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Full Length Research Paper

Diagnostic value of an IFN-γ ELISPOT assay to detect latent tuberculosis infection in hepatitis C patients

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The rapid diagnosis of tuberculosis (TB) and latent tuberculosis infections (LTBI) is a significant problem in clinical practice. The aim of this study was to evaluate the diagnostic value of an enzyme-linked immunosorbent spot (ELISPOT) assay measuring interferon-γ in hepatitis C patients with LTBI. A total of 160 hepatitis C patients at the Jilin University Hospital, Changchun, China, were prospectively enrolled from January 2009 to December 2010; 43 had been positively diagnosed with TB, 38 with non-TB diseases, and 79 with a history of TB. All patients were evaluated by the tuberculin skin test (TST) and ELISPOT assays. Among the 43 diagnosed TB patients, the ELISPOT assay had a sensitivity of 92.1%, compared to a sensitivity of 60.5% for the TST. Among the 79 TB exposure patients, the ELISPOT assay was more sensitive (90%) than the TST (61.5%), the specificity of the ELISPOT assay was 90%, and the specificity of the TST was 61.5% in LTBI. Among the 38 subjects with non-TB diseases, the specificity of the ELISPOT was better than the TST's. In conclusion, this ELISPOT assay could provide useful support in diagnosing LTBI in hepatitis C patients and may provide guidance regarding the treatment of LTBI and hepatitis C co-infection.

Key words: Latent tuberculosis infections (LTBI), Hepatitis C virus (HCV), enzyme-linked immunosorbent spot (ELISPOT) assay, CFP-10/ESAT-6.

INTRODUCTION

Hepatitis C virus (HCV) is a major public health issue, and approximately 200 million people are infected with HCV worldwide, representing about 3.3% of world's population (Berger, 2011). Surveys have found that the patients with chronic hepatitis C are often elderly, and patients at this stage often have a high incidence of latent tuberculosis infection (LTBI) (Grasso et al., 2008). Interferon is the immune enhancers with broad-spectrum anti-viral effect, which can inhibit viral replication and block the spread of hepatitis viruses. Interferon can stimulate the body's own anti-viral effect of interferon system, but also can improve the body's natural anti-viral ability to change the body's immune response to hepatitis virus infection, to reduce or block viral pathological immune hepatitis injury. Interferon treatment of hepatitis C was considered the only effective drug, but not all patients are applicable to treat with interferon, when a patient suffered from TB and LTBI, the interferon could speed up TB's condition. It is necessary to determine whether the patients with chronic hepatitis C suffered from latent tuberculosis infection at the time of hepatitis detection or whether the LTBI was directly related to the treatment of hepatitis C. Interferon is an effective treatment for hepatitis C.

However, interferon may convert latent tuberculosis into active tuberculosis if hepatitis C patients also suffer from LTBI (Vaddady et al., 2010; Corstjens et al., 2007).

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Table 1. Study participants.

Characteristics	TB	TB exposure	No. TB exposure
Age, mean years ± SD	30.7±17.6	42.9±18.6	37.8±17.6
Male sex	24	42	21
Clinical diagnosis			
HCV	43	79	38
Pulmonary TB	25	4	1
Disseminated TB	15	6	0
Possible TB	0	29	1
Underlying condition or illness			
Heart disease	2	3	2
Diabetes	4	7	4
Liver cirrhosis	8	10	5
Solid tumor	2	3	0
Chronic renal failure	0	2	0
No underlying illness	27	55	21

Interferon therapies may accelerate the progression of tuberculosis. Therefore, it is very important to diagnose latent tuberculosis in hepatitis C patients. The tuberculin skin test (TST) is the predominant means of diagnosing LTBI; however, the specificity of this test is limited because the purified protein derivative used as the antigen broadly cross-reacts with antigens derived from a number of mycobacterial species, including the vaccine strain Bacillus Calmette-Guerin (BCG) and other non-tuberculous mycobacteria (NTM) (Chen et al., 2011). The TST has poor specificity and the misdiagnosis rates are very high in diagnosing LTBI. Therefore, there is an urgent need for a more sensitive and specific immunological tool for the diagnosis of latent tuberculosis infections.

