Iranian Journal of Microbiology
Volume 5 Number 4 (December 2013) 383-390

Evaluation of FLASH – PCR forrapid detection of *Mycobacterium tuberculosis* from clinical specimens

Abbas Ali Imani Fooladi[1], Yousef Tarvedi zadeh[2], Rezvan Mehrab[1], Raheleh Halabian[1], Taghi Azizi[3]

[1]Applied Microbiology, Research Center Baqiyatallah University of Medical Sciences, Tehran, Iran. [2]Department of Biology, Faculty of Basic Sciences, Imam Hussein University, Tehran, Iran. [3]Department of Pathology, Schoo of Medical Sciences, Baqiyatallah University of Medical Sciences,Tehran, Iran.

Received: December 2012, **Accepted:** August 2013.

ABSTRACT

Background and Objectives: Tuberculosis (TB) is the oldest known bacterial disease in humans. Due to the rise of morbidity in recent years, early diagnosis of the disease is necessary.
Materials and Methods: In this study we used Fluorescent Amplification–Based Specific Hybridization (FLASH) PCR to target*IS6110* for rapid detection of *M. tuberculosis* (MTB). To investigate the important factors influencing the risk of TB, data from patients and their medical records were analyzed.
Result: The sensitivity and specificity of FLASH-PCR for detecting MTB were determined as 93.33% and 92.5%, respectively. The findings of this study have suggested that removal of the contaminants in FLASH-PCR sign ificantly reduced the detection time, and MTB was much more rapidly detected in the clinical specimens compared to the conventional culture and smear examination. Results of the medical survey showed that the majority of TB patients were males, over 51 years old, smokers, with pulmonary TB and normal chest X-ray (CXR).
Conclusion: MTB can be rapidly detected inclinical specimens using FLASH-PCR in comparison with culture and smear examination.

Keywords: *Mycobacterium tuberculosis*, FLASH-PCR, Rapid detection,Tuberculosis

INTRODUCTION

Mycobacterium tuberculosis (MTB) is one of the important causes of millions ofdeaths among adults annually (1). Recently, in many regions of the world, increase in multi drug resistant tuberculosis (MDR-TB) has become an emerging problem for public health (1, 2). Rapid detection of MDR-TB is necessary andcruicial for treatment management (3, 4). MDR-TB causes higher mortality rates than drug susceptible tuberculosis and is even more important in patients with immunodeficiency (2, 5). Moreover, early diagnosis of MTB infection is essential to the strategy selection for control of the spread and effective treatment (6, 7). Staining and direct microscopy of the pulmonary and extra pulmonary specimens lack sufficient sensitivity and specificity. The conventional techniques of MTB diagnosis are unreliable and time consuming (8, 9). Researchers are interested in devising rapid tests for detection of MTB (9-11). In recent years, themain focus has been on the development of PCR techniques (6) and many primer sets have been developed for specific detection of MTB genes (11, 12). Fuorescent amplification-based specific hybridization PCR (FLASH-PCR) with the power of fluorescence detection after the amplification has been introduced as a rapid and sensitive method for identification of pathogenic bacteria (12, 13). Abramova *et al.* applied FLASH–

* Corresponding author: Abbas Ali Imani Fooladi Ph.D
Address: IApplied Microbiology, Research Center Baqiyatallah University of Medical Sciences, Tehran, Iran.
Tel/Fax: +98-21-88068924
E-mail: imanifouladi.a@gmail.com

PCR for specific identification of wheat pathogenic fungi *S. tritici* and *S. nodorum* (14). Ryazantsev *et al.* diagnosed toxigenic fungi of the genus *Fusarium* with the same PCR technique (12). Moreover, Ryazantsev and Zavriev have developed an efficient diagnostic method for the major potato viral pathogens based on FLASH–PCR (15). It should be noted that FLASH–PCR requires no expensive equipment and the results are obtained within a few hours (12). On the other hand, this method decreases the risk of the bench-top contamination (15), whereas contamination is inevitable indetection by electrophoresis (14).

