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LIOFeron®TB/LTBI: A novel and reliable test for LTBI and tuberculosis

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ABSTRACT

Objectives: High accuracy diagnostic screening tests for tuberculosis (TB) are required to improve the diagnosis of both active TB and latent *Mycobacterium tuberculosis* (MTB) infection (LTBI). The novel IGRA LIOFeron®TB/LTBI assay was tested and its accuracy was compared to the QuantiFERON®-TB Gold Plus assay.

Methods: A total of 389 subjects were enrolled in two cohorts and classified as healthy, active TB or LTBI persons. The blood of all the patients was tested with LIOFeron®TB/LTBI assay, containing MTB alanine dehydrogenase, able to differentiate active TB from LTBI diagnosis. The results obtained with both IGRAs, performed on the same 250 samples, were finally compared.

Results: The two assays demonstrated an excellent concordance of their results with patients' diagnosis of MTB infection. ROC analysis for QuantiFERON®-TB Gold Plus showed sensitivity and specificity respectively of 98% and 97% in diagnosing active TB patients and 85% and 94% in diagnosing LTBI subjects. LIOFeron®TB/LTBI assay showed sensitivity and specificity respectively of 90% and 98% in diagnosing active TB patients and 94% and 97% in diagnosing LTBI subjects.

Conclusions: The two IGRAs displayed the same high accuracy in diagnosing MTB infection/TB disease, and LIOFeron®TB/LTBI assay demonstrated higher sensitivity than QuantiFERON®-TB Gold Plus test in LTBI detection.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB) *complex* organisms, is still one of the most prevalent human infections worldwide with 1.2 million deaths among HIV-negative people and an additional 251,000 deaths from TB among HIV-positive people, estimated in 2018. Moreover, 1.7 billion people in the world's population harbour a latent tuberculosis infection (LTBI) and thus they are at risk of developing active TB disease during their lifetime (World Health Organization, 2019) The preventive treatment of LTBI subjects is a key point to reduce TB at the individual level, according to the WHO post 2015 End TB strategy. The early identification and treatment of patients with LTBI can reduce the burden of active TB, especially if the screening is performed on populations at high risk for progression to active disease, such as people with HIV infection or other immune deficiency, children

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aged under 5 years, and household contacts with widespread exposure (all ages) to someone who has bacteriologically confirmed pulmonary TB. (World Health Organization, 2018; World Health Organization, 2014; Rangaka et al., 2015).

Thus, urgent action is required to improve the diagnosis of both active TB and latent infection in order to set-up new screening tests for MTB that can be used for public health to detect and treat individuals who are at high risk of developing active TB (World Health Organization, 2018; Amicosante et al., 2017).

The MTB infection elicits a T cell immune response: class II MHC-restricted $CD4^+$ T cells are essential for immunity against MTB, however also efficient $CD8^+$ T cells are required for optimum resolution of the infection. Effector helper and cytotoxic T cells produce type 1 cytokines; the IFN-γ released by T cells triggers microbicide activity in macrophages which kill, or at least suppress, the bacterial growth (Behar, 2013).

Interferon-γ release assays (IGRAs) are based on the IFN-γ detection by enzyme-linked immunosorbent assay (ELISA) into plasma samples from MTB antigens stimulated whole blood. The amount of IFN-γ produced is an index of the cell-mediated immune response to MTB infection.

The TST (Tuberculin skin test) and the Interferon-gamma release assay (IGRA) are current indirect methods for TB screening; neither IGRAs nor TST can distinguish active TB from LTBI (de Lima Corvino and Kosmin, 2019; Gualano et al., 2019). Current IGRA tests based on ESAT-6 and CFP-10 antigens, unlike TST, are more specific laboratory in-vitro tests that have been optimized by using MTB-specific antigens. In particular IGRAs don't show cross-reactions with Bacillus Calmette-Guérin (BCG) vaccine in immunized subjects and with *Non tubercular Mycobacteria* (NTM) infection (except for *Mmarinum*, *M. kansasii*, and *M. szulgai,* for which a small potential for cross-reaction therefore remains) (Bastian and Coulter, 2017; Andersen et al., 2000; Qiagen, 2016)

