Comparison of a commercial and an in-house T cell-based assay for the diagnosis of *Mycobacterium tuberculosis* infection

Paola Mantegani[1], Federica Piana[2], Laura Galli[1], Paolo Scarpellini[1], Luigi Codecasa[4], Adriano Lazzarin[1,3], Daniela Cirillo[2], Claudio Fortis[1]

[1]*Clinic of Infectious Diseases and* [2]*Emerging Bacterial Pathogens Unit, San Raffaele Scientific Institute, Milan, Italy;*
[3]*"Vita-Salute" San Raffaele University, School of Medicine, Milan, Italy;*
[4]*Villa Marelli Institute, Lombardy Regional Reference Center for Tuberculosis, Niguarda Hospital, Milan, Italy*

SUMMARY

Identification of individuals with a tuberculosis infection is a very important element for the control of tuberculosis. The currently used tuberculin skin test has poor sensitivity and specificity. Recently, an important advance in tuberculosis diagnosis occurred with the development of *in vitro* T cell-based IFN-γ release assays.
The aim of this study was to compare a RD1-based in-house ELISPOT-IFN-γ assay with a commercial (T-SPOT.*TB*) assay for the diagnosis of tuberculosis infection.
The results showed an almost complete concordance between the two assays, confirming that our restricted but highly selected pool of peptides is sufficient to detect tuberculosis infection.

KEY WORDS: Tuberculosis, ELISPOT, Bacillus Calmette-Guérin, IFN

Mycobacterium tuberculosis is one of the most successful pathogens and remains a leading cause of morbidity and mortality worldwide.

The course of a *M. tuberculosis* infection is very complex. Most people control the initial infection by mounting a strong cell-mediated immune response that prevents disease and the infection remains latent (Jasmer *et al.*, 2002).

However latent tuberculosis infection (LTBI) has the potential to develop into tuberculosis (TB) at any time, from months to decades after infection, and 5-10% of individuals who become infected will subsequently develop active disease (Bloom and Murray, 1992, Graham *et al.*, 2003). This course can occur in response to perturbation of the immune response like infection with human immunodeficiency virus (HIV), malnutrition, use of steroids, aging, alcohol or drug abuse (Flynn and Chan, 2001).

So the estimated one-third of the world's population with LTBI represents an infection reservoir and future source of disease transmission. Treatment of LTBI reduces the probability that TB will develop, therefore a key component of TB control is being able to diagnose and treat LTBI (Jasmer *et al.*, 2002).

The tuberculin skin test (TST) has been used for decades to detect LTBI but its major drawback is lack of sensitivity and specificity (Andersen *et al.*, 2000). In fact *M. tuberculosis* purified protein derivative (PPD), used as antigen in the test, cross-reacts with antigens derived from the vaccine strains of Bacillus Calmette-Guérin (BCG) and from several non-tuberculous mycobacteria (NTM) (American Thoracic Society, 2000). In addition, TST sensitivity may be low in individuals with depressed immunity or advanced TB (Jasmer *et al.*, 2002, Huebner *et al.*, 1993, Andersen *et al.*, 2000, Pai *et al.*, 2004).

Corresponding author
Claudio Fortis
Clinic of Infectious Diseases
San Raffaele Scientific Institute
Via Stamira D'Ancona 20, 20127 Milano, Italy
E-mail: fortis.claudio@hsr.it

Recently, progress has been made in TB diagnosis due to the development of *in vitro* T cell-based IFN-γ release assays. The ex vivo enzyme-linked immunospot (ELISPOT) assay that enumerates IFN-γ producing T cells which respond specifically to *M. tuberculosis* antigens is more specific than the TST. Particularly this assay uses, as antigen, two proteins, early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10), that are encoded by genes located within the region of difference (RD) 1 of the *M. tuberculosis* genome that are absent in all vaccine strains of BCG and in the majority of environmental isolates (Andersen *et al.,* 2000, Pai *et al.,* 2004). Therefore the RD1-specific antigens can overcome some of the TST drawbacks.

We have recently developed and validated an in-house ELISPOT-IFN-γ assay, based on a restricted pool of highly immunogenic peptides derived from ESAT-6 and CFP-10 proteins, that resulted highly specific (87%) and sensitive (93%) (Scarpellini *et al.,* 2004). Furthermore, the test was more accurate than TST in identifying subjects with LTBI (Codecasa *et al.,* 2006).

To further validate our assay we have compared it with a commercial assay, the T-SPOT.*TB*™ (Oxford Immunotec, Oxford, UK, currently approved for use in Europe and awaiting US FDA approval) (Lalvani, 2003) that uses synthetic overlapping peptides corresponding to the full length of ESAT-6 and CFP-10 proteins.

To this end, 86 subjects were recruited at the Villa Marelli Institute, Lombardy's Regional Reference Center for Tuberculosis, Milan, Italy, including 38 BCG-vaccinated persons and 65 household contacts of contagious (sputum smear-positive) TB-infected patients (for patients' characteristics see Table 1). Each subject was submitted to Mantoux TST and to our in-house ELISPOT-IFN-γ assay and, in another laboratory, to the commercial T-SPOT.*TB*™ assay under double blind conditions. Blood samples were collected from all enrolled individuals after informed consent; peripheral blood mononuclear cells (PBMCs) were obtained by blood centrifugation over a density gradient (Ficoll), separated into two aliquots and tested.