An IFN-γ ELISPOT assay is based on detecting the interferon-γ (IFN-γ) released by activated T lymphocytes (T cells) that are stimulated by a number of secretory proteins encoded by the RD1 locus of the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex but are absent from the majority of environmental isolates, including BCG strains (Eisenhut, 2010). The early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) proteins were initially tested and shown to elicit a strong T cell response in subjects with active TB or LTBI (Girlanda et al., 2010). Therefore, in this study, we evaluated the clinical value of this ELISPOT assay for the diagnosis of LTBI in hepatitis C patients.

MATERIALS AND METHODS

Participants

One hundred and sixty hepatitis C patients were recruited at the Jilin University Hospital, Changchun, China, from January 2009 to December 2010. TB exposure group (n= 79) comprised patients who had a history of exposure to tuberculosis and did not do clinical diagnosis of TB, the clinical symptoms were obvious; non-TB exposure group (n= 38) comprised patients who had no history of exposure to tuberculosis and no clinical symptoms; TB group (n= 43) comprised patients who were clinical diagnosed with TB and the clinical symptoms were apparent. These 160 patients were selected as the subjects of our research and divided into three groups. The TB group contained 43 TB patients with a mean age of 30.7 ± 17.6, including 24 males and 19 females. In this group, 27 patients were retrospectively confirmed to be smear-positive by the TST test, and 16 patients were retrospectively confirmed to be smear-negative by other detection methods (chest X- ray, PPD test, bronchoscopy and bacteriological test). The TB exposure group contained 79 subjects with a mean age of 42.9 ± 18.6, including 42 males and 37 females. The non-TB exposure group contained 38 subjects with a mean age of 37.8 ± 17.9, including 21 males and 17 females. Details of patients and controls enrolled in the study are shown in Table 1.

Tuberculin skin test

A standard TST was performed by intradermal injection (Mantoux method) of 0.1 mL (5 U) of PPD according to current recommend-dations for LTBI prophylaxis in candidate patients for anti-TNF-α therapy (Kobashi et al, 2010). The diameter of cutaneous induration was measured with a ruler by a trained physician 72 h after injection. The TST was considered positive when the transverse diameter of induration was ≥5 mm.

IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay (Beijing Gaoke Life and Technology Inc., China) was performed according to the manufacturer's recommendations. Briefly, 200,000 peripheral blood mononuclear cells (PBMC) were plated for 24 h in 96-well plates that had been pre-coated with mouse anti-human IFN-γ antibody. Cells were left unstimulated (negative control), stimulated with 50 µl PHA (positive control), or with 50 µl of CFP-10 and ESAT-6 peptides in separate

