT-6- and 27 (0.7–93.8) SFCs for CFP-10-specific lymphocytes in peripheral blood, while in BAL they had a median (IQR) of 0 (0–0) SFCs for ESAT-6- and 0 (0–0) SFCs for CFP-10-specific lymphocytes per 1 000 000 lymphocytes. Figure 3 and supplementary figure S2 show the number of SFCs per 1 000 000 lymphocytes in PBMCs and BALCs in patients with culture-confirmed pulmonary TB but negative AFB microscopy/negative GeneXpert results compared with patients without TB.

Differentiation of active TB from non-TB

To improve the discrimination between patients without TB and those with culture-confirmed pulmonary TB but undetectable AFB on microscopy and negative GeneXpert results in BAL, different cut-offs were investigated. A cut-off of >50 SFCs for ESAT-6- or CFP-10-specific cells from 250 000 BALCs provides a sensitivity of 100% and a specificity of 91% (95% CI 84–98%). Considering the proportion of lymphocytes, a cut-off of >4000 antigen-specific lymphocytes for either ESAT-6 or CFP-10 per 1 000 000 BAL lymphocytes had a sensitivity of 89% (95% CI 68–100%) and a specificity of 97% (95% CI 93–100%). To account for the different percentages of lymphocytes in BAL and peripheral blood, a recruitment factor was defined as SFCs in BALCs/SFCs in PBMCs multiplied by the lymphocyte factor (% of lymphocytes in PBMCs/% lymphocytes in BALCs). A recruitment factor of at least 6 for ESAT-6 or CFP-10 had a sensitivity of 67% (95% CI 36–97%) and a specificity of 83% (95% CI 74–92%).

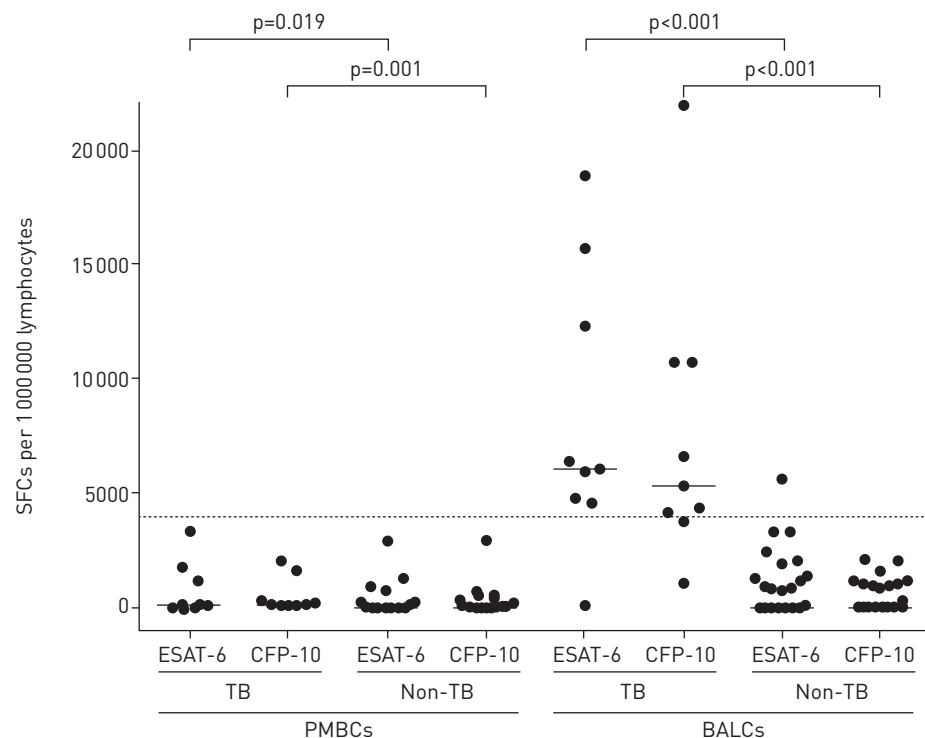


FIGURE 3 Number of spot-forming cells (SFCs) of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10-specific peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage cells (BALCs) per 1 000 000 lymphocytes in individuals where tuberculosis was excluded (non-TB) and in patients with culture-confirmed pulmonary TB with undetectable acid-fast bacilli on microscopy and negative GeneXpert results (n=9). A suggested cut-off >4000 SFCs per 1 000 000 lymphocytes is indicated.