In 2019, Lionex GmbH (Braunschweig, Germany) has introduced a novel IGRA test called LIOFeron®TB/LTBI which, for the first time, contains alanine dehydrogenase (Ala-DH) of MTB (Lionex propriety). This antigen is not produced by BCG (Jungblut et al., 1999) and it is known that MHC class I-restricted T $CD8^+$ lymphocytes epitopes are present in Ala-DH (Dong et al., 2004). Another interesting property of this antigen is that it has been reported to be involved in adaptation of MTB to the anaerobic dormant stage in LTBI (Tripathi and Ramachandran, 2008; Agren et al., 2008). We have recently reported that, based on IL-2 measurement, Ala-DH can be used to differentiate active TB from LTBI diagnosis (Della Bella et al., 2018).

The QuantiFERON®-TB Gold Plus (Qiagen, Hilden, Germany) and the LIOFeron®TB/LTBI are two-component kits consisting of a four human blood stimulation tubes kit (positive control, negative control and two test tubes containing TB antigens) and an IFN-γ ELISA. Both these assays are capable of measuring the T cell response to specific MTB antigens.

The QuantiFERON®-TB Gold Plus assay includes a TB-1 vial containing ESAT-6 and CFP-10 peptides stimulating $CD4^+$ T cell response and a second antigen tube (TB-2) with further shorter peptides from ESAT-6 and CFP-10, designed to stimulate $CD8^+$ T-cells too. Indeed, although $CD4^+$ T cells are the predominant cell type producing IFN-γ in response to MTB infection, cytotoxic T lymphocytes (CTL) can also contribute to control contamination by lysing infected cells and releasing IFN-γ upon recognition of their target (Rueda et al., 2010; Jasenosky et al., 2015).

The LIOFeron®TB/LTBI test is designed like QuantiFERON®-TB Gold Plus, but it differs strongly since in LIOFeron®TB/LTBI the first antigen tube (TB-A) contains full-length ESAT-6, CFP-10 and TB7.7, and in the second antigen tube (TB-B), the highly purified recombinant Ala-DH is included. No other antigen or peptide is included in the second antigen tube (TB-B).

The interpretation of the results of both tests is summarised in Table 1.

The aim of this study was to test the performance of the novel IGRA LIOFeron®TB/LTBI test in samples from active TB and latent TB infected patients and in comparison to the QuantiFERON®-TB Gold Plus assay which is currently widely used.

Materials and methods

Study participants

Ethics statements

The study design and all procedures were reviewed and approved by Florence and Almaty Local Ethical Committees. Informed consent was obtained for all enrolled subjects of both cohorts and the privacy rights of all participants were observed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Florence cohort

A total of 250 subjects were consecutively enrolled in Florence University Hospital: 151 healthy subjects, 66 active TB patients and 33 subjects with LTBI. Diagnosis of active TB cases relied on clinical, microbiological and radiological findings, in accordance with current guidelines (World Health Organziation, 2018; Hoppe et al., 2016; Lewinsohn et al., 2017); latent TB infection was defined by a previous positive tuberculin skin test (TST) or a previous positive

Table 1
How to interpret test results. The test is recorded as positive if the IFN-γ release in the antigen tube minus the negative control tube is ≥0.35 IU/mL and ≥25% of the negative control. A test is considered negative if the IFN-γ release of the antigen tube minus the negative control is <0.35 IU/mL or <25% of the negative control and the positive control (mitogen tube) is ≥0.5 IU/mL. A test is considered indeterminate if the IFN-γ release of the negative control is >8 IU/mL or the antigen tube minus the negative control is <0.35 IU/mL or <25% of the negative control with a mitogen control of <0.5 IU/mL (Lionex GmbH, 2018).