The repetitive insertion sequence *IS6110* has been one of the excellent targets for PCR amplification (16-18) and DNA probes for diagnosis of MTB and epidemiological investigations since early 1990s (19-21). Multiplecopies of *IS6110* (10 to 20 copies) exist in MTB (6, 22), whereas strains of *M. bovis* BCG contain only a single copy (16, 22). Therefore, targeting *IS6110* is helpful to increasethe sensitivity of PCR over that obtained in the amplification of single-copygenes.

In this study, we aimed to develop a FLASH-PCR system as a rapid and sensitive method for the detection and identification of MTB. The results obtained by FLASH-PCR technique was compared with those of culture and smear microscopy. Also, the important factors influencing the risk of TB disease such as age, gender, history of contact with TB patient, undelying diseases, and smoking were investigated in the patients.

MATERIALS AND METHODS

Sample collection and bacterial isolation. During April 2009 until March 2010, 255 pulmonary and extra-pulmonary specimens were collected from patients suspected with TB hospitalized in at eaching hospital in North of Tehran. Clincal specimens included sputum, bronchoalveolar lavage (BAL), pleural fluids, pulmonary aspiration, tracheal aspirates, ascites, wound samples, urine, gastric fluids, synovial fluids, throat swabs, and abscesses. The specimens were tested directly by Ziehl–Neelsen staining and cultured on Lowenstein-Jensen (LJ) medium as the standard methods (17, 24).

DNA extraction with Sorbent method. Mucolysin solution was used in the purification step to prevent the inhibitory effects of mucosal materials on PCR. Qiagen kit was used for DNA extraction as instructed by the supplier (Qiagen, Germany).DNA was eluted in 50µl NSP-5 (elution buffer), intensely vortexed, and incubated at 55°C for 15 min. Finally, 30 µl of supernatant containing DNA was used for PCR reaction.

DNA amplification. DNA amplification of the 123 bp *IS6110* insertion element was performed with above mention primers (25). Briefly, PCR reaction was designed with 25 µl total reaction mixture containing 10 µl of 2X master mix reaction buffer (1 mM of each dNTP, fluorescent probes, and 2 mM $MgCl_2$; Takarra, Japan), 1 µL of Taq polymerase (Roche, Germany), 5 µL (500 ng) product of DNA extraction (DNA sample), 5 µL DDW, 1µL forward and reverse primers at final concentration of 10 pmol and 3 µL of internal control (IC). The primers and the probe were used from a kit (DNA Technology, Russia). The GeneAmp PCR system 9600 (PerkinElmer, Wellesley, MA, USA) was used for DNA amplification. The amplification cycles contained: 1 cycle, 3min at 94°C for initial denaturation, 5 cycles, each of 1min at 94°C, and 1min at 67°C for denaturation and primer annealing, 45 cycles each of 30s at 94°C, 50s at 67°C for extension. PCR product tubes were transferred to Fluorescent Detector FD-12 (DNA Technology, Russia) and each tube was read and analyzed with GeneXpert software.

Survey of important factors influencing the risk of TB disease. A questionnaire was developed to collect social and demographic information of all TB cases (pulmonary and extra-pulmonary) such as age, gender, history of contact with TB patient, smoking and narcotics. Furthermore, medical documents of these patients were studied that contained a range of illnesses including bronchiectasis, cancer, sarcoidosis, pneumonia, fibrosis, pulmonary embolism, hydatid cyst, asthma, chronic obstructive pulmonary disease (COPD), chemical injury, symptoms of TB, normal or abnormal CXR, PPD skin test, and old or new TB (26).

Statistical Analysis. Data were processed and analyzed using statistical software such as Microsoft Excel 2010 and SPSS version 16.0. Comparisons between groups were made using chi-square and Fisher's exact test.

Fig. 1. FLASH-PCR; upper Fig: chart of flash PCR that detected with GeneXpert software.
Lower fig: gel electrophoresis; M: 100 bp ladder, 2 & 7 were negative sample, 1 was low positive sample.

