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LIODetect®TB-ST: Evaluation of novel blood test for a rapid diagnosis of active pulmonary and extra-pulmonary tuberculosis in IGRA confirmed patients

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ABSTRACT

Because of the current limits of immunological tests in the diagnosis of tuberculosis there is a need to identify new and rapid tests that can be carried out on a large scale in endemic countries and useful in the identification of infected subjects, but also able to discriminate those with latent infection from subjects with active. We have taken into consideration and analysed the LIODetect®TB-ST Tuberculosis Rapid Test, a membrane test for the qualitative detection of specific IgG, IgA, and IgM antibodies against *Mycobacterium tuberculosis*, performed on serum, plasma, or whole blood.85 samples positive to QuantiFERON TB-GOLD PLUS test were processed using this test and the results obtained were concordant with clinical diagnosis.To our knowledge, the LIODetect®TB-ST Tuberculosis Rapid Test is the only test; that identifies active tuberculosis disease with high sensitivity and specificity and its use might be of help in the diagnosis of tuberculosis, especially in endemic countries.

1. Introduction

Tuberculosis (TB) is a serious infectious disease caused by *Mycobacterium tuberculosis* (MTB) which usually affects the lungs (Pulmonary TB), but can also affect other organs (extrapulmonary TB). TB has always been considered one of the leading causes of death in the world. For this reason, since 1993 World Health Organization (WHO) has been reporting every year on all statistical data relating to the incidence and prevalence of this disease and the most effective prevention techniques against infection. According to the latest WHO report globally in 2018, 10 million people fell ill with TB, most of them being in Africa (24%) and Western Pacific Region (18%). Lower percentages of cases occurred in Eastern Mediterranean (8.1%), American states (2.9%), and Europe (4%) [1].

Approximately 75% of people exposed to MTB do not become infected, while in the remaining 25% only 5–10% of people develop the disease within a few weeks (active TB). Conversely, 90–95% of people infected with MTB will have an asymptomatic infection, called latent tuberculosis infection (LTBI) without clinically manifested evidence of active TB. However, 5–10% of people with LTBI risk having the disease reactivation throughout their life. Furthermore, if not treated in a preventive way, TB can be fatal especially in immunocompromised subjects such as HIV positive patients. In fact, HIV affects T lymphocytes that protect against MTB, and in these subjects, the percentage of active progression to TB increases by 5–10% [2,3].

The main limit of the currently available diagnostic tests is that they do not discriminate infection from disease. For this reason, there is a need to search for simple and cheap diagnostic methods to discriminate patients with active TB disease from LTBI. This would allow healing subjects with active disease and to block the spread of the pathogen from sputum positive TB individuals [4].

Th1 $CD4^+$ T cells and type-1 cytokines mainly guarantee protection against MTB, and the frequency of IFN-γ producing cells has been widely used as a correlate of protection against MTB [5].

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The currently used screening and diagnostic tests include: tuberculosis skin test (TST), chest x-ray and Interferon-Gamma Release Assay (IGRAs tests). IGRAs tests have been used as diagnostic tests since 2000 and are based on the ability of T lymphocytes to produce IFN-γ following recognition of specific MTB complex antigens [6–10]. TST suffers from low specificity and is being discontinued in several countries. To overcome the limitations of IGRA tests, new techniques have been introduced such as microarrays, flow cytometry, and Luminex cytokine/chemokine assays. These tests have shown promising but often discordant results because several factors in addition to the pathogen can influence their results [11].

Another approach to overcome these limitations relies on a modified QFT TB GOLD, which exploits a combination of soluble markers as EGF, sCD40L, MIP-1β, VEGF, and TGF-α to develop a rapid and sensitive test for active TB [12].

Even though the recommendation by the WHO clearly addressed the tests which were on the market, the scientific community and the manufacturers regarded it as aban on serology of tuberculosis.

Regarding serological tests for the identification of tubercular infection, the array of commercial kits is currently lacking. These tests suffer from an incomplete understanding of the humoral immune response against MTB. In addition, multiple humoral response patterns towards different MTB antigens seem to overlap depending on the stage of infection or disease. This determines the presence of a repertoire of antibodies directed against a wide range of MTB antigens, some shared with other species of environmental mycobacteria, a condition that has so far determined a low specificity of serological tests [13].

However, the identification of numerous antigens derived from MTB since 2011 has led to the production of extremely specific monoclonal Ab, but their use in anti-TB tests has a weakness in its poor sensitivity.

