

Full Length Research Paper

Serological diagnosis of tuberculosis using proteins expressed from the region of difference 1 (RD1) and RD2 of mycobacterium tuberculosis as antigens

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Accepted 18 May, 2012

Tuberculosis is a well known public health problem. However, the diagnose of this disease usually takes too long time. And, the specificity is not good enough. So it is extremely urgent to develop a diagnose method to detect tuberculosis accurately. In this study, we used rEC (recombinant of ESAT-6 and CFP10 fusion protein) and rCM (recombinant of CFP21 and MPT64 fusion protein) as antigens in enzyme-linked immunosorbent assays (ELISA) to detect tubercular, healthy people and household contacts serum. We used fusion protein rCM and rEC to detect different group serum. We established an ELISA detecting system based on this two fusion proteins. We find an efficacious way to detect the latent *Tuberculosis* infection, providing a new method to detect tuberculosis.

Key words: Household contacts, serodiagnosis, tuberculosis.

INTRODUCTION

Tuberculosis (TB) causes a major public health problem in the world. Approximate one-third of the population is infected with *Mycobacterium tuberculosis* (M.tb), more

than 90% of which are asymptomatic with latent M. tb infection (LTBI), since the pathogen can persist at a latent state for a long time. Under specific conditions, 5-10% of TB may be activated by some factors. Currently there are some factors causing drug-resistant strains generating, such as drug side effects and unsatisfactory compliance of patients. Human immunodeficiency virus co-infection can also lead to the increasing mortality of patients with combined TB infections (Jassal and Bishai, 2009). Therefore, novel effective diagnose methods of TB are needed.

The main types of TB pathogens include human M. tb and bovine M. tb. TB can be diagnosed by bacterial culture, and can be treated by using the sensitive drugs. However, the diagnosis rate is low in bacteriology detection. Furthermore, M. tb grows slowly, so it will be a time-consuming process. The techniques such as gene chip technology, DNA fingerprinting etc. are used in TB diagnosis. However, there are still many problems to be

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Abbreviations: BCG, Bacille calmette-guerin; CFP, culture filtrate protein; CFP21, culture filtrate protein 21; ELISA, enzyme-linked immunosorbent assay; EPTB, extrapulmonary tuberculosis; ESAT-6, 6-kDa Early Secretory Antigenic Target; IPTG, isopropylthio- β -D-galactoside; kDa, kilo-Dalton; LTBI, latent tb infection; M.tb, *Mycobacterium tuberculosis*; MPT64, major protein of mycobacterium tuberculosis 64; OD, optical density; PTB, pulmonary tuberculosis; rCM, recombinant of CFP21 and MPT64 fusion protein; RD1, t region of difference 1; RD2, region of difference 2; rEC, recombinant of ESAT-6 and CFP10 fusion protein; SD, standard deviation; SEM, standard error of mean; TB, *Tuberculosis*.

resolved, such as more advanced equipments needed and high detection costs. The serological diagnostic techniques are simple and easy to be operated (Boehme et al., 2010; Sougakoff et al., 2004; Liu et al., 2006). If the specificity of serum detection antigen can be developed, it will be a promising diagnostic method. Presently, the genome sequences of *M.tb* have been identified. The culture filtrate protein 21 (CFP21) and the major protein of *M. tuberculosis* 64 (MPT64), encoded by the region of difference 2 (RD2) of *M.tb* genome, and the 6-kDa early secretory antigenic target protein (ESAT-6) and the culture filtrate protein 10 (CFP10) encoded by the the region of difference 1 (RD1), are considered as important candidates of TB-specific diagnostic antigens. These RD region-specific antigens are present in *M. tb*, but not in bacille calmette-guerin (BCG) vaccines. The antigen in RD2 region is also missing in BCG strain. The BCG strain vaccinated universally in China was transmuted from the late Danish strains. In developed countries such as Europe and USA, RD1 antigens ESAT-6 and CFP-10 have been made of T-cell immune diagnostic kits (Zachary, 2005; Arend et al., 2007). It has been demonstrated that EAST-6 used in *M.tb* cutantest is more effective for latent infection (Guinn et al., 2004). However, CFP10, when used as an antigen to detect the specific antibodies in TB serum, shows low sensitivity (Maue et al., 2007). MPT64 and CFP21, two antigens in RD2 region with strong immunogenicity, have been used as TB vaccines (Kobashi et al., 2008; Wang et al., 2011; Fu et al., 2009). In previous experiments, we found that the sensitivty to detect LTBI is up to 90.9%. The specificity of single MPY64 antigen detecting TB antigen is slightly higher than the purified protein derivative, but the sensitivity is low.