Negative control (IU/ml)	First antigen tube minus Negative control (IU/ml)	Second antigen tube minus Negative control (IU/ml)	Positive control minus Negative control (IU/ml)	Test result
≤8.00	<0.35	<0.35	≥0.50	NEGATIVE
	≥0.35 and <25% of Negative control value	≥0.35 and <25% of Negative control value		
	<0.35	≥0.35 and <25% of Negative control value		
	≥0.35 and <25% of Negative control value	<0.35		
	≥0.35 and ≥25% of Negative control value	Not relevant	Not relevant	POSITIVE
	Not relevant	≥0.35 and ≥25% of Negative control value		
	<0.35	<0.35	<0.50	INDETERMINATE
	≥0.35 and <25% of Negative control value	≥0.35 and <25% of Negative control value		
	<0.35	≥0.35 and <25% of Negative control value		
	≥0.35 and <25% of Negative control value	<0.35		
>8.00	Not relevant	Not relevant	Not relevant	

QuantiFERON®-TB Gold test ("In tube" or "Plus"), obtained a few weeks or months before their enrollment in the study, in subjects without clinical symptoms nor radiological abnormalities at a chest X-ray.

The blood drawn by venipuncture was pre-tubed in lithium-heparin and then LIOFeron®TB/LTBI test and QuantiFERON®-TB Gold Plus assay were performed simultaneously, on the same sample, to better compare the two results.

Thirty-one subjects (24 healthy and 7 MTB infected) with LIOFeron®TB/LTBI test and 24 subjects (19 healthy and 5 MTB infected) with QuantiFERON®-TB Gold Plus assay had an indeterminate test result, so they were excluded from statistical analysis.

Almaty cohort

A group of 139 blood donors was recruited in Blood Center, Almaty, Kazakhstan; they were designated as "conditionally healthy" since all were negative by annual fluorographic examination according to The Instruction on Organization of Medical Care for Tuberculosis (Annex 1 to the Order of the Health Minister of the Republic of Kazakhstan, 2017).

Total number of subjects enrolled in two cohorts was 389.

LIOFeron®TB/LTBI assay

Whole-blood samples of all participants were pre-tubed in lithium-heparin vials and, within 16 h, 1 ml was pipetted into each of the four tubes (negative control, TB-A, TB-B and positive control) (Lionex, 2018). After gently shaking upside down ten times, tubes were upright incubated for 20 h at 37 °C. Following this overnight incubation, tubes were centrifuged for 15 min at 2500 RCF (g) to facilitate harvesting of plasma (200 μl). Harvested plasma samples were immediately frozen at −20 °C.

The second step was to quantify by human IFN-γ ELISA the T cells IFN-γ production that occurred during blood stimulation tubes incubation, in response to MTB antigens. The four plasma samples obtained from each subject were thawed; the plasma samples of 22 subjects were tested in one ELISA plate. IFN-γ standards were reconstituted and diluted 1:4 with incubation buffer to finally set four concentration points in the standard curve (4.0 IU/ml, 1.0 IU/ml, 0.25 IU/ml and the blank). 50 μl of detection antibody, ready to use, were distributed together with 50 μl of the standard or of the sample into each well of a 96-well plate anti-IFN-γ pre-coated. After 1 h of incubation and washing six times with the kit wash buffer, conjugate solution was added and incubated for 1 h more; then after six more washes, substrate solution was put inside each well. Colour reaction occurred within 10 min, thus the kit stop solution was dispensed to fix the result. Procedure time for this is approximately 2.5 h for one full ELISA plate and 15 min more for each extra plate. The optical density (OD) was quantified at 450 nm with a spectrophotometer (Multiskan Go, Thermo Scientific, Massachusetts, USA). ELISA data were computed with the validated LIOferon® software (free downloaded from the Lionex web page) to obtain the automatic generation of the standard curve, the interpolation of samples data to define their IFN-γ concentration (IU/ml), the blank subtraction and the 25% of negative value calculation.