RESULTS

From April 2009 until March 2010, 255 TB suspected cases were examined, of which 30 subjects were positive in MTB FLASH-PCR assay. Most of the positive specimens were BAL samples (25 out of 30, 83%). The patients ranging from 1 to 97 years of age (average 56 years) were studied and most of them were 50 years old (164 out of 255, 64%). The age breakdown of 30 positive cases were as follows: 5 cases ≤ 30 (16.6%), 1 case 31-40 age (3.3%), 5 cases 41-50 (16.6%) and 19 cases ≤ 51 (63.5%). 106 out of total 255 subjects were females (41.6%) and 149 males (58.4%); in 30 positive cases, 9 cases were females (30%) and 21 males (70%).

Comparison between FLASH-PCR and microscopic and culture examination. FLASH-PCR was performed with *IS6110* as specific target gene for MTB detection. For detection of FLASH-PCR products, PCR product tubes were transferred to Fluorescent Detector FD-12. The PCR reaction was validated by detection of fluorescent IC. In positive samples (MTB patients) fluorescence of MTB probe was detected (Fig. 1). Therefore, the products are easily identified without the extra step of electrophoresis and the risk of contamination.

Maximum positive detection was obtained by FLASH-PCR in 30 cases (11.7% of total subjects), followed by culture (15 cases, 5.8%) and smear examination (11 cases, 4.3%) (Fig. 2).

Comparison of PCR results with both culture and microscopy examination showed that FLASH-PCR technique is more sensitive than other tests (Fig. 3) ($p < 0.05$), but specificity of this type of PCR was less than the one in gold standard test (culture) **(**Fig., $p > 0.05$).

Documentation of medical records. In the second part, we studied the factors that might play an

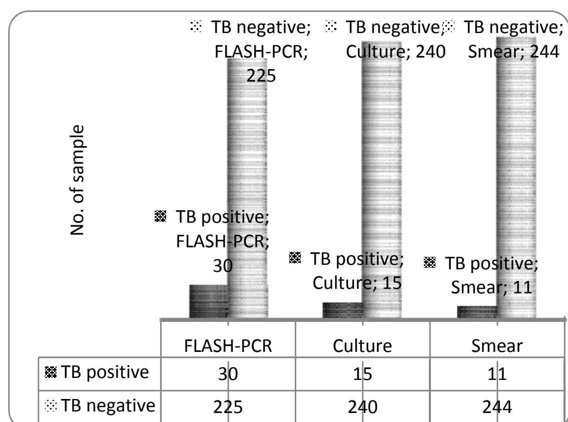


Fig. 2. Frequency of the positive samples (FLASH-PCR, culture, and smear examination).

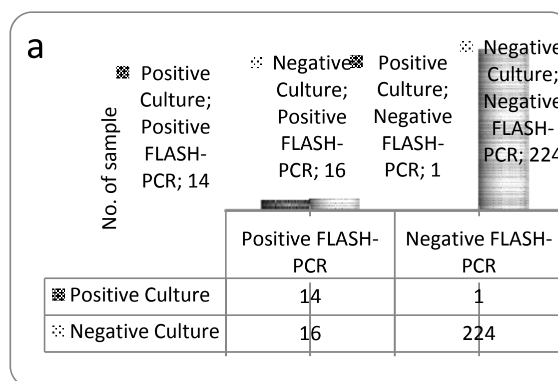
important role in the incidence of TB disease (Fig. 4). According to the laboratory results, among the 30 TB patients, 28 (93.3%) were pulmonary and 2 (6.66%) were extra-pulmonary cases (1 case urinary tract TB, and 1 case pleural TB).

Six out of 255 subjects had a history of contact with TB patients in their familiesor relatives of whom only one case was reported positive for clinical tuberculosis (0.39%). Among 249 cases that had no previous contact with a TB patient, 29 (1.13%) were positive for TB.