The ELISPOT-IFN-γ assay was performed as previously described (Scarpellini *et al.,* 2004). Briefly, PBMCs were seeded in duplicate and stimulated with:

1) a pool of six synthetic *M. tuberculosis* specific peptides (MTP) (PRIMM Srl, Milan, Italy) derived from the sequences of ESAT-6 and CFP-10 proteins (each at 2 µg/ml);

2) heat-inactivated and sonicated whole-cell BCG (Organon Teknika, Boxtel, The Netherlands) at a diluition of 1:10;

3) phytoemagglutinin (PHA) (Sigma, St. Louis, MO) at 5 µg/ml as positive control; in addition cells in medium alone were used as negative control. The responses were scored as positive if the test wells had a higher mean number of spot-forming cells (SFC) than the mean number plus two standard deviations in the negative control wells, and the number of SFC per million PBMC in the stimulated wells (minus the values of the negative control well) was ≥20.

The T-SPOT.*TB* assay was performed in accor-

TABLE 1 - *Patients' characteristics.*

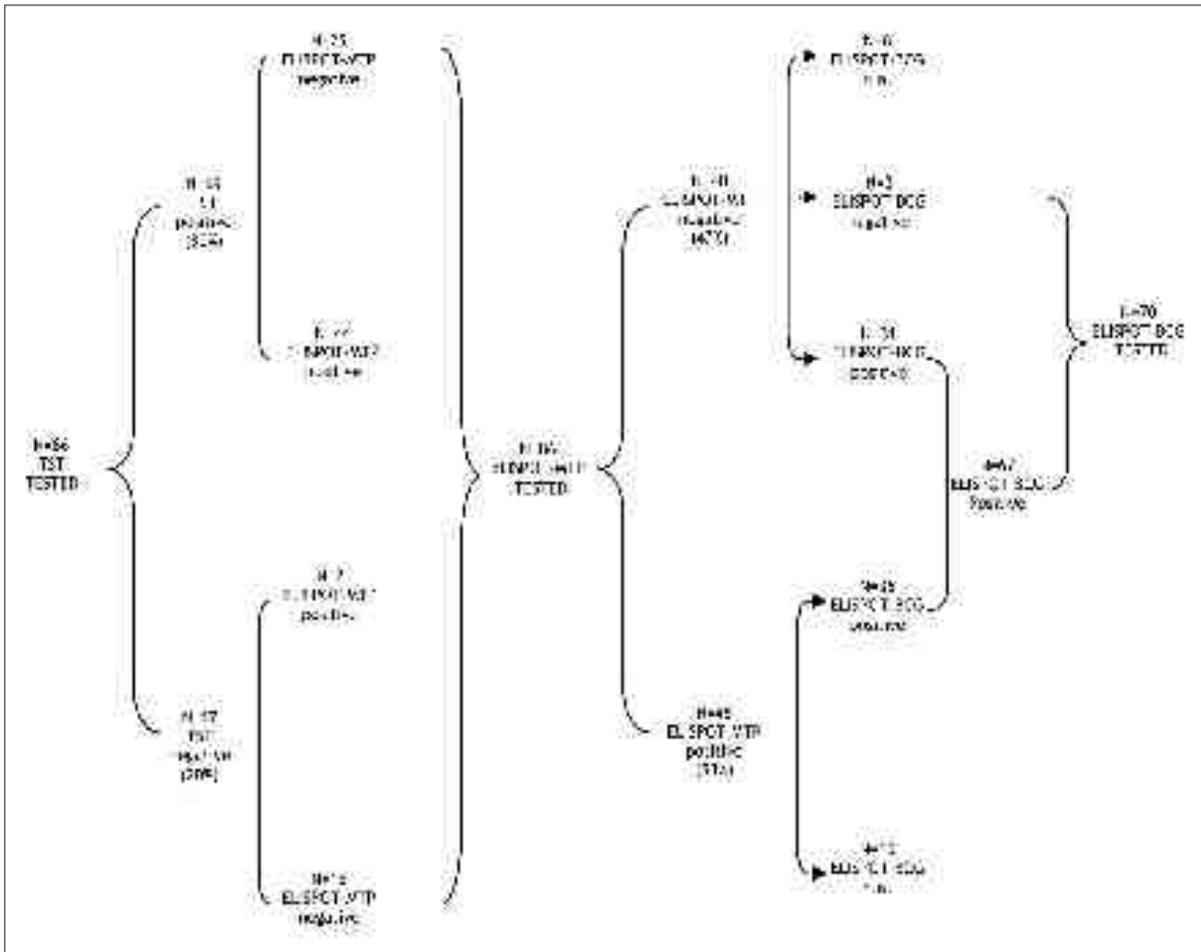| Origin	N	M/F	Age range	Household contacts	BCG Vaccinated	TST positive
Africa	2	2/0	20-29	2	2	2
Asia	5	2/3	13-51	3	5	4
Central and South America	26	13/13	8-69	19	22	23
Eastern Europe	7	3 4	0.5-60	6	5	7
Western Europe	46	26 20	2-93	35	4	33
Total	86	46/40	0.5-93	65	38	69