The goal of this type of test is to analyse a large number of antigens to select the right antigen/antibody combinations capable of intercepting the different antibody profiles found in infected subjects. Extensive testing of these antigens with sera of healthy and TB patients led to the development of a cocktail of five protein antigens including the well know-known 38kd antigen [14]. Finally, LIODetect®TB-ST test was developed which contains the cocktail of the five protein antigens in line T1 and a highly purified cell wall antigen in line T2. This test provides results in 20 min and aims at detecting the presence of IgG, IgA, and IgM antibodies against MTB antigens in serum, plasma, or whole blood. It is considered an aid test for the discrimination of LTBI subjects from patients with active TB disease.

In this study, we have evaluated the performance of LIODetect®TB-ST test in a cohort of LTBI, active TB, and healthy donors without any contact with TB patients (HD).

In particular, LIODetect, to date is the only test able to detect the active phase of TB, and can give a very important diagnostic contribution especially in endemic countries where could be more useful to identify TB progressors subjects among LTBI and household contacts.

2. Materials and methods

2.1. Study cohort

We analysed 184 subjects grouped as follows: 99 HD, 55 LTBI subjects, and 30 active TB patients. According to WHO guidelines, the diagnosis of active TB cases was made on clinical, microscopy, microbiological, biomolecular (GeneXpert), and radiological findings [1]. LTBI subjects were defined by a previous positive result to IGRA test without any clinical finding or microbiological, biomolecular, and microscopy positive test. For the LIODetect®TB-ST test, we tested 85 QFT-PLUS positive subjects divided as follows: 55 LTBI subjects positive for QFT-PLUS test with no evidence of active TB, 30 microbiologically confirmed active TB and QFT-PLUS positive patients. Moreover, 99 healthy donors without any contact with TB patients (HD) and who tested negative for QFT-PLUS were included.

The characteristics of the analysed subjects are shown in Table 1. The Ethical Committee of the University Hospital in Palermo, where the patients were recruited, approved the study (approval number 13/2013). All participants signed informed consent. In this study, all the tests were conducted according to the manufacturer's instructions.

2.2. Sample collection and blood stimulation

Peripheral blood was collected by venepuncture at the same time but for QFT-PLUS test. Blood was collected directly in QFT tubes: NIL, which contains heparin only; TB1, containing peptides belonging to the RD1 proteins ESAT6 and CFP10 which are able to stimulate CD4 T cells; TB2, which contains peptides belonging to ESAT6 and CFP10 but the length of these peptides can stimulate both CD4 and CD8 T cells; the last tube named MIT, contains PHA and is used as a positive control.

2.3. IGRA test QFT-PLUS

QFT-PLUS test was performed routinely according to the manufacturer's protocol.

Briefly, blood samples were collected as above described and, after at least 10 gentle inversions, the tubes were incubated at 37 °C for 18–20 h and the plasma collected by centrifugation was frozen at −20 °C for at most one week. All the collected plasma samples were tested with QFT-PLUS ELISA and the results were analysed and validated by a QuantiFERON software provided by the manufacturer and available from www.quantiFERON.com website. The spectrophotometer for the measurement of absorbance was set at wavelength 450 nm with reference at 630 nm, according to manufacturer's instructions.

2.4. LIODetect®TB-ST

The LIODetect® TB-ST Tuberculosis Rapid Test is an *in-vitro* diagnostic rapid test for the qualitative detection of IgG, IgA, and IgM antibodies to MTB in serum, plasma, or whole blood within 20 min (www.lionex.de).

The test consists of one test strip, which is integrated into a test cassette. The test strip consists of a special antibody-binding protein, coupled to coloured particles (conjugate), and a membrane with two test lines (T1 and T2) and one control line (C). The test lines contain TB antigens: T1 contains highly purified recombinant protein antigens PstS1 and PstS3, while T2 contains highly purified lipoglycan of MTB cell wall; C consists of an antibody binding protein. After the sample is pipetted into the sample well (S) followed by the LIODetect®TB-ST Diluent, the diluted sample passes through the conjugate and the antibodies in the sample bind to the conjugate. The antibody-conjugate complex migrates due to the capillary action to the site of the membrane where the TB antigens are immobilized (test lines T1 and T2). If antibodies against MTB antigens are present in the sample, they bind to the test lines. Then one or two coloured lines appear in the test zone ("T"). The remaining complex migrates further across the membrane to the control zone ("C"). Again, a coloured line appears, indicating that the test was performed correctly.

LIODetect test was performed according to the manufacturer's instructions: briefly, a drop of the sample was pipetted into the sample well, followed by two drops of diluent. The results were recorded according to the instructions given in the manual.

A positive result is considered in those cassettes in which T1 or T2 or both T1 and T2 lines give positive colouring after 20 min. In our tests, the T2 line was far more frequently coloured both in the true and false positive samples. This was expected since T2 contains a purified cell wall antigen which has lower specificity than the highly purified protein antigens present in the T1 line.

Table 1
Characteristics of subjects and patients.