Since the initial diagnosis of pulmonary tuberculosis is often based on abnormal findings in the CXR images of patients plus respiratory signs and symptoms, the key to the clinicaldiagnosis of tuberculosis is often under suspecion. In this study, 22 out of 255 cases had tuberculosis specific report of CXR with fibrotic lesions. 2 cases (0.78%) were positive for TB, 140 of 255 cases had non specific report of CXR for tuberculosis,26 of whom (1.01%) were TB positive. 93 of 255 cases had a normal CXR, 2 cases (0.78%) were TB positive.

Our results of 30 TB positive patients, revealed that 3 cases (10%) had positive PPD, and PPD was not reported in many patients. In total,16 subjects (7.1%) had positive PPD test among 225 cases that were TB negative.

Furthermore, in the present study, 10 common symptoms of tuberculosis were selectedincluding: fever, chills, sweating, night sweating, weight loss, dry cough, cough with secretions, coughing up blood, asthma, and chest pain (Fig. 3a). Most of people with TB symptoms were asthmatic (225 cases, 83.3%), and showed symptoms such as weight loss (18 cases, 60%), fever (17 cases, 56.7%), dry cough (17 cases, 56.7%),



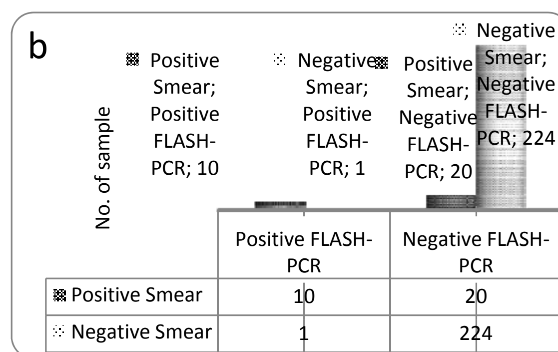


Fig 3. Comparison of FLASH-PCR with: a. Culture and b. Smear.

and cough with secretions (15 cases, 50%). In addition, 11 common lung diseases of the patients were studied in their medical documents. (Fig. 3b). Among 30 TB positive cases, 4 cases suffered from bronchiectasis (13.3%), 4 cases had lung cancer (13.3%), 1 case had co-infection with sarcoidos is (3.3%), 11 cases with pneumonia (36.7%), 5 cases with chemical injury (16.7%), 1case with hydatid cyst (3.3%), 1 case with asthma (3.3%), and 8 cases with COPD (26.7%). Considering the known effects of smoking, narcotics and alcohol on the lung diseases, we investigated the relationship between these factors and lung diseases. Out of the 255 cases studied, 193 were non-smokers, 41 smokers, 8 smokers and consumer of narcotics, 3 smokers and consumer of alcohol, and 10 consumers of narcotics. Among 30 TB positive patients, 21 were nonsmokers (70%), 7 smokers (23.3%), and 2 smokers and narcotic consumers (6.7%).

DISCUSSION

The World Health Organization (WHO) declared TB asone of the the greatest health problems for the global public health (27, 28). Since early detection