Many studies are needed to very carefully assess the sensitivity and specificity at different geographical locations for appropriate clinical populations using appropriate diagnostic gold standard. Some methods showed a better sensitivity, but lower specificity. Sensitivity, specificity, and ease of use should be maximized, even if with increases in the cost of detection (Lawn, 2012). Therefore, it is necessary to enhance the stability, sensitivity and specificity to replace the traditional purified protein derivative detection serum antibody.

In this study, we used the fusion antigen rCM of MPT64 and CFP21 and the fusion antigen rEC of ESAT-6 and CFP10 to perform ELISA-based serological diagnostic tests. The aims are to establish a sensitive, specific, and rapid approach to detect TB infection and investigate the sensitivity and specificity of this test.

MATERIALS AND METHODS

Preparation of antigens rEC and rCM

Plasmids pPro610 and pPro2164, which express proteins rEC and

rCM, respectively, were constructed previously (Wang et al., 2011; Fu et al., 2009). Proteins rEC and rCM were expressed in pPro610 and pPro2164-transformed *Escherichia coli* and purified using a Ni-NTA purification system and detected by separation on SDS-PAGE gels followed by comassie staining.

Serum collection

There are 55 cases of TB patients totally, including 51 cases of pulmonary *Tuberculosis* (PTB) patients' serum (age 33±16 years; male/female 31:20). There are 4 cases of extrapulmonary *Tuberculosis* (EPTB) patients' serum (age 43 ± 20 years; male/female 3:1). The patients has given informed consent. They were devided into BCG vaccinated healthy people group and non-BCG vaccinated healthy people group. PTB patients were all confirmed by physical examination, sputum acid-fast staining, cultivation, and chest X-ray diagnosis. EPTB patients were diagnosed based on medical history and lesion-specific clinical symptoms and signs, combined with X-ray, CT, B ultrasound, bacterial culture, puncture, biopsy and other laboratory examinations, and meanwhile by effective anti-tuberculosis treatment, in which there were 1 case of tuberculous pleurisy, 1 case of renal tuberculosis, 1 case of lymphatic system and 1 case of bone tuberculosis. Before the patients receiving anti-TB treatment, 5 ml of peripheral blood was collected. The upper serum after centrifugation were stored at -20 °C.

Totally there are 92 cases of healthy group serum, including 82 cases of BCG-vaccinated healthy people (age 37±12 years; male/female 45:37), and 10 cases of non-BCG vaccinated healthy people (age 66±8 years; male/female = 6/4). Serum samples were obtained from health examination, which were identified by physical examination to be without human immunodeficiency virus infection, pregnancy, use of immunosuppressive agents or enhancers, severe kidney failure, oboslete tuberculose focus and other affections. Active TB was also excluded by sputum acid-fast staining, cultivation and chest X-ray examination. There were 14 cases of close contacts' serum that were confirmed to be IGRA positive and saved in our lab.

ELISA

The anti-TB antibodies were detected in serum samples by indirect detection. Two kinds of purified *M.tb* antigens, Pro rEC and rCM (5 µg/ml), respectively, were used to coat plates in 96-well microtiter plates. The OD values at 450 nm were measured using ELISA (Bio-tek, USA).

