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Rapid Direct Detection and Differentiation of *Mycobacterium tuberculosis* complex in Sputum by Real-Time PCR

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Abstract

Tuberculosis status as the second leading causes of significant morbidity and mortality from an infectious disease worldwide, after human immunodeficiency virus *(*HIV). Sample collection was conducted at the Institute of Chest and Respiratory Diseases/Baghdad Medical City in Baghdad. The collection interval was from August to October 2014, 629 suspected TB patients were examined during this period. The results revealed among total 629 specimens, 56 (8.9%) of the specimens were positive by direct examination and 573 (91.1%) negative specimens by smear microscopy. Fifty six DNA samples were extracted from positive ZN smears of sputum specimens and 40 samples from healthy persons (as control) were subjected to molecular diagnosis by real time PCR to detect and differentiate of *M. tuberculosis*, *M. bovis* and *M. bovis* BCG. The results were clarified that the 48 samples (85.72%) were *M. tuberculosis*, 2 samples (3.57%) were mixed of *M. tuberculosis* and *M. bovis*, no *M. bovis* BCG was detected, and 6 (10.71%) were negative. These findings propose that *M. bovis* plays a minor role compared to *M. tuberculosis* in the etiology of pulmonary tuberculosis in Baghdad.

Keywords: *Mycobacterium tuberculosis* complex, Ziehl Neelsen Stain, Real time PCR, Sputum.

الكشف والتمييز المباشر السريع لعصيات السل المركبة في القشع بأستخدام تفاعل البوليميراز المتسلسل اللحظي

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الخلاصة

يعتبر مرض السل المسبب الرئيسي الثاني للهلاك و الوفاة في جميع انحاء العالم من بين الأمراض المعدية، بعد فيروس نقص المناعة المكتسب (HIV). تم جمع العينات في المختبر المرجعي التابع لمعهد الأمراض الصدرية و التنفسية/مدينة بغداد الطبية في بغداد، للفترة من بداية شهر آب الى نهاية شهر تشرين الأول 2014، أذ تم فحص 629 من المرضى المشكوك بأصابتهم بمرض السل، أكدت النتائج وجود 56 (8.9%) عينة موجبة للفحص المجهري المباشر، بينما 537 (91.1%) عينة كانت سالبة لهذا الفحص. تم

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أختيار 56 عينة الموجبة للكشف المجهري المباشر و 40 عينة سالبة للكشف المجهري المباشر لأغراض الدراسة الجزيئية بأستخدام تفاعل البوليميراز المتسلسل اللحظي و بعد أستخلاص الجينوم (DNA) منها لتشخيص عائدية البكتريا الى جنس *Mycobacterium* كذلك لمعرفة عائدية هذه الجراثيم لمجموعة عصيات السل المركبة complex *Mycobacterium tuberculosis* وهي *M. tuberculosis* ، *M. bovis* ، *M. bovis* BCG . أظهرت النتائج وجود 48 (85.72%) مريض مصاب بـ *M. tuberculosis*، 2 (3.57%) مريض مصاب بخليط من *M. tuberculosis* و *M. bovis*، ولم تشخص اي حالة أصابة بـ *M. bovis* BCG، بينما أظهرت 6 (10.71%) عينات نتيجة سالبة لهذا الفحص. و تشير هذه النتائج الى أن *M. tuberculosis* تلعب دوراً كبيراً و بارزاً في التسبب في مرض السل الرئوي في بغداد مقارنة بـ *M. bovis*.

Introduction

Tuberculosis (TB) infection is a contagious disease; the majority of cases of human TB are caused by *Mycobacterium tuberculosis* (MTB), a species among the genus *Mycobacterium* that are acid-fast, non-motile, slow-growing aerobic bacilli. Pulmonary TB is the usual form of infection but other sites can also be affected (extra-pulmonary TB). The disease is spread through the air, and only from patients with pulmonary TB. Therefore, it is important to diagnose MTB from pulmonary specimens to prevent spread of the disease [1, 2, 3]. According to the World Health Organization (WHO), TB infection is the second highest mortality causing infectious disease worldwide [4]. Despite advances and developments in diagnosing and treating TB, TB remains a major health burden around the globe. An estimated 8.6 million new cases and 1.3 million deaths occurred in 2012 [3]. Iraq is considered among eight high TB burden countries in Eastern Mediterranean Region (EMR) [5]. The key aspect of TB control is rapid diagnosis, which for many years has been based on the staining of smears for the presence of acid-fast bacilli (AFB) [6]. Molecular methods have been increasingly used in the diagnosis of TB [7]. Nucleic acid amplification based techniques are potentially the most rapid and sensitive methods for detection, and identification [8].

The aim of this study was evaluate the using of traditional and molecular methods for identification of causative agent of tuberculosis from sputum of Iraqi patients in Baghdad.