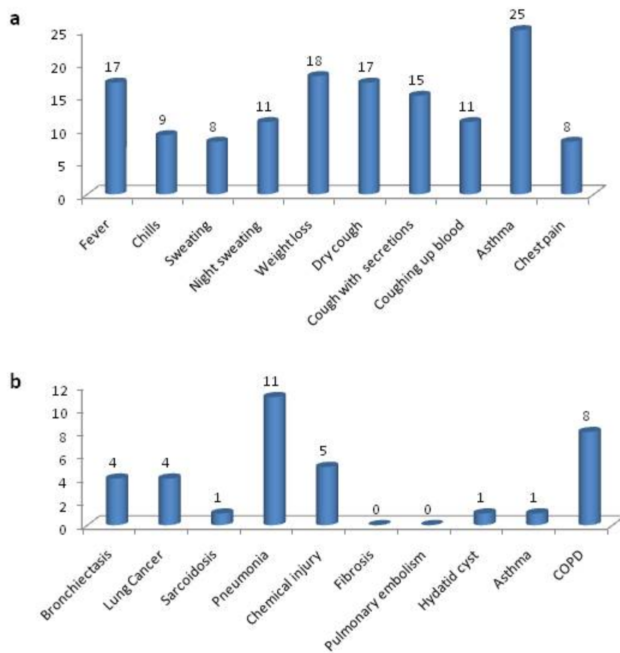


Fig 4. a. Symptoms of TB disease in the patients, b. Background of diseases of patients.

of MTB is an important emergency procedure for the control of tuberculosis (16), many researchers from different laboratories have used the PCR method for early diagnosis of MTB. In several studies, sensitivity of PCR with different DNA extraction methods has been reported between 80-90% for MTB complex diagnosis. The discrepancy in the findings could be related to the different type of PCR and primer sets. Various molecular methods have been applied to detect *M. tuberculosis* and gene mutations. The molecular methods such as PCR single stranded conformation polymorphism (PCR SSCP) assay, multiplex specific PCR assay, PCR RFLP analysis, PCR based dot blot hybridization strategy, molecular beacon assay, and direct sequencing assay (29-34) are each capable of detecting the bacterium, however they are time consuming, expensive and labor intensive. Real time PCR and GeneXpert MTB/RIF assays are highly sensitive and specific for the detection of *M. tuberculosis and* rifampicin resistance (35, 36). Importantly, one of the features of the MTB/RIF assay is its potential utility in resource poor settings. In such settings, there may be limited, if any, access to confirmatory phenotypic or genotypic drug susceptibility testing. In real time PCR technique the probe is used for detection and quantitative analysis. However, quantitative analysis is not as important as detection of MTB. In addition, real time PCR and MTB GeneXpert methods are dependant on specific real time PCR teromcycler. Therefore, an accurate, cost effective, rapid and easy-to-perform approach for identifying mycobacterial species is necessary.In recent studies FLASH-PCR technique has been used to detect various pathogens rapidly and specifically. Abramova *et al.* (14), used FLASH-PCR to detect the phytopathogens *Septoria tritici* and *Stagonospora nodorum*. Ryazantsev et al. (12) optimized FLASH-PCR for diagnosis of the toxigenic fungi *F. sporotrichioides* and obtained similar to those gained by the electrophoretic analysis. On the other hand, their results were similar to Ryazantsev and Zavriev's studyfor diagnosis of potato viral pathogens (15), and detection of *Septoria tritici* in wheat seeds by Consolo *et al.* (10). In this study FLASH-PCR concomitant with culture and smear examination were carried out for rapid detection of MTB in clinical specimens. Compared with the culture and smear results, sensitivity and specificity of the FLASH-PCR method for MTB detection was 93.33% and 92.5%, respectively. This demonstrates that FLASH-PCR test has a higher sensitivity than conventional examination and can be performed in less than 2 days (17). In our study, the primer specific from *IS6110* fragment was used in the FLASH-PCR for MTB detection, because there are multiple copies of this sequence in the genome of MTB which increases the accuracy, sensitivity, and specificity of this technique (23). Some culture-negative samples turned positive in PCR (18 samples). The loss of mycobacteria in cultures may be due to the decontamination process or structural damage to the bacteria or the death caused by drug therapy. It has been shown in previous studies that the bacterial count significantly reduced if the bacterial culture has not been performed immediately. Therefore, it is recommended that laboratories that perform MTB culture, must pay attention to the "time" factor (37). Exceptionally in the present study we gained negative PCR for a culture positive sample, which actually was a false negative result. This may be related to the low number of copies of *IS6110* in the genome of the samples or non uniform distribution of bacteria in the sputum samples.