The reference wavelength was set to 630 nm. The ROC curve was made with SPSS 17.0 software. According to the formula sensitivity=true positive/(true positive+false negative) × 100%, specificity=true negative/(true negative + false positive) × 100%, two kinds of recombinant proteins and their combined sensitivity and specifcity were calculated, respectively.

Statistical analysis

SPSS 17.0 software was used to analyse stastically the related experiments data (test level α=0.05). Two different groups of data were compared by T test. Two or more groups of data were obtained by pairwise comparison and post hoc analysis. Two different antigens of the same crowd were compared by T test. Fourfold table analyses were done using chi-square test. Correlation analysis was performed using pearson analysis. Scatter was drawn by GraphPad Prism 5.

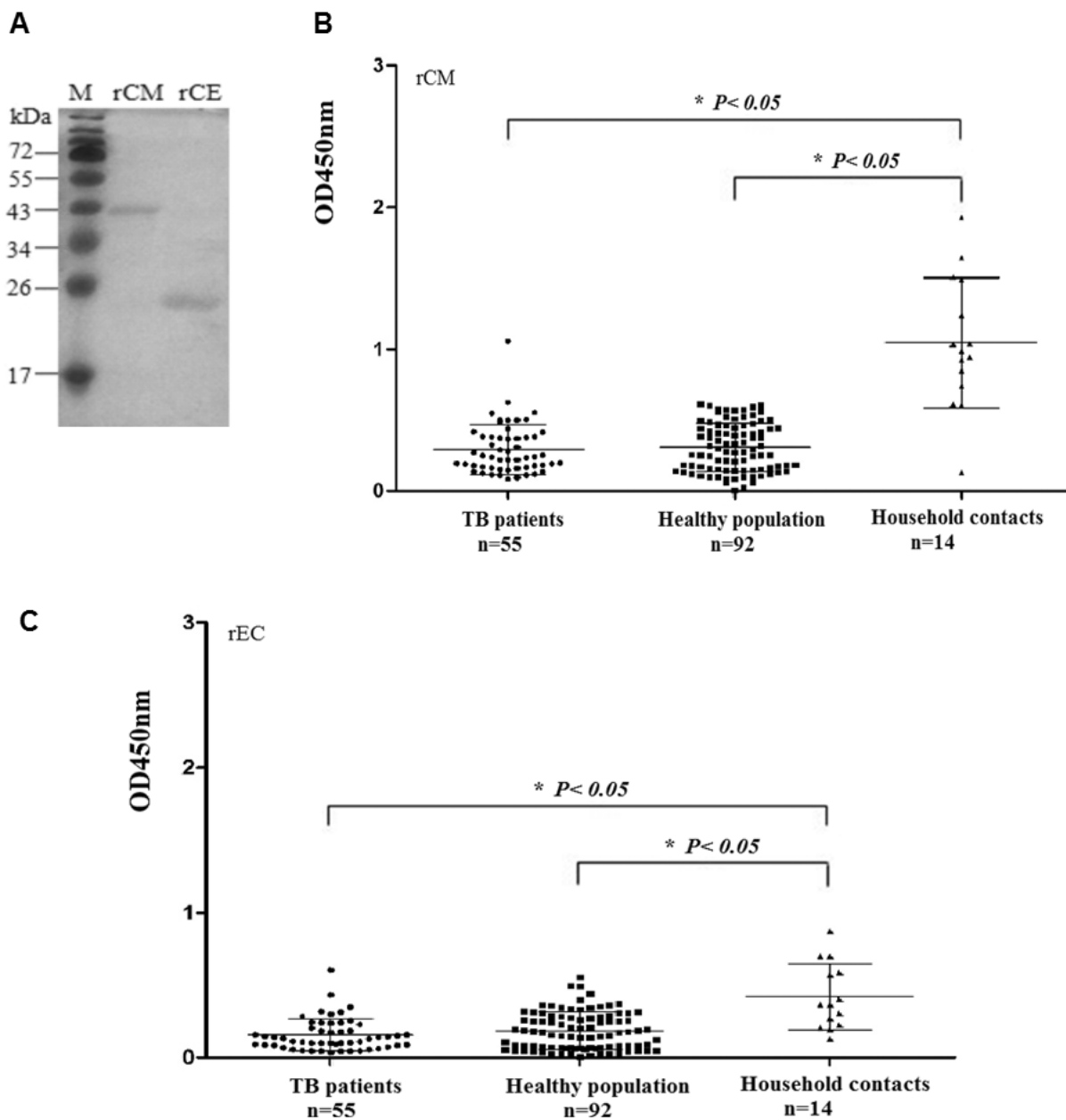


Figure 1. (A) SDS-PAGE gel electrophoresis of fusion protein rCM and rEC. M: protein molecular weight standards; rCM: rCM purified protein (43kDa); rEC: rEC purified protein (22kDa). (B) Detection results of rCM antibodies in serum of TB patients, healthy people, and the closer contacts. * indicates that it is statistically different when $p < 0.05$. (C) Detection results of rEC antibodies in serum of TB patients, healthy people, and the closer contacts. * indicates that it is statistically different when $p < 0.05$.