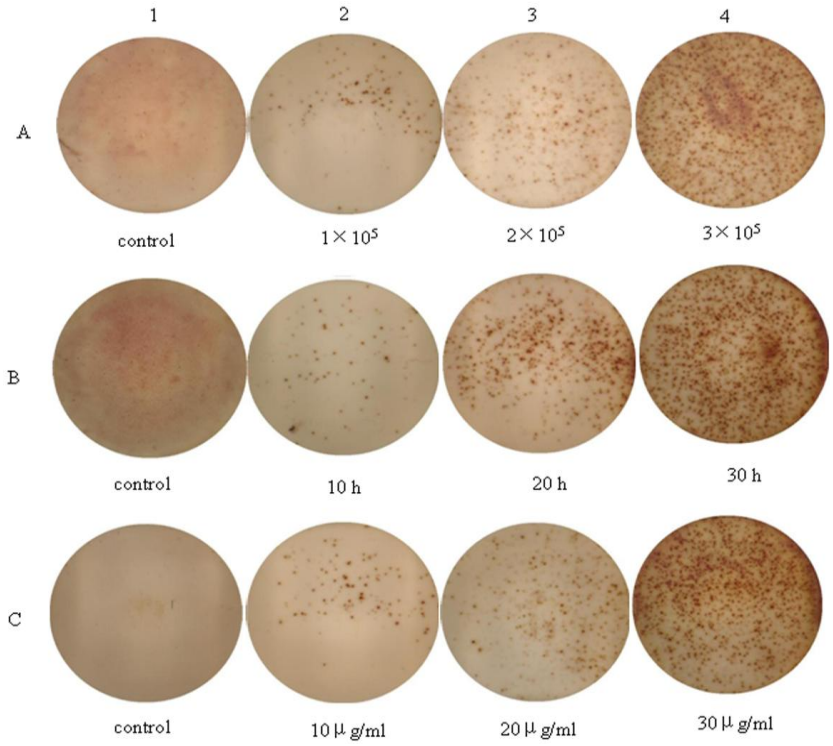

Figure 1. The optimized experimental conditions of the ELISPOT assay. (A) The comparison of different PBMC numbers for the TB patient in the ELISPOT assay. Lane 1 is the negative control. Lanes 2-4 are test wells in which 1×10^5, 2×10^5, or 3×10^5 PBMC were stimulated with 20 μg/ml CFP-10/ESAT-6 for 20 h. (B) Comparison of different incubation times when 2×10^5 PBMC/well were stimulated with CFP-10/ESAT-6 protein in the ELISPOT assay. Lane 1 is the negative control. Lanes 2-4 are test wells in which 2×10^5 PBMC/well were stimulated with 20 μg/ml CFP-10/ESAT-6 for 10, 20, or 30 h. (C) Comparison of different CFP-10/ESAT-6 protein concentrations when 2×10^5 PBMC/well were stimulated in the ELISPOT assay. Lane 1 is the negative control. Lanes in the figure, 2-4 are test wells in which 2×10^5 PBMC/well were stimulated with 10, 20, or 30 μg/ml of CFP-10/ESAT-6 antigen for 30 h.

wells. The response of stimulated cultures was considered positive when one or both test wells contained at least six more spots than the negative control wells or had at least twice as many SFCs as the negative control wells (Taniguchi et al, 2010).

Statistical analysis

Diagnostic performance was expressed in terms of sensitivity, specificity, positive predictive value, and negative predictive value. Analyses were performed using the commercial statistical software SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA). Continuous variables were compared using the Manne-Whitney *U* test or Student's *t* test. All tests of significance were 2-tailed, and $P<0.05$ was considered to be significant.

RESULTS

ELISPOT assay in TB group

We used 43 TB patients to optimize the ELISPOT assay to detect latent tuberculosis infection in chronic HCV hepatitis patients, and we evaluated multiple experimental conditions. First, we determined the optimal number of peripheral blood mononuclear cells (PBMC) per well from a TB patient. We used 1×10^5, 2×10^5, and 3×10^5 cells/well of PBMC in a 30 h experiment, and the results demonstrate that 2×10^5 cells/well produced the optimal number of SFC (Figure 1A). We then determined the optimal incubation time, testing 10, 20, and 30 h with 2×10^5 cells/well of PBMC stimulated with CFP-10/ESAT-6 protein; the results show that 30 h of incubation produced an optimal number of SFC (Figure 1B). We then determined the optimal concentration of the CFP-10/ESAT-6 protein for use in this assay. We tested 10, 20, and 30 μg/ml using 2×10^5 cells/well of PBMC for 30 h, and the results show that 20 and 30 μg/ml of CFP-10/ESAT-6 protein resulted in no significant difference in SFC (Figure 1C). These optimization studies led us to our final set of conditions; 2×10^5 PBMC/well, an incubation time of 30 h, and 20 μg/ml of CFP-10/ESAT-6 protein were used in all subsequent experiments.

Table 2. Comparison of the ELISPOT assay and the TST in TB and non-TB subjects. The sensitivity of the ELISPOT assay was 74.4%, which was higher than the TST (62.8%) in sputum test-positive subjects; * denotes a significant difference from controls (P< 0.05). In the non-TB group, the specificity of the ELISPOT assay was 92.1%, and the specificity of the TST was 60.5%.The specificity was calculated as follows: (negative sample / total sample) × 100%.