In this study, there was no significant difference between the number of male (58.4%) and female (41.6%) participants. These findings are consistent with previous studies (26), that reported a higher frequency of pulmonary tuberculosis in men, which

is due to job and smoking and narcoticuse in male cases. In general, the diagnosis of pulmonary TB is performed with identification of acid-fast bacilli from bronchial secretions, chest radiography and evidence of patient's physical signs (38). The advantage of this study is that majority of patients had non-specific CXR report for tuberculosis or had normal CXR (39, 40). Normal CXR and a negative smear is a challenge for the early diagnosis of pulmonary TB, and we recommend FLASH-PCR as a more sensitive diagnostic tool to avoid the delay in the specific diagnosis of tuberculosis. In the present survey, most of the patients were smokers and some of them were drinking alcohol or consumed narcotics. In several studies a relationship between smoking and TB has been shown and was reported that the prevalence of smoking is higher in males than in females. Thus, smoking has been known as a risk factor for pulmonary tuberculos is because of various deleterious effects on the lungs (especially in the upper zones of the lung) (41). Smoking and alcohol consumption (53.6% and 36.5%, respectively) were the important risk factors for the development of tuberculosis (3). Interestingly, 5 out of 30 TB-positive patients (16.7%) were chemically injured. This finding implies that immune-compromised patiens may be more prone to tuberclosis.

In conclusion, the findings of this study have suggested that MTB in clinical specimens can be rapidly detected using of FLASH-PCR associated with culture and smear examination. FLASH-PCR as rapid diagnosis can be used for HIV-infected patients because of a higher susceptibility to MTB infection and so can be a great help for controlling MDR-TB. Moreover, FLASH-PCR technique allows results to be recorded without opening the test tubes and this means the removal of the contaminants in a conventional PCR, while contamination occurs during electrophoresis. Another advantage of this technique is the addition of specific primers including fluorescent probes that increased sensitivity and specificity of this technique.

ACKNOWLEDGMENT

This study was part of the dissertation of *Yousef Tarvedizadeh*, submitted to Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran in partial fulfillment of the requirements for the MSc in Microbiology.

REFERENCES

1. Aragón LM, Navarro F, Heiser V, Garrigó M, Español M, Coll P. Rapid detection of specific gene mutations associated with isoniazid or rifampicin resistance in *Mycobacterium tuberculosis* clinical isolates using non-fluorescent low-density DNA microarrays. *J Antimicrobl Chemother* 2006; 57: 825-831.
2. Watterson SA, Wilson SM, Yates MD, Drobniewski FA. Comparison of three molecular assays for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* 1998; 36: 1969-1973.
3. Maurya V, Vijayan VK, Shah A. Smoking and tuberculosis: an association overlooked. *Int J Tuberc Lung Dis* 2002; 6: 942-51.
4. Sekiguchi J, Miyoshi-Akiyama T, Augustynowicz-Kopeć E, Zwolska Z, Kirikae F, Toyota E. Detection of multidrug resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* 2007; 45: 179-192.
5. Musial CE, Tice LS, Stockman L, Roberts GD. Identification of mycobacteria from culture by using the Gen-Probe Rapid Diagnostic system for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 1988; 26: 2120-2123.
6. Dalovisio JR, Montenegro-James S, Kemmerly SA, Genre CF, Chambers R, Greer D, et al. Comparison of the Amplified *Mycobacterium tuberculosis* (MTB) Direct Test, Amplicor MTB PCR, and IS6110-PCR for detection of MTB in respiratory Specimens. *Clin Infect Dis*1996; 23: 1099-106.
7. Mäkinen J, Marttila HJ, Marjamäki M, Viljanen MK, Soini H. Comparison of Two Commercially Available DNA Line Probe Assays for detection of multidrug-Resistant *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006; 44: 350-352.
8. Negi SS, Basir SF, Gupta S, Pasha ST, Khare S, Lal S. Comparative study of PCR, smear examination and culture for diagnosis of cutaneous tuberculosis. *J Commun Dis* 2005; 37: 83-92.
9. Negi S, Gupta S, Khare S, Lal S. Comparison of various microbiological tests including polymerase chain reaction for the diagnosis of osteoarticular tuberculosis. *Indian J Med Microbiol* 2005; 23: 245-248.
10. Consolo V, Albani C, Berón C, Salerno G, Cordo C. A conventional PCR technique to detect & lt; i & gt; Septoria tritici in wheat seeds. *Australasian Plant Pathology* 2009; 38: 222-7.
11. Liu Y, Han J-X, Huang H-Y, Zhu B. Development and evaluation of 16S rDNA Microarray for detecting bacterial pathogens in cerebrospinal fluid. *Exp Biol Med (Maywood)* 2005; 230: 587-91.
12. Ryazantsev D, Abramova S, Evstratova S, Gagkaeva T, Zavriev S. FLASH-PCR diagnostics of toxigenic fungi of the genus & lt; i & gt; Fusarium & lt;/i & gt. *Bioorg Khim* 2008; 34: 716-24.
13. Abd-Elsalam K, Bahkali AH, Moslem M, De Wit PJGM, Verreet J-A. Detection of *Mycosphaerella graminicola* in Wheat Leaves by a microsatellite dinucleotide specific-primer. *Int J Mol Sci* 2011; 12: 682-93.