RESULTS

Expression and purification of recombinant antigens

We conducted the prokaryotic expression system of the fusion proteins rEC and rCM in our previous work. We

expanded culture of *E. coli* BL21(DE3) containing recombinant expression plasmids, pPro610 and pPro2164, induced with IPTG and collected the thallus. After ultrasonication, we purified proteins. As shown in Figure 1A, SDS-PAGE gel electrophoresis of fusion protein rCM and rEC indicated that the molecular weight of rCM and

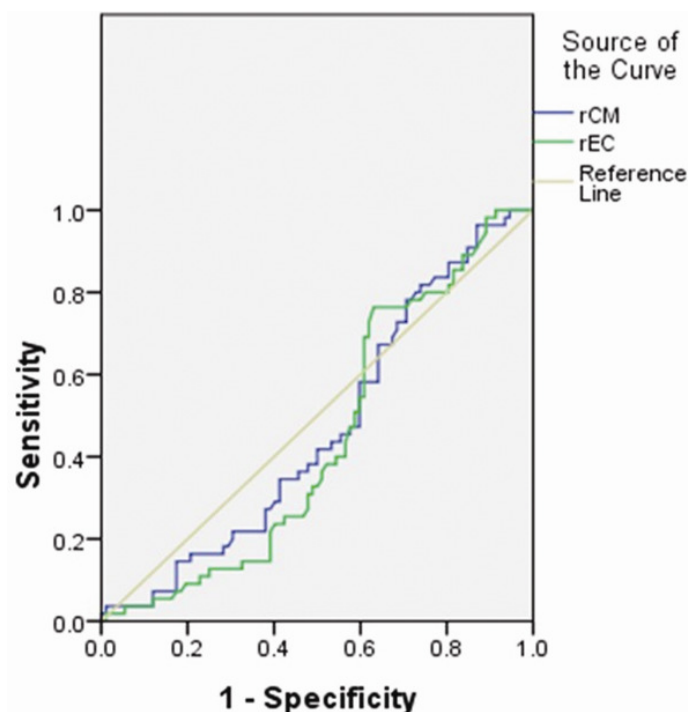


Figure 2. ELISA detection of rCM and rEC proteins.

rEC are about 43 and 22 kDa, respectively.

The influences on ELISA results for healthy and TB patient samples vaccinated with BCG

We divided the healthy people and TB patients into the BCG vaccinated group and the non-BCG vaccinated group. The results were shown in Table 1. There was no difference among the same specific antibodies in the sera of both BCG inoculators and non-BCG inoculators, in both the healthy group and TB patients. Therefore, in the following experiments, the effects of BCG vaccination will not be calculated.