Results were reported as positive, negative and indeterminate as explained in Table 1.

QuantiFERON®-TB Gold assay

According to the manufacturer's guidelines (Qiagen, 2016), patients blood samples were collected by venipuncture in a lithium-heparin tube and, within 16 h, 1 ml was transferred into each of the four stimulation tubes (Nil, TB-1, TB-2 and mitogen). After gently shaking, tubes were incubated for 20 h at 37 °C and, the day after, plasma was harvested and stored at −20 °C.

In order to measure T cells IFN-γ production in response to MTB antigen in the stimulation tubes, all plasmas were thawed and tested by QFT-plus ELISA kit. Validated QuantiFERON® software, available online on the manufacturer's website, was used to calculate results after photometric analysis at 450 nm. The results interpretation is summarised in Table 1.

Statistical analysis

Descriptive statistic was used for the calculation of absolute frequencies and percentages of qualitative data, as well as for mean and standard deviation of quantitative data.

The accordance of both IGRAs results with patient diagnosis was assessed by calculating the percentage of concordant results and by computing the Cohen's Kappa coefficient. There will be slight concordance for the value of $k = 0.2$, poor concordance for k value in the range 0.2 ± 0.4, moderate concordance for k value in the range 0.4 ± 0.6, substantial concordance for a k value in the range 0.6 ± 0.8, good concordance for k value in the range 0.8 ± 1 (Fleiss and Cohen, 1973).

Test performance in terms of sensitivity (ability of the test to identify the true positive subjects) and specificity (ability of the test to identify the true negative subjects) was evaluated for each IGRA by a Receiving Operating Characteristic (ROC) curve. The Area under the curve (AUC) is a parameter to assess the assay as not accurate for AUC = 0.5, poorly accurate for $0.5 \leq$ AUC <0.7, moderately accurate for $0.7 \leq$ AUC <0.9, highly accurate for $0.9 \leq$ AUC <1 and perfect for AUC = 1 (Swet, 1988). Youden's index (= Sensitivity ± [1- Specificity]) was also applied to evaluate the best cutoff in our experimental data distributions.

Analyses were carried out with the SPSS Statistics software package for Windows, version 20.0 (SPSS Italia SRL, Bologna, Italy).

Results

LIOFeron®TB/LTBI: sensitivity and specificity determination in Florence cohort

A total of 250 subjects were enrolled in this study and their characteristics are shown in Table 2.

31 subjects (24 healthy and 7 MTB infected) in LIOFeron®TB/LTBI test and 24 subjects (19 healthy and 5 MTB infected) in

Table 2
Sex and age of the Florentine selected study participants grouped by MTB infection diagnosis.

		Healthy subjects	MTB infected subjects	
			Active TB	LTBI
Total (N = 250)		151 (60.4%)	66 (26.4%)	33 (13.2%)
Sex	Male	72 (47.7%)	41 (62.1%)	18 (54.5%)
	Female	79 (52.3%)	25 (37.9%)	15 (45.5%)
Age (over 18) (Median-IQR)		48 (16)	38 (11)	54 (21)

Table 3
Agreement of both IGRAs results with patient diagnosis. Results are expressed as percentage of concordant results.

QuantiFERON®-TB Gold Plus			
Diagnosis of MTB infection	Tested samples (N)	Positive result (%)	Negative result (%)
Negative (Healthy)	132	0 (0%)	132 (100%)
Positive (Active TB)	61	59 (97%)	2 (3%)
Positive (LTBI)	33	28 (85%)	5 (15%)
TOTAL	226		
LIOFeron®TB/LTBI			
Diagnosis of MTB infection	Tested samples (N)	Positive result (%)	Negative result (%)
Negative (Healthy)	127	0 (0%)	127 (100%)
Positive (Active TB)	59	55 (93%)	4 (7%)
Positive (LTBI)	33	31 (94%)	2 (6%)
TOTAL	219		

QuantiFERON®-TB Gold Plus assay were excluded from statistical analysis because of their indeterminate test results.