	HD		*Active TB*		*LTBI*		*Total*	
Subjects (%)	*99*	*(53.8%)*	*30*	*(16.3%)*	*55*	*(29.9%)*	*184*	*(100%)*
Median age	*49*		*44*		*54*			
Range	*13–87*		*20–61*		*17–77*			
Male gender (%)	*56*	*(56.5%)*	*23*	*(77%)*	*24*	*(44%)*	*104*	*(56.5%)*
Origin (%)								
Western Europe	*83*	*(83.8%)*	*13*	*(32.5%)*	*51*	*(92.7%)*	*147*	*(79.8%)*
Eastern Europe	*1*	*(1.01%)*	*1*	*(3%)*	*2*	*(3.6%)*	*4*	*(2.2%)*
Asia	*2*	*(2.02%)*	*4*	*(12%)*	*0*		*6*	*(3.3%)*
Africa	*10*	*(10.1%)*	*11*	*(27.5%)*	*2*	*(3.6%)*	*23*	*(12.5%)*
Unknown	*3*	*(3.03%)*	*1*	*(3%)*	*0*		*4*	*(2.2%)*
TB (%)								
Pulmonary TB			*21*	*(70.0%)*				
Extrapulmonary TB			*3*	*(10.0%)*				
Disseminated TB			*1*	*(3.0%)*				
Lymph-nodal TB			*5*	*(17.0%)*				

2.5. Statistical analysis

The Cohoen's Kappa coefficient was used to calculate the concordance of QFT-PLUS and LIODetect®TB-ST with diagnosis of active TB. The sensitivity and specificity of LIODetect test was calculated as follows:

Sensitivity = active TB patients with a positive test/all active TB patients;
Specificity = LTBI + non-infected subjects with a negative test/all LTBI + non-infected subjects.

Statistical analysis was performed using Graph Pad PRISM 9®.

3. Results

We analysed the IGRA test results in samples from 99 HD, 30 active TB patients, and 55 LTBI subjects. QFT-PLUS test was able to recognize correctly all the non-TB subjects. Regarding the TB infected subjects, QFT-PLUS recognized 83/85 (55 LTBI and 28 active TB). The overall concordance between QFT-PLUS response and the diagnosis was 98.91% and Cohen's Kappa was 0.98 which means an almost perfect concordance with the diagnosis (Table 2).

We then tested the same 184 samples with the LIODetect®TB-ST cassettes. The results show that the LIODetect®TB-ST test was negative in all the HD and in 51/55 LTBI subjects. The remaining four subjects diagnosed as LTBI had a positive result in the T2 band only (3/4) and in the T1 band only (1/4).

Amongst the 30 patients with active TB, the LIODetect®TB-ST test was positive in 22 cases, but negative in 8 patients. The overall concordance between LIODetect®TB-ST test and diagnosis was 97.82% with a Cohen's K of 0.90, which means an almost perfect concordance (Table 3). The overall sensitivity and specificity of this test were calculated as 73.3% and 97.4% respectively, close to the values reported in the kit by the manufacturer.

Further analysis of positive results based on the data shown in Table 3 revealed that 18 out of 21 (equal to 85.7%) of pulmonary TB patients had a positive LIODetect®TB-ST test, while in the combined group of paucibacillary patients (extra-pulmonary, disseminated, and lymph-nodal TB) only 4 out of 9 patients (44,4%) were positive.

Table 2
Number of subjects tested for QFT-PLUS, concordance with diagnosis and Cohen's K coefficient.

Tested subjects' diagnosis		QFT _PLUS	
		Negative	Positive
HD	**99**	**99**	**0**
LTBI	**55**	**0**	**55**
Active TB	**30**	**2**	**28**
TOTAL	**184**	**101**	**83**
Concordance with diagnosis		**98.91%**	
Cohoen's K		**0.98**	

Table 3
Number of subjects tested for LIODetect®TB-ST, concordance with diagnosis and Cohen's K coefficient.

Tested subjects' diagnosis	NEGATIVE	POSITIVE	Only T1 band positive	Only T2 band positive	T1 and T2 bands positive
HD 99	**99**	**0**			
LTBI 55	**51**	**4**	**1**	**3**	
Active TB 30	**8**	**22**	**1**	**19**	**2**
•Pulmonary 21	***3***	***18***	***1***	***15***	***2***
•Extra-pulmonary 3	***1***	***2***		***2***	
•Disseminated TB 1	***1***	***0***			
•Lymph-nodal TB 5	***3***	***2***		***2***	
TOTAL 184	**158**	**26**			
Concordance with diagnosis		**97.82%**			
Cohoen's K		**0.90**			

4. Discussion

This study is a survey on the possibility of the use of the LIODetect®TB-ST test as a diagnostic tool for active TB patients. To assess the characteristics of this new test, we have compared the LIODetect®TB-ST and the QFT-PLUS tests with diagnosis of TB infection/disease.