ELISA detection of serum rCM and rEC antibodies in TB patients, healthy people and close contacts

ELISA detection of serum rCM and rEC antibodies in TB patients, healthy people and close contacts were performed. According to the rCM test results shown in Figure 1B, the OD mean of close contacts ($n = 14$) were statistically different from the two other groups, the TB patient group ($n = 55$) and the healthy population group ($n = 92$). According to rEC test results shown in Figure 1C, the OD mean of close contacts were also statistically

different from the other two groups, the TB patient group ($n = 55$) and the healthy population group ($n = 92$).

Detection of the cut-off value in system

The OD values of pro rCM and pro rEC in ELISA detection system into TB+ healthy people group, TB+ close contacts group and healthy crowd + close contacts group were calculated. The ROC curve with the sensitivity as the Y-axis, with the false-positive rate as the X-axis, was generated. As shown in Figure 2, the cut-off values were 0.1085, 0.6025 and 0.5800. The area under the curve were 0.466, 0.995 and 0.986, according to the rCM protein detection. The cut-off values were 0.084, 0.3635 and 0.1910. The area under the curve was 0.443, 0.813 and 0.886, according to the rEC protein detection.

Sensitivity and specificity

Using the cut-off values mentioned above, the positive and negative cases of rCM and rEC specific antigens were detected, listed in fourfold table, and calculated (Table 2). Among them, the parallel detection of two antigens are identified as that it is confirmed to be positive when the antibody in either of the two antigens detection serum was positive. The serial detection of two antigens is identified as that it is positive when both antibodies in two antigens detection serum were positive. We can see from Table 2 that the P value was less than 0.05 whether it's rCM or rEC, or both used in conjunction, which indicates it's unable to meet the need to distinguish TB patients. However, rCM and rEC showed high sensitivity and specificity when distinguished between closer contacts and healthy people group or between closer contacts and TB patients, which was highly consistent with the real situation identified by complicated means before. The detection method based on rCM was superior to the one based on rEC.

The relationship between the level of rCM specific antigens and that of rEC specific antigens in the same category population sample

In order to investigate antigen's immune dominance, we compared the levels of two kinds of antibodies in TB patients, healthy people and close contacts. The results were shown in Table 3. In TB patients, healthy people and close contacts, the rCM specific antibodies levels were significantly higher than rEC specific antibodies levels.

Among TB patients, the two kinds of antibodies' coefficient correlation was 0.602 ($P < 0.001$). The two antibodies showed positive correlation. The coefficient correlation among healthy people was 0.846 ($P <$

Table 1. ELISA results of TB patients and healthy individuals inoculated with BCG.

Result	Antigens	BCG inoculation	Samples	Means	SD	P
TB patients	rCM	+	44	0.3015	0.1849	0.4028
		-	11	0.2516	0.1253	
	rEC	+	44	0.1572	0.1129	0.7851
		-	11	0.1470	0.0962	
Healthy individuals	rCM	+	82	0.3036	0.1711	0.2218
		-	10	0.3767	0.2265	
	rEC	+	82	0.1850	0.1327	0.1521
		-	10	0.2572	0.2531	

BCG, Bacille Calmette-Guerin; ELISA, Enzyme-Linked Immunosorbent Assay; rCM, recombinant of CFP21 and MPT64 fusion protein; rEC, recombinant of ESAT-6 and CFP10 fusion protein; TB, *Tuberculosis*.

Table 2. TB detection of TB patients and healthy individuals.

TB	rCM		Total	rEC		Total
	+	-		+	-	
+	53	2	55	42	13	55
-	80	12	92	58	34	92
Total	133	14	147	100	47	147

$p < 0.05$.

0.001, positive correlation). The coefficient correlation among close contacts was 0.938 ($P < 0.001$, positive correlation).