Both IGRAs detected all (100%) the healthy subjects as negative. Focusing on QuantiFERON®-TB Gold Plus results, 87/94 (92.5%) patients with MTB infection (59/61 with active TB and 28/33 with LTBI) had a positive result and 7/94 (7.5%) were identified as false negative (2/61 with active TB and 5/33 with LTBI). In LIOFeron®TB/LTBI 86/92 (93.5%) MTB infected patients had a positive response and 6/92 (6.5%) were wrongly assessed as negative (4/59 with active TB and 2/33 with LTBI); results are shown in Table 3. Cohen's k coefficient was computed to evaluate the accordance between the diagnosis and the test result (positive/negative): a K = 0.93 was obtained for QuantiFERON®-TB Gold Plus, a K = 0.94 for LIOFeron®TB/LTBI, reaching for both assays an excellent concordance of their results with patients' diagnosis of MTB infection.

The overall accuracy (95% confidence interval, CI) was calculated for each IGRA by ROC analysis, first by adding tubes 1 + 2 results for QuantiFERON®-TB Gold Plus assay and tubes A + B results for LIOFeron®TB/LTBI assay in healthy subjects versus infected population groups. Then we focused on each tube and on active TB or LTBI. The IFN-γ release results (IU/ml) were calculated as the IFN-γ release into the antigen tube (TB-1, TB-2, TB-A, TB-B) minus the IFN-γ production into the negative control tube.

The QuantiFERON®-TB Gold Plus ROC curve analysing healthy subjects compared to MTB infected ones (tube 1 + 2) showed an AUC = 0.96 (highly accurate assay), with a sensitivity of 93% and a specificity of 94% at the best cutoff (0.51 IU/ml). Focusing only on active TB patients the sensitivity was 98% and the specificity 97% at the best cutoff (0.62 IU/ml); on LTBI patients the sensitivity was 85% and the specificity 94% at the best cutoff (0.51 IU/ml). The statistical analysis between healthy and infected enrolled persons was performed on TB-1 or TB-2 single tube data distributions with the following results respectively calculated at the manufacturer's recommended cutoff (0.35 IU/ml): tube 1 ROC curve showed an AUC = 0.96 with a sensitivity of 86% and a specificity of 98%; tube 2 ROC curve showed an AUC = 0.97 with a sensitivity of 89% and a specificity of 66%.

The LIOFeron®TB/LTBI ROC curve analysing healthy persons versus MTB infected patients (tube A + B) showed an AUC = 0.96 (highly accurate assay), with a sensitivity of 91% and a specificity of 97% at the best cutoff (0.59 IU/ml). Focusing only on active TB patients the sensitivity was 90% and the specificity 98% at the best cutoff (0.6 IU/ml); on LTBI subjects the sensitivity was 94% and the specificity 97% at the best cutoff (0.59 IU/ml). The statistical analysis between healthy and infected enrolled persons was performed on TB-A or TB-B single tube data distributions with the following results respectively calculated at the manufacturer's recommended cutoff (0.35 IU/ml): tube A ROC curve showed an AUC = 0.93 with a sensitivity of 91% and a specificity of 62%; tube B ROC curve showed an AUC = 0.94 with a sensitivity of 92% and a specificity of 60%. Finally, with a focus on LTBI patients, the ROC analysis on tube B indicated AUC = 0.96 with a sensitivity of 94% and a specificity of 61%.

LIOFeron®TB/LTBI: specificity determination on Almaty healthy population

A total of 139 healthy subjects were enrolled in this cohort and their characteristics are shown in Table 4.

Results presented in Table 5 confirmed the very high specificity of LIOFeron®TB/LTBI since 137 phytohemoagglutinin (PHA), positive control tube, positive subjects gave negative results.