FIGURE 1 - *TST, ELISPOT-MTP and ELISPOT-BCG results; n.a. = not analyzed.*

dance with the procedures recommended by the manufacturer. The PBMCs were seeded in single-well and stimulated with ESAT-6 and CFP-10 overlapping peptides; a negative control (cells alone) and a positive control (cells plus PHA) were included. The responses were scored positive when the number of spots in the test wells (against at least one of the two tested antigens) was ≥6 times the number of spots in the negative control wells or, if the negative control had more than 6 SFCs, at least twice that number. At screening, 69 of the 86 subjects tested (80%) had a positive TST reaction (a 5 mm induration cut-off was used for the household contacts and a 10 mm cutoff was used for the other classes) (Figure 1). Of the 69 TST-positive subjects, 25 (36%) showed a negative response to the in-house ELISPOT-MTP assay and 2 (12%) of the TST-neg-

ative subjects were ELISPOT-MTP positive (64% were TST/ELISPOT double-positive and 88% were double negative).

We observed an almost complete concordance (99% concordance: K=0.977, 95% CI 0.931-1.022, P=ns) between the in-house ELISPOT-IFN-γ assay and the commercial T-SPOT.*TB* assay (the data were analyzed using SAS software version 8.2). In fact, 46 subjects (53%) had a positive response to the in-house ELISPOT and 45 (52%) to the commercial assay. Moreover, of the 70 individuals tested for BCG (ELISPOT-BCG), 67 (96%) were positive and these included all the 30 individuals with positive response to MTP (ELISPOT-MTP), whereas 31 of 40 subjects with a negative response to MTP (78%) were positive to ELISPOT-BCG, suggesting that most of them had received a BCG vaccination or have been infect-

ed with NTM. In addition, of the 38 BCG vaccinated subjects (individuals with a detectable BCG scar or documentation confirming previous vaccination), 37 had a positive TST reaction (≥5 mm) but 10 showed a negative response to MTP.

We also observed a significant difference in the size (expressed in mm) of the TST induration between the subjects with a positive or negative ELISPOT-MTP response (MTP negative: median=6.5; q1=0, q3=13.5; MTP positive: median=16; q1=11, q3=20; $P<0.0001$).

In conclusion, our study showed a very high concordance between the results obtained with the in-house assay and the commercial T-SPOT.*TB* assay, confirming that our restricted but highly selected pool of peptides is sufficient to detect acute TB infection.

Moreover, our assay is more accurate than the TST in identifying subjects with suspected latent TB infection.

REFERENCES

AMERICAN THORACIC SOCIETY (2000). Targeted tuberculin testing and treatment of latent tuberculosis infection. *Am J Respir Crit Care Med.* **161**, S221-S247.

ANDERSEN, P., MUNK, M.E., POLLOCK, J.M., DOHERTY, T.M. (2000). Specific immune-based diagnosis of tuberculosis. *Lancet.* **356**, 1099-1104.

BLOOM, B.R. & MURRAY, C.J. (1992). Tuberculosis: commentary on a reemergent killer. *Science.* **257**. 1055-1064.

CODECASA, L., MANTEGANI, P., GALLI, L., LAZZARIN, A., SCALPELLINI, P., FORTIS, C. (2006). An in-house RD1-based enzyme-linked immunospot-gamma interferon assay instead of the tuberculin skin test for diagnosis of latent *Mycobacterium tuberculosis* infection. *J Clin Microbiol.* **44**, 1944-1950.

FLYNN, J.L. AND CHAN, J. (2001). Immunology of tuberculosis. *Ann Rew Immunol.* **19**, 93-129

HUEBNER, R.E., SCHEIN, M.F., BASS, J.B. JR. (1993). The tuberculin skin test. *Clin Infect Dis.* **17**, 968-975.

JASMER, R.M., NAHID, P. AND HOPEWELL, P.C. (2002). Clinical Practice. Latent Tuberculosis Infection. *N Engl J Med.* **347**, 1860-1866.

LALVANI A. (2003). Spotting latent infection: the path to better tuberculosis control. *Thorax.* **58**, 916-918.

PAI, M., RILEY, L.W. AND COLFORD, M. JR. (2004). Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infect Dis.* **4**, 761-776.

SCARPELLINI, P., TASCA, S., GALLI, L., BERETTA, A., LAZZARIN, A., FORTIS C. (2004). Selected pool of peptides from ESAT-6 and CFP-10 proteins for detection of *Mycobacterium tuberculosis* infection. *J Clin Microbiol.* **42**, 3469-3474.

STEWART, GR., ROBERTSON, BD. AND YOUNG, DB. (2003). Tuberculosis: a problem with persistence. *Nat Rew Microbiol.* **1**, 97-105.