DISCUSSION

In this study, we discovered that RD1 region encoding fusion protein rCM and RD2 region encoding fusion protein rEC are recognized specifically by the serum antibodies in TB patients, healthy people and close contacts. Surprisingly, based on the serum specific antibodies of close contacts detected by two fusion proteins higher than other two groups, there were 3 reasonable explanations for this phenomenon. Firstly, the test samples were limited and the sample size may be increased in the future studies. Secondly, rCM and rEC were related with *M.tb*'s latent infection (Hoff et al., 2007; McConkey et al., 2002; Kassa-Kelembho et al., 2006). Thirdly, mpt64 and other genes were present in minority of BCG vaccine and non-*M.tb* genome (Behr and Small, 1999), which may lead to low specificity.

We established a method depending on two fusion antigens rCM and rEC to detect the specific antibodies in serum by ROC curve, and made further analysis of two antigens detection system detecting samples of different

crowds. ROC curve is a method that we derived the diagnostic test results into several critical points, and calculated all the possible values, thus demonstrating the correlation between sensitivity and specificity. Therefore it is good to apply ROC curve into this experiment, which not only ensures the reliability of the test results but also make comprehensive evaluation of this attempt as an effective tool. Our test is more advantageous than results reported previously. For example, the T-cell response of the vast majority of TB immune advantage antigens such as ESAT-6 and CFP-10, can not distinguish between active TB and latent TB infection (Pai et al., 2008). Our fusion antigens' humoral immune response can identify the infection is active TB or latent TB, which is also an advantage than the previous publication. Our research gave an encouraging answer. Two fusion antigens of rCM and rEC can not distinguish between TB patients and healthy people in this study, but can distinguish between TB patients and close contacts or healthy people and close contacts. The sensitivity and specificity of the latter two groups are up to a high level. The areas under the curve exceed 0.8, which is statistically significant and has considerable potential for detection of LTBI. In addition, rCM's immune dominance is higher than rEC overall. There was positive correlation between the two antigens on the level of specific antibodies detected in the same category crowd. Furthermore, based on the rCM detection in close contacts and TB patients, the sensitivity and specificity were up to 100 and 96.4% respectively, which was more effective than the detection method based on rEC (the sensitivity and specificity were up to 100 and 96.4%, respectively).

Generally, TB patients' antibody response was against multiple TB antigens. Any kind of antigens detect all the TB antibodies of *Tb* patients serum. Therefore we need combined multiple antigens to detect, which will help improve the diagnostic sensitivity on the basis of ensuring

Table 3. Immuno-potitive detection of rCM and rEC.

Variable	Antigens	Means	Sample	SD	SEM	P
TB patients	rCM	0.2915	55	0.1748	0.0236	<0.001
	rEC	0.1551	55	0.1090	0.0147	
Healthy	rCM	0.3115	92	0.1779	0.0185	<0.001
	rEC	0.1929	92	0.1501	0.0156	
Closer contacts	rCM	1.1099	14	0.3995	0.1068	<0.001
	rEC	0.4211	14	0.2276	0.0608	

SD, Standard Deviation, SEM, Standard Error of Mean.

specificity. It is a meaningful attempt for this that we applied fusion protein rCM and rEC. In this study we established ELISA immune system based on these two fusion proteins in order to detect more antibodies of TB patients *in vivo*. We found an effective direction for LTBI diagnostic method, and provided theoretical support to promote TB serological diagnostic level and TB epidemic control.