Discussion

The present manuscript is the first describing the novel IGRA LIOFeron®TB/LTBI test; the purpose of this study was to evaluate the accuracy of the new LIOFeron® TB/LTBI IGRA in clinically confirmed patients and also to compare the performances of LIOFeron® TB/LTBI IGRA and the QuantiFERON®-TB Gold Plus test in a population of healthy subjects and patients with a definite diagnosis of MTB infection. The IFN-γ release from blood of each enrolled patient was simultaneously measured by the two IGRAs. Results showed that both assays had the same high accuracy (AUC = 0.96), considering IFN-γ production into both stimulation tubes (TB-1+TB-2 and TB-A + TB-B): QuantiFERON®-TB Gold Plus test had a sensitivity of 93% (98% in the active TB group, 85% in LTBI patients) and a specificity of 94% (97% in active TB patients and 94% in LTBI subjects); LIOFeron® TB/LTBI had a sensitivity of 91% (90% in active TB patients and 94% in LTBI ones) and a specificity of 97% (98% in the active TB group and 97% in LTBI subjects). The specificity of 98% was also confirmed on Almaty cohort. Thus, LIOFeron® TB/LTBI assay can diagnose MTB infection with a similar sensitivity to QuantiFERON®-TB Gold Plus test, again without differentiating between active TB and LTBI. Interestingly, focusing on the latent MTB infected group, LIOFeron® TB/LTBI sensitivity was 94% in tube B, the one containing Ala-DH antigen, showing that alanine dehydrogenase alone has higher sensitivity for LTBI than the well-known antigens ESAT-6 and CFP-10.

The most critical limitation in designing a study about IGRAs' accuracy is that TB infection has no gold standard test, because IGRAs results are associated on memory T-cell response, thus, active TB is used as a surrogate to measure the sensitivity of these assays. Furthermore IGRAs cannot distinguish between TB infection and active TB disease. Nevertheless, they can show cross

Table 4
Sex and age of the Almaty study healthy participants.

Total (N = 139)		
Sex	Male	96 (69.1%)
	Female	43 (30.9%)
Age (over 18) (Median-IQR)		30 (14)

Table 5
Summary table of Almaty healthy population (N = 139) tested using the LIOFeron® TB/LTBI test

	Results			PC minus NC (according to instruction)		NC (according to instruction)	
Group, N	Negative results	Indeterminate results	Positive results	≥0.50 IU/ml	≤0.50 IU/ml	≤8.0 IU/ml	≥8.0 IU/ml
Blood donors	137	2	0	137 (98.6%)	2	139 (100%)	0
N = 139	(98.6%)	(1.4%)	(0 %)		(1.4%)		(0%)

reactivity with some NTM and this must be better assessed in LIOFeron® TB/LTBI test, especially focusing on Ala-DH response. Consequently, as well as for QuantiFERON®-TB Gold Plus assay, whose reported sensitivity and specificity are not consistent across studies (Sotgiu et al., 2019; Takasaki et al., 2018; Auguste et al., 2017), wider research works are needed to validate the accuracy of the LIOFeron® TB/LTBI assay and to better define its contribution as an adjunctive method for the early diagnosis of both active and latent TB infected individuals. A further purpose for the future is to understand LIOFeron® TB/LTBI assay accuracy in risk groups not enrolled in this study.

To conclude, the LIOFeron® TB/LTBI assay displayed the same high accuracy as the QuantiFERON®-TB Gold Plus test in diagnosing MTB infection/TB disease, and demonstrated higher sensitivity than QuantiFERON®-TB Gold Plus test in LTBI detection. The already shown characteristics of the LIOFeron® TB/LTBI test can lead it to have a role in WHO strategies for ending TB.

Declaration of interest

None.

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Author contributions

The conceptualization and study design was by D'EMM.

DBC, SM, AHFM, BF, TS, MJ, BM, GA, D'ES, AA, KD, BE, TAn, SEA, BA and D'EMM conducted the acquisition of data and investigation.

DBC, TA and SEA analysed data.

DBC, SM, BA and D'EMM wrote the original draft, review and editing was by D'EMM.

Final approval to the submitted version was obtained from all the authors.

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