TABLE 3 Performance of different cut-offs of culture filtrate protein (CFP)-10 or early secretory antigenic target (ESAT)-6 for *Mycobacterium tuberculosis*-specific bronchoalveolar lavage cell (BALC) ELISPOT for the diagnosis of active tuberculosis

CFP-10 or ESAT-6 cut-off	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	CC %	LR+ % (95% CI)	LR- % (95% CI)	False positive (treated unnecessarily)	AUC	
									ESAT-6	CFP-10
>50 BALCs	100 (100–100)	91 (84–98)	60 (35–85)	100 (100–100)	91.9	10.8 (5.1–23.2)	0 [#]	6/100 (6%)	0.968	0.985
>4000 BAL lymphocytes	89 (68–100)	97 (93–100)	80 (55–100)	98 (95–100)	95.9	28.9 (7.2–115.3)	0.1 (0.02–0.7)	2/100 (2%)	0.973	0.978
Recruitment factor $\geq 6^{\ddagger}$	67 (36–97)	83 (74–92)	35 (13–58)	95 (89–100)	80.8	3.9 (1.9–7.9)	0.4 (0.2–1)	11/100 (11%)	0.879	0.910
Ratio $\geq 2^*$	78 (51–100)	84 (75–93)	41 (18–65)	96 (92–100)	83.6	5 (2.6–9.7)	0.26 (0.08–0.9)	10/100 (10%)	0.892	0.915

The test performance was computed for the 100 individuals who underwent bronchoscopy with BAL. PPV: positive predictive value; NPV: negative predictive value; CC: correctly classified; LR: likelihood ratio; AUC: area under the receiver operating characteristic curve. [#]: 95% CI cannot be calculated; [†]: spot-forming cells (SFCs) in BALCs/SFCs in PBMCs multiplied by the lymphocyte factor (% of lymphocytes in PBMCs/% lymphocytes in BALCs); ^{*}: SFCs in BALCs/SFCs in PBMCs.

The area under the ROC curve (AUC) to differentiate patients without TB from the subgroup of patients with culture-confirmed pulmonary TB but undetectable AFB on microscopy and negative GeneXpert results was 0.968 for ESAT-6- and 0.985 for CFP-10-specific BALCs, while the AUC for PBMCs was 0.760 for ESAT-6 and 0.841 for CFP-10. When evaluating the 1 000 000 lymphocytes from the BALCs only, the AUC was 0.973 for ESAT-6 and 0.978 for CFP-10.

Table 3 shows the performance of ELISPOT on BALCs using these different cut-offs. Supplementary figure S3 shows the graphs of the respective AUCs.

In summary, among 166 patients who were admitted with a presumptive diagnosis of pulmonary TB, the diagnosis was confirmed by *M. tuberculosis* culture in 81 patients (48.8%). 66 out of 81 (81.5%) initially had a positive GeneXpert result from sputum and in addition six out of 81 (7.4%) were diagnosed by BAL GeneXpert (together 72 out of 81 (88.9%)). In eight out of the nine out of 81 (88.8%) patients with negative results on sputum and BAL by microscopy and GeneXpert, BAL ELISPOT identified patients with culture-confirmed TB correctly with a specificity of 97%.

With a stepwise analysis of GeneXpert (2 h) and BAL ELISPOT only for patients with a negative BAL GeneXpert test result, the combined sensitivity and specificity for a rapid diagnosis of pulmonary TB was 98.8% and 97.6%, respectively (table 4).

Discussion

We evaluated the accuracy of a stepwise approach consisting of an initial molecular test, *i.e.* GeneXpert, followed by a *M. tuberculosis*-specific BAL ELISPOT for the diagnosis of pulmonary TB. Using BAL GeneXpert and BAL ELISPOT sequentially, the diagnostic accuracy for sputum smear- and GeneXpert-negative pulmonary TB was 97%. The diagnosis became available within the first days of presentation to the hospital.