Materials and Methods:

Samples Collection and DNA Extraction

During August - October 2014, the Institute of Chest and Respiratory Diseases/Baghdad Medical City in Baghdad received 629 patients with suspected pulmonary tuberculosis lesions. Each randomly selected patient instructed to inhale deeply 2-3 times, cough out deep from the chest and spit the sputum into sterile universal container. Two samples were collected from each patient. First, one was taken from patient when he or she just reached the institute; second sample collected at early morning before breakfast. Collected specimens were stored at –20°C until use [9, 10]. Ziehl-Neelsen Stain sputum smears were examined for the presence of pulmonary acid fast bacilli. Of them, 56 specimens were acid fast bacilli smear positive pulmonary tuberculosis. These 56 specimens were selected for evaluation of the assay, and 40 specimens from healthy persons were collected as negative control. DNA extraction carries out according to the manufacture's instruction of DNA-Sorb-B Kit (Sacace – Italy) with some modifications and stored at –20°C until use. Concentration of dsDNA was determined using the Quantus Fluorometer with QuantiFluor dsDNA System (Promega, USA) according to the manufacture's instruction.

Real time PCR for detection and differentiation of *M. tuberculosis* complex

All positive Ziehl Neelsen Stain sputum smears were confirmed by MTB Diff Real-TM kit (Sacace, Italy) with real time PCR, and carries out according to the protocol provided by the kit.

Results and Discussion:

As in many developing countries, direct sputum microscopy is the gold standard method for the diagnosis of pulmonary TB in Iraq. Only 56 from 629 patients were positive by using ZN smears (Ziehl-Neelsen stain technique), they were therefore regarded as presence of *Mycobacterium* in the specimen and further investigation was performed for those 56 samples. Stained by ZN showed acid-fast bacilli, which were appear red, straight or slightly curved rods, occurring singly or in clumps and the size ranges from 0.3-0.6 µm x 0.6-1µm [11]. Acid-fast staining of sputum is a rapid, inexpensive method for diagnosing pulmonary tuberculosis. Despite of low sensitivity of ZN stain as diagnostic tool for TB, it is still an essential process for diagnosis of high bacillary load patients where sputum

smears would be positive [12, 13]. It requires 10^4 CFU/ml of sputum to be visualized by a light microscope [14]. As a conclusion, the examination through Ziehl- Neelsen stain is commonly missed in sputum specimen may be due to the very few number of acid fast bacilli.

Fifty six DNA samples were extracted from positive ZN smears of sputum specimens and 40 samples from healthy persons (as control) were subjected to molecular diagnosis by real time PCR to determine *Mycobacterium* species identity and confirmation result gained from Ziehl Neelsen stain test, using MTB Diff Real-TM kit (Sacace, Italy). This assay was successful in the qualitative detection and differentiation of *M. tuberculosis, M. bovis* and *M. bovis* BCG.

Differentiation of *M. tuberculosis* complex members by biochemical test profiles of pure culture is time consuming require 3 to 6 weeks and not practical for control purposes, it has been evaluated that possibly 5-10% of the global tuberculosis burden may be due to *M. bovis.* The PCR based methods have been used extensively to differentiate TB from Bacillus Calmette Guerin (BCG) [15, 16].

Because the recent global resurgence of mycobacterial infections, there is an increasing demand for rapid, sensitive, and specific diagnostic methods for the detection and identification of *Mycobacterium tuberculosis* complex (MTBC) and nontuberculous mycobacteria (NTM) in a clinical setting. In addition, mixed infections of MTBC and NTM have been reported; therefore, it has become important to be able to differentiate between the two during the early stage of the diagnostic procedure. Conventional clinical estimation, Ziehl Neelsen staining and culture methods accompanied with biochemical identifications are not satisfactory for accurate and precise diagnosis, while the molecular identification using PCR based methods have been more effective to differentiated and diagnosis mycobacteria [17].

The diagnosis of the positive ZN smears by real time PCR demonstrated that the greater number of positive ZN smears belongs to *Mycobacterium* genus, it was important to distinguish the *M. tuberculosis* complex (MTBC) species from *Mycobacterium* other than tuberculosis (MOTT) also known as nontuberculous mycobacteria (NTM) (NTM refers to all the species in the family of mycobacteria that may cause human disease, but do not cause tuberculosis), so that using the MTB Diff Real-TM kit (Sacace, Italy) which contains specific primers and fluorescent reporter dye probes for *M. tuberculosis, M. bovis* and *M. bovis* BCG. The results were clarified that the 48 samples (85.72%) were *M. tuberculosis* Figure-1, 2 samples (3.57%) were mixed of *M. tuberculosis* and *M. bovis*, no *M. bovis* BCG was detected, and 6 (10.71%) were negative. These findings propose that *M. bovis* plays a minor role compared to *M. tuberculosis* in the etiology of pulmonary tuberculosis in Baghdad. Current results were in accordance with study in Egypt by [18] which found that among the 45 *M. tuberculosis* complex isolates from sputum samples, 44 were identified as *M. tuberculosis* and one as *M. bovis*. Unlike with study that showed the absence of *M. bovis* in Iraq [19].

Difference in the number of positive patient's samples when diagnosed by Ziehl Neelsen staining test and real time PCR technique may be due to the direct smear microscopy cannot discriminate between *M. tuberculosis* (MTB) and *Mycobacterium* other than tuberculosis (MOTT), and the real time PCR has high specificity and greater sensitivity than Ziehl Neelsen staining [20].