14. Abramova S, Ryazantsev D, Voinova T, Zavriev S. Diagnostics of phytopathogen fungi; *Septoria tritici* and *Stagonospora nodorum* by fluorescent amplification-based specific hybridization (FLASH) PCR. *Bioorg Khim* 2008; 34: 97-102.
15. Ryazantsev D, Zavriev S. An efficient diagnostic method for the identification of potato viral pathogens. *Molecular Biology* 2009; 43: 515-23.
16. Noordhoek GT, Kolk AH, Bjune G, Catty D, Dale JW, Fine PE, et al. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. *J Clin Microbiol* 1994; 32: 277-84.
17. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 1997; 35: 907-914.
18. Hosseini MJ, Imani Fooladi AA. Miliary tuberculosis with empyema, a case report. *Jundishapur J Microbiol* 2010; 3: 129-132.
19. Al-Hajoj SAM, Zozio T, Al-Rabiah F, Mohammad V, Al-Nasser M, Sola C, et al. First Insight into the population structure of *Mycobacterium tuberculosis* in Saudi Arabia. *J Clin Microbiol* 2007; 45: 2467-73.
20. de Boer AS, Borgdorff MW, de Haas PEW, Nagelkerke NJD, van Embden JDA, van Soolingen D. Analysis of rate of change of IS6110 RFLP patterns of *Mycobacterium tuberculosis* based on serial patient isolates. *J Infect Dis* 1999; 180: 1238-1244.
21. Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PWM, Martín C, et al. Comparison of Methods Based on Different Molecular Epidemiological Markers for Typing of *Mycobacterium tuberculosis* Complex Strains: Interlaboratory study of discriminatory power and reproducibility. *J Clin Microbiol* 1999; 37: 2607-2618.
22. Cave MD, Eisenach KD, Templeton G, Salfinger M, Mazurek G, Bates JH, et al. Stability of DNA fingerprint pattern produced with IS6110 in strains of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1994; 32: 262-266.
23. Negi SS, Anand R, Pasha ST, Gupta S, Basir SF, Khare S, et al. Diagnostic potential of IS6110, 38kDa, 65kDa and 85B sequence-based polymerase chain reaction in the diagnosis of *Mycobacterium tuberculosis* in clinical samples. *Indian J Med Microbiol* 2007; 25: 43-49.
24. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid Molecular Detection of Tuberculosis and Rifampin Resistance. *N Engl J Med New England Journal of Medicine* 2010; 363: 1005-1015.
25. Narayanan S, Parandaman V, Narayanan PR, Venkatesan P, Girish C, Mahadevan S, et al. Evaluation of PCR using TRC (4) and IS6110 primers in detection of tuberculous meningitis. *J Clin Microbiol* 2001; 39: 2006-2008.
26. Nguyen Phuong Hoa, Thorson AEK, Nguyen Hoang Long, Diwan VK. Knowledge of tuberculosis and associated health-seeking behaviour among rural Vietnamese adults with a cough for at least three weeks. *Scand J Public Health Suppl* 2003; 31: 59-65.
27. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* 1998; 79: 3-29.
28. Von Mutius E, Pearce N, Beasley R, Cheng S, von Ehrenstein O, Björkstén B, Weiland S. International patterns of tuberculosis and the prevalence of symptoms of asthma, rhinitis, and eczema. *Thorax* 2000; 55: 449-453.