TABLE 4 Performance of a combined approach of GeneXpert and bronchoalveolar lavage cell (BALC) ELISPOT for the diagnosis of active tuberculosis

Cut-off	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	CC %	LR+ % (95% CI)	LR- % (95% CI)	False positive (treated unnecessarily)
Combined GeneXpert and ELISPOT >4000 BAL lymphocytes	99 (96–100)	98 (94–100)	98 (94–100)	99 (96–100)	98.2	42 (10.7–165.2)	0.01 (0–0.1)	2/166 (1.2%)

Test performance of this stepwise approach of combined GeneXpert and ELISPOT refers to 166 individuals (100 BAL and 66 sputum). PPV: positive predictive value; NPV: negative predictive value; CC: correctly classified; LR: likelihood ratio.

GeneXpert has greatly improved the diagnosis of TB. If GeneXpert is performed on two different sputum samples, >90% of adult patients with pulmonary TB are correctly identified by GeneXpert within the first days of presentation to a healthcare facility [25]. However, the sensitivity of GeneXpert is lower in sputum smear-negative and in extrapulmonary TB [25, 26]. If results from GeneXpert tests are negative, patients could remain undiagnosed for several weeks until results of *M. tuberculosis* cultures become available.

M. tuberculosis-specific ELISPOT performed on cells from BAL is able to close this diagnostic gap. In the past decade several studies have shown that *M. tuberculosis*-specific ELISPOT performed on specimens from the site of the disease, e.g. BAL in pulmonary TB, pleural or pericardial effusion in TB pleurisy or TB pericarditis, respectively, ascites in TB peritonitis, or cerebrospinal fluid in TB meningitis [27–30], can improve the case detection rate of TB in paucibacillary disease, but the added value of a stepwise approach of GeneXpert and ELISPOT had not been investigated so far.

In active TB, *M. tuberculosis*-specific lymphocytes are recruited from the blood into the disease-affected organ [31, 32]. Active disease is more likely with increasing concentration of *M. tuberculosis*-specific cells at the site of infection compared with peripheral blood [24]. HOFLAND *et al.* [33] suggested a ratio of SFCs in BAL/SFCs in blood with a cut-off >1. However, one needs to consider that lymphocytes predominate in peripheral blood, whereas the proportion in BAL is considerably lower [34]. These differences in percentages of lymphocytes in PBMCs and BALCs should be acknowledged individually by calculating a lymphocyte-dependent recruitment factor of ESAT-6- and CFP 10-specific T-cells between blood and BALCs or by normalising the SFCs per 250 000 cells to 1 000 000 lymphocytes [11, 24].

Optimal result were obtained in this study with a cut-off of >4000 lymphocytes per 1 000 000 lymphocytes in BAL. When applying this cut-off, patients with paucibacillary pulmonary TB could be identified with a sensitivity of 89% and a specificity of 97%. This cut-off provided an excellent negative predictive value of 98% and a high positive likelihood ratio of 28.9.

As with most studies from low TB incidence countries, our study is limited by the sample size of patients with culture-confirmed active TB and negative GeneXpert results from sputum and BAL. Performing BAL ELISPOT and flow cytometry is also substantially more labour intensive and requires considerably more technician time than automated *M. tuberculosis*-specific nucleic acid amplification by GeneXpert. However, most patients who are suspected of having pulmonary TB in low TB incidence countries with sufficient economical resources will undergo bronchoscopy when results of sputum smear microscopy and sputum GeneXpert analysis are negative, and also to exclude alternative diseases such as sarcoidosis, cryptogenic organising pneumonia, lung cancer and others. Furthermore, the results cannot be generalised to high TB burden countries, where repeated exposure to *M. tuberculosis* may alter BAL ELISPOT responses [14] and performance of ELISPOT would be challenging due to limited laboratory capacity.

Due to the high sensitivity for pulmonary TB of GeneXpert performed on respiratory specimens, the performance of *M. tuberculosis*-specific BAL ELISPOT can be limited to patients where the GeneXpert results from sputum and BAL are negative and when the differential diagnosis of active pulmonary TB is still to be considered.

In conclusion, in low TB incidence countries, nearly all patients with active pulmonary TB can be identified within the first few days of clinical presentation by using a stepwise testing strategy with GeneXpert and BAL ELISPOT.

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