Diagnosed case	Smear	No. of case	No. of positive (positive rate)	
			TST (%)	ELISPOT assay (%)
TB group	+	27	20 (74.1)	23 (85.2)
	-	16	7 (43.75)	9 (56.25)
	Total	43	27 (62.8)	32 (74.4)*
Non-TB group		38	15 (39.4)	3 (7.9)

Table 3. Comparison of the ELISPOT assay and the TST in LTBI. In 79 TB exposure cases in HCV hepatitis patients, 9 cases were TB-positive, 31 cases were suspected to have TB but did not have the symptoms of TB, and 39 cases were non-TB. The sensitivity of the ELISPOT assay was 71%, which was higher than the TST (61.3%), * denotes a significant difference from controls (P< 0.05). In the non-TB group, the sensitivity of the ELISPOT assay was 90% and the sensitivity of the TST was 61.5%, making the false positive rate of the TST higher than that of the ELISPOT assay. The sensitivity was calculated as follows: (positive patient sample / total patient sample) × 100%.

Diagnosed case	Smear	No. of case	No. of positive (positive rate)	
			TST	ELISPOT assay
TB exposure group	+	9	5 (55.6)	7 (77.8)
	- (suspected TB)	31	19 (61.3)	22 (71)
	Total	40	18 (45)	29 (72.5)*
	-	39	15 (38.5)	4 (10.3)*

Comparison of the ELISPOT assay with the TST for detecting TB

Using clinical data from our retrospective survey, we selected 43 confirmed TB cases (TB group) and 38 non-TB cases (non-TB group) of HCV hepatitis patients and compared available data from the ELISPOT assay and the TST performed at the same time point. The results are shown in Table 2 for the 43 TB cases, which were classified as having either smear-positive (62.8%, 27/43) or smear-negative (37.2%, 16/43) TB. For these TB cases, the diagnostic sensitivity of the ELISPOT assay was 74.4%, and the diagnostic sensitivity of the TST was 62.8%. The ELISPOT assay was more sensitive than the TST in smear-positive TB cases. In non-TB cases, the diagnostic specificity of the ELISPOT assay was 92.1%, and the diagnostic specificity of the TST was 60.5%. These results suggested that the sensitivity and specificity of the ELISPOT assay were better than the TST, the false positive rate of the TST was higher than the ELISPOT assay and that the ELISPOT assay was more sensitive than the TST in diagnosing TB.

Comparison of the ELISPOT assay with the TST in LTBI

LTBI is a special case in which the host is infected with *M.* tuberculosis but does not have the symptoms of TB. We selected 79 cases of hepatitis C patients who had a history of TB contact (TB exposure group), 9 of who had TB and 31 of who had suspected TB but without symptoms of TB, and 39 of who had non-TB diseases. We compared available data from the ELISPOT assay and the TST performed at the same time point. As the results in Table 3 show, the diagnostic sensitivity of the ELISPOT assay was 71%, and the diagnostic sensitivity of the TST was 61.3%. In suspected TB cases (smear-negative), the specificity of the ELISPOT assay was 90%, and the specificity of the TST was 61.5%. The results showed that the ELISPOT assay had a high sensitivity in diagnosing LTBI. In non-TB cases, as shown in Figure 2A, data from one representative patient who was smear-positive are shown in which the diameter of the TST lesion was 11.5 mm and the ELISPOT assay was positive. Data from another representative patient are shown in Figure 2B, in which the smear was negative, the diameter of the TST lesion was 2 mm and the ELISPOT assay was positive. Data from a third representative patient are shown in Figure 2C; in this case, the smear was negative, the diameter of the TST lesion was 10 mm, and the ELISPOT assay was negative. The above results show that the TST had a higher false positive rate than the ELISPOT assay such that compared to the available data from the TST; the ELISPOT assay had a high diagnostic sensitivity and low false positive rate for the