REFERENCES

- Arend SM, Thijsen SF, Leyten EM, Bouwman JJ, Franken WP, Koster BF, Cobelens FG, van Houte AJ, Bossink AW (2007). Comparison of twointerferon-gamma assays and tuberculin skin test for tracing tuberculosis contacts. *Am. J. Respir. Crit. Care. Med.* 175(6):618-627.
- Behr MA, Small PM (1999). A historical and molecular phylogeny of BCG strains. *Vaccine* 17(7-8):912-922.
- Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, Allen J, Tahirlir R, Blakemore R, Rustomjee R, Milovic A, Jones M, O'Brien SM, Persing DH, Ruesch-Gerdes S, Gotuzzo E, Rodrigues C, Alland D, Perkins MD (2010). Rapid Molecular Detection of Tuberculosis and Rifampin Resistance. *N. Engl. J. Med.* 363(11):1005-1015.
- Fu R, Wang C, Shi C, Lu M, Fang Z, Lu J, Wang F, Fan X (2009). An improved whole-blood gamma interferon assay based on the CFP21-MPT64 fusion protein. *Clin. Vaccine Immunol.* 16(5):686-691.
- Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, Lewinsohn DM, Smith S, Sherman DR (2004). Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*. *Mol. Microbiol.* 51(2):359-370.
- Hoff ST, Abebe M, Ravn P, Range N, Malenganisho W, Rodrigues DS, Kallas EG, Søborg C, Mark DT, Andersen P, Weldingh K (2007). Evaluation of *Mycobacterium tuberculosis*-specific antibody responses in populations with different levels of exposure from Tanzania, Ethiopia, Brazil, and Denmark. *Clin. Infect. Dis.* 45(5):575-582.
- Jassal M, Bishai WR (2009). Extensively drug-resistant tuberculosis. *Lancet. Infect. Dis.* 9(1):19-30.
- Kassa-Kelembho E, Kassa E, Zandanga G, Service YB, Ignaleamoko A, Talarmin A (2006). Poor performance of a novel serological test for diagnosis of pulmonary tuberculosis in Bangui, Central African Republic. *Int. J. Tuberc. Lung Dis.* 13(6):702-703.
- Kobashi Y, Mouri K, Yagi S, Obase Y, Miyashita N, Okimoto N, Matsushima T, Kageoka T, Oka M (2008). Clinical utility of the QuantiFERON TB-2G test for elderly patients with active tuberculosis. *Chest.* 133(5):1196-1202.
- Lawn SD (2012). Point-of-care detection of lipoarabinomannan (LAM) in urine for diagnosis of HIV-associated tuberculosis: a state of the art review. *BMC. Infect. Dis.* 12(1):103-107.
- Liu LC, Lu XY, Fan XL (2006). Serological diagnosis of tuberculosis. *Microorganisms Infect.* 1(5):170-172.
- Maue AC, Waters WR, Palmer MV, Nonnecke BJ, Minion FC, Brown WC, Norimine J, Foote MR, Scherer CF, Estes DM (2007). An ESAT-6:CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. Bovis*. *Vaccine* 25(24):4735-4746.
- McConkey SJ, Youssef FG, Azem E, Frenck RW, Weil GJ (2002). Evaluation of a rapid-format antibody test and the tuberculin skin test for diagnosis of tuberculosis in two contrasting endemic settings. *Int. J. Tuberc. Lung Dis.* 6(3):246-252.
- Pai M, Zwerling A, Menzies D (2008). Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Ann. Int. Med.* 149(3):177-184.
- Sougakoff W, Rodrigue M, Truffot-Pernot C, Renard M, Durin N, Szpytma M, Vachon R, Troesch A, Jarlier V (2004). Use of a high-density DNA probe array for detecting mutations involved in rifampicin resistance in *Mycobacterium tuberculosis*. *Clin. Microbiol. Infect.* 10(4):289-294.
- Wang C, Chen Z, Fu R, Zhang Y, Chen L, Huang L, Li J, Shi C, Fan X (2011). A DNA vaccine expressing CFP21 and MPT64 fusion protein enhances BCG-induced protective immunity against *Mycobacterium tuberculosis* infection in mice. *Med. Microbiol. Immunol.* 200(3):165-175.
- Zachary T (2005). Guidelines for the Investigation of Contacts of Persons with Infectious Tuberculosis Recommendations from the National Tuberculosis Controllers. *MMWR. Recomm. Rep.* 54(15):1-47.