Our results show a perfect concordance with the diagnosis in HD subjects, and QFT-PLUS well recognizes infected subjects with 98.91% concordance with diagnosis.

The most relevant limitation of QFT-PLUS remains its inability to distinguish active TB from LTBI. Therefore, this test is unsuitable for confirming a positive diagnosis of active TB disease. Moreover, a negative QFT result could not be useful in ruling out TB infection [15–18].

Serological tests based on detection of MTB-specific Abs, potentially can recognize a wider repertoire of antigens thanks to the ability of antibodies to recognize non only linear peptides but also conformational antigens. It was shown already that some MTB proteins, as the DNA-binding protein 1 (MDP1), are recognized by B cells in their native form leading to the production of Abs able to recognize conformational antigens [19]. Novel MTB peptides with higher immunogenicity compared to those directed against classical antigenic proteins have been considered for new generation Ab-based tests.

Among these, RV0310c- or RV1255c-derived peptides induce the production of IgG whose serum levels can identify with good accuracy subjects with MTB infection. Moreover, the evaluation of Ab levels against the proline-proline glutamic acid protein 17 or the mycobacterial DNA binding protein, make it possible to distinguish between LTBI and active TB patients [20,21]. Another interesting approach to the use of Abs in the diagnosis of TB is the analysis of Abs' glycosylation pattern, which distinguishes between active TB patients and LTBI subjects [22,23].

However, further extensive work is needed before any of the above-mentioned antigens can be put into practice for the manufacturing of low-cost rapid test with high sensitivity and specificity.

The use of LIODetect®TB-ST has shown multiple advantages. It requires a small quantity of sample and the results are immediately available, it does not require specialized personnel or laboratories, and can be run under non-sterile conditions.

Finally, LIODetect®TB-ST assay exploits specific MTB antibodies in a very easy, fast, and cheap way. Certainly, this test showed encouraging and clearly better results, in terms of sensitivity and specificity, than other rapid tests [24,25].

The LIODetect®TB-ST Tuberculosis Rapid test allows identifying the very active phase of the TB disease. It also showed encouraging results, as we found 97.8% of agreement with the diagnosis of active TB.

This test shows a relatively low sensitivity but a very good specificity, comparable to IGRAs test.

Our data display also a different ability of LIODetect test to recognize active TB patients associated to clinical manifestations of TB. In fact, while 18 out of 21 patients with pulmonary TB scored positive, only 4 out of 9 extrapulmonary TB patients scored positive. One possible explanation is the different amounts of IgG and IgA antibodies produced in paucibacillary *versus* multibacillary patients. In fact, it has been shown that the levels of IgG specific for 38- and 16-kDa mycobacterial proteins are significantly higher in culture-positive than in culture-negative patients; moreover, levels of IgG specific for 38-kDa protein and lipoarabinomannan (LAM) are higher in pulmonary TB compared to disseminated TB. Finally, also IgA levels specific for 3–8 kDa protein and LAM are associated with the extent of pulmonary TB [26]. Moreover, several diagnostic tests based on the detection of the anti-TB antibody have shown a higher sensitivity in pulmonary TB compared to extra-pulmonary TB [27]. On the basis of these findings, the LIODetect®TB-ST test is an additional tool to identify patients with multibacillary TB disease.

To our knowledge, this is the only test able to detect the active phase of the TB disease to date, and it can be useful to broaden the choice of immunological tests and overcome the limitations of the current IGRAs test about the discrimination between LTBI and active TB disease patients' status, making a particularly important diagnostic contribution, especially in endemic countries.

This test was created for the rapid identification of patients with active disease in developing countries where TB is endemic and there is comorbidity with HIV, but before the routinely use, it is clear that this serodiagnostic kit would require a 'prospective' epidemiological 'case finding' evaluation of its potentials for preventing transmission, i.e., whether test positivity could identify infectious subjects, before being diagnosed by conventional primary TB diagnostic health care measures.

Author contributions

M.P.LM., B.T., V.O., G.D.B., performed the experiments and analysed the data. P.DC. and A.C. recruited patients and provided blood samples. M.S. provided LIODetect®TB-ST kits and helped in manuscript preparation but had no role in data generation or interpretation of results. F.D. and N.C. planned studies and wrote the manuscript. All the author read and approved the final manuscript.

Declaration of competing interest

M.P.LM., B.T., V.O., G.D.B., P.DC., A.C., F.D. and N.C. declare that they have no disclosures. M.S. is the managing Director of LIONEX GmbH which provided the LIODetect®TB-ST tests free of cost.

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