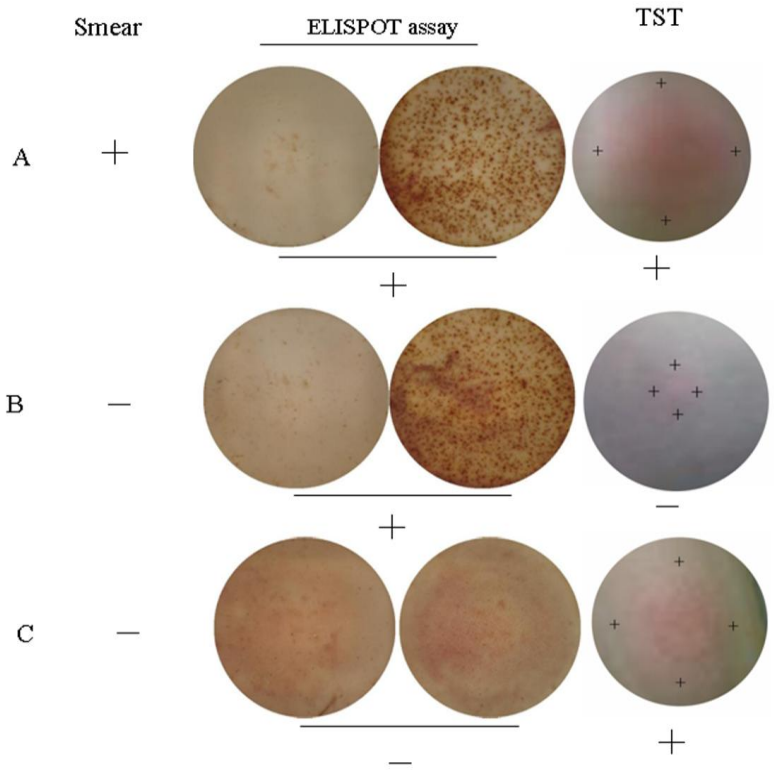

Figure 2. Image comparison of the ELISPOT assay and the TST in LTBI. This figure shows representative results of the ELISPOT assay from three patients. (A) Patient in which the smear was positive, the TST was positive, and the ELISPOT assay was positive; (B) patient in which the smear was negative, the TST was negative, and the ELISPOT assay was positive; (C) patient in which the smear was negative, the TST was positive, and the ELISPOT assay was negative. This figure suggests that the ELISPOT assay has a higher diagnostic sensitivity and lower false positive rate in the diagnosis of LTBI.

diagnosis LTBI, making it an effective diagnostic tool for LTBI in HCV hepatitis patients.

DISCUSSION

Interferon is an effective way to treat hepatitis C that can effectively control the disease progress. Hepatitis C patients often have other infectious diseases (Drumright et al, 2011). TB is one of the most common diseases co-occurring in hepatitis C patients. TB cases consist of latent tuberculosis infections (LTBI) and active tuberculosis infections. LTBI is the special case in which the host is infected with *M.* tuberculosis but does not have the symptoms of TB. If hepatitis C patients suffer from LTBI, this will directly affect the choice of treatment (Hoshino, 2010; Mack et al., 2009; Sismanidis and Williams, 2008). Interferon can convert latent tuberculosis into active tuberculosis and interferon therapies can accelerate the progression of tuberculosis.

The TST is the diagnostic method for TB and LTBI; however because cross-reactivity exists between *M.* tuberculosis and Mycobacterium BCG, TST cannot distinguish between TB infection and BCG immunization, and it has poor specificity for the diagnosis of TB and LTBI (Figures 2B and 2C) (Diel et al., 2009). Therefore, it is very important to have fast and accurate diagnosis of LTBI in hepatitis C patients to provide guidance for the treatment of hepatitis C.