29. Ahmad S, Jaber AA, Mokaddas E. Frequency of embB codon 306 mutations in ethambutol-susceptible and resistant clinical *Mycobacterium tuberculosis* isolates in Kuwait. *Tuberculosis (Edinb)* 2007; 87: 123-129.
30. Chan RC, Hui M, Chan EW, Au TK, Chin ML, Yip CK, et al. Genetic and phenotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates in Hong Kong. *J Antimicrob Chemother* 2007; 59: 866-873.
31. Hazbón MH, Bobadilla del Valle M, Guerrero MI, Varma-Basil M, Filliol I, et al. Role of embB codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. *Antimicrob Agents Chemother* 2005; 49: 3794-3802.
32. Rinder H, Mieskes KT, Tortoli E, Richter E, Casal M, Vaquero M, et al. Detection of embB codon 306 mutations in ethambutol resistant *Mycobacterium tuberculosis* directly from sputum samples: a low-cost, rapid approach. *Mol Cell Probes* 2001; 15: 37-42.
33. Ramaswamy SV, Amin AG, Göksel S, Stager CE, Dou SJ, El Sahly H, et al. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2000; 44: 326-336.
34. Victor TC, Jordaan AM, van Rie A, van der Spuy GD, Richardson M, van Helden PD, et al. Detection of mutations in drug resistance genes of *Mycobacterium tuberculosis* by a dot-blot hybridization strategy. *Tuber Lung Dis* 1999; 79: 343-348.
35. Scott LE, McCarthy K, Gous N, Nduna M, Van Rie A, Sanne I, et al. Comparison of Xpert MTB/RIF with other nucleic acid technologies for diagnosing pulmonary tuberculosis in a high HIV prevalence setting: a prospective study. *PLoS Med* 2011; 8: 235-240.
36. Lawn SD, Brooks SV, Kranzer K, Nicol MP, Whitelaw A, Vogt M, et al. Screening for HIV-associated tuberculosis and rifampicin resistance before antiretroviral therapy using the Xpert MTB/RIF assay: a prospective study. *PLoS Med* 2011; 8: e1001067.
37. Banavaliker JN, Bhalotra B, Sharma DC, Goel MK, Khandekar PS, Bose M. Identification of *Mycobacterium tuberculosis* by polymerase chain reaction in clinical specimens. *Indian journal of tuberculosis* 1998; 45: 15-18.
38. Zheng W, Blot WJ, Liao ML, Wang ZX, Levin LI, Zhao JJ, et al. Lung cancer and prior tuberculosis infection in Shanghai. *Br J Cancer* 1987; 56: 501-504.
39. Aderaye G, Bruchfeld J, Assefa G, Feleke D, Källenius

G, Baat M, et al. The relationship between disease pattern and disease burden by chest radiography, M. tuberculosis Load, and HIV status in patients with pulmonary tuberculosis in Addis Ababa. *Infection* 2004; 32: 333-338.

40. Marciniuk DD, McNab BD, Martin WT, Hoeppner VH. Detection of pulmonary tuberculosis in patients with a normal chest radiograph. *Chest* 1999; 115: 445-452.
41. Leung CC, Li T, Lam TH, Yew WW, Law WS, Tam CM, et al. Smoking and tuberculosis among the Elderly in Hong Kong. *Am J Respir Crit Care Med* 2004; 170: 1027-1033.