The ELISPOT assay is a new generation of diagnostic TB assay that uses *M.* tuberculosis-specific antigens encoded by genes located in region of difference 1 (RD1) (Ryan et al., 2010). In this study, we used the ELISPOT assay to evaluate the sensitivity of TB diagnosis and obtained promising results on the diagnosis of latent TB infection and active TB in hepatitis C patients. We used 43 TB patients to optimize the ELISPOT assay for TB in HCV hepatitis patients and determine the best experimental conditions (Figure 1). We used the optimal experimental conditions (2×10^5 PBMC/well, 20 μg/ml of CFP-10/ESAT-6 protein incubated for 30 h) in all subsequent experiments. We used 79 HCV hepatitis cases that had a history of TB contact (TB exposure group) and found that the diagnostic sensitivity of the ELISPOT

assay was 71%, and the diagnostic sensitivity of the TST was 61.3%. In suspected TB cases (smear-negative), the specificity of the ELISPOT assay was 90%, and the specificity of the TST was 61.5%, the false positive rate of the TST was higher than of the ELISPOT assay, as shown in Table 3 and Figure 2. The ELISPOT assay was more sensitive than the TST for the diagnosis of TB. In Korea, Kim et al. (2007) found that the sensitivity of the ELISPOT assay was 94% in a study of 72 patients with suspected extrapulmonary TB, and Cho et al. (2007) found that the sensitivity of the ELISPOT assay for active TB osteoarthritis was 100%. Although our findings for the ELISPOT assay in Chang chun are not as good as these two studies in Korea, they suggest that the ELISPOT assay appears to be a useful supplementary tool for diagnosing skeletal TB. These results are related to the release of IFN-γ in the ELISPOT assay (Chambers et al., 2010).

The release of IFN-γ was induced by the specific tuberculosis antigens ESAT-6 and CFP-10. ESAT-6 and CFP-10 are present in the RD1 region of all *M.* tuberculosis strains, whereas BCG lacks the RD1 region. The majority of mycobacterial species do not have this RD1 region. Therefore, ESAT-6 and CFP-10 induce specific IFN-γ release that is a highly effective method to distinguish between healthy and infected individuals. Therefore, the ELISPOT assay has strongly specificity in the diagnosis of TB and LTBI (Zhang et al., 2010). However, the TST could not distinguish between TB infection and BCG immunization (Figure 2C), which resulted in a higher false positive rate in the diagnosis of TB and LTBI.

In summary, our results demonstrate that the ELISPOT assay has a high diagnostic sensitivity and low false positive rate in the diagnosis of LTBI, suggesting that it may be effective in diagnosing LTBI and providing treatment guidance for HCV hepatitis to prevent latent TB from turning into active TB.

REFERENCES

Berger DS (2011). A new era in HCV treatment. Posit Aware, 23(3): 42-43.

Chambers IR, Cone TR, Oswald-Richter K, Drake WP (2010). Enzyme-linked immunospot assay (ELISPOT): Quantification of Th-1 cellular immune responses against microbial antigens. J. Vis. Exp., (45): 65-67

Chen J, Sun J, Zhang R, Liu L, Zheng Y, Shen Y, Wang Z, Sun F, Li L, Lu H (2011). T-SPOT.TB in the diagnosis of active tuberculosis among HIV-infected patients with advanced immunodeficiency. AIDS Res. Hum. Retroviruses, 27 (3): 289-294.

Cho OH, Park SJ, Park KH, Chong YP, Sung H, Kim MN, Lee SO, Choi SH, Woo JH, Kim YS, Kim SH (2010). Diagnostic usefulness of a T-cell-based assay for osteoarticular tuberculosis. J. Infect., 61: 228–234.

Corstjens PL, Chen Z, Zuiderwijk M, Bau HH, Abrams WR, Malamud D, Sam Niedbala R, Tanke HJ (2007). Rapid assay format for multiplex detection of humoral immune responses to infectious disease pathogens (HIV, HCV, and TB). Ann. N Y Acad. Sci., 1098: 437-445.

Diel R, Schaberg T, Loddenkemper R, Welte T, Nienhaus A (2009). Enhanced cost-benefit analysis of strategies for LTBI screening and INH chemoprevention in Germany. Respir. Med., 103(12): 1838-1853.

Drumright LN, Hagan H, Thomas DL, Latka MH, Golub ET, Garfein RS, Clapp JD, Campbell JV, Bonner S, Kapadia F, Thiel TK, Strathdee SA (2011). Predictors and effects of alcohol use on liver function among young HCV-infected injection drug users in a behavioral intervention. J. Hepatol., 55(1): 45-52.

Eisenhut M (2010). Significance of reduced reactivity in ELISPOT assay to RD antigens of Mycobacterium tuberculosis in BCG vaccinated individuals exposed to a patient with tuberculosis. Virulence, 1(2): 105-106.

Girlanda S, Mantegani P, Baldissera E, Aiello P, Ratti M, Sabbadini MG, Fortis C (2010). ELISPOT-IFN-gamma assay instead of tuberculin skin test for detecting latent Mycobacterium tuberculosis infection in rheumatic patients candidate to anti-TNF-alpha treatment. Clin. Rheumatol., 29(10):1135-1141.

Grasso A, De Leo P, Malfatti F, Toscanini F, Anselmo M, Menardo G (2008). Late occurrence of pleural and peritoneal effusion due to Mycobacterium tuberculosis infection (TB) in a patient with posttransplantation recurrent HCV chronic hepatitis: safety of peginterferon and ribavirin treatment after recovery of TB: a case report. Transplant Proc., 40(5): 1783-1785.

Hoshino H (2010). [Present activity of BCG and LTBI treatment for infants in Japan]. Kekkaku., 85(11): 783-786.

Kim SH, Choi SJ, Kim HB, Kim NJ, Oh MD, Choe KW (2007). Diagnostic usefulness of a T-cell based assay for extrapulmonary tuberculosis. Arch. Int. Med., 167: 2255–2259.

Kobashi Y, Abe M, Mouri K, Obase Y, Miyashita N, Oka M (2010). Rapid diagnosis of tuberculous pericarditis by ELISPOT assay. Scand. J. Infect. Dis., 42(9): 712-715.

Mack U, Migliori GB, Sester M, Rieder HL, Ehlers S, Goletti D, Bossink A, Magdorf K, Holscher C, Kampmann B, Arend SM, Detjen A, Bothamley G, Zellweger JP, Milburn H, Diel R, Ravn P, Cobelens F, Cardona PJ, Kan B, Solovic I, Duarte R, Cirillo DM (2009). LTBI: latent tuberculosis infection or lasting immune responses to M. tuberculosis? A TBNET consensus statement. Eur. Respir. J., 33(5): 956-973.

Ryan C, Giguere S, Hagen J, Hartnett C, Kalyuzhny AE (2010). Effect of age and mitogen on the frequency of interleukin-4 and interferon gamma secreting cells in foals and adult horses as assessed by an equine-specific ELISPOT assay. Vet. Immunol. Immunopathol., 133(1): 66-71.

Sismanidis C, Williams B (2008). Detecting LTBI: old techniques, new approaches. Int. J. Tuberc. Lung. Dis., 12(8):867-868.

Taniguchi K, Shimazaki C, Ochiai N, Maruya E, Akatsuka Y, Ashihara E, Maekawa T, Taniwaki M, Saji H (2010). Modified ELISPOT assay may predict T-cell hyporesponsiveness to non-inherited maternal antigens. Int. J. Lab. Hematol., 32(1 Pt 1): e163-168.

Vaddady PK, Lee RE, Meibohm B (2010). *In vitro* pharmacokinetic/pharmacodynamic models in anti-infective drug development: focus on TB. Future Med. Chem., 2(8):1355-1369.

Zhang M, Wang H, Liao M, Chen X, Graner M, Zhu X, Zhang J, Yang Q, Lu H, Zhou B (2010). Diagnosis of latent tuberculosis infection in bacille Calmette-Guerin vaccinated subjects in China by interferon-gamma ELISpot assay. Int. J. Tuberc Lung Dis., 14(12): 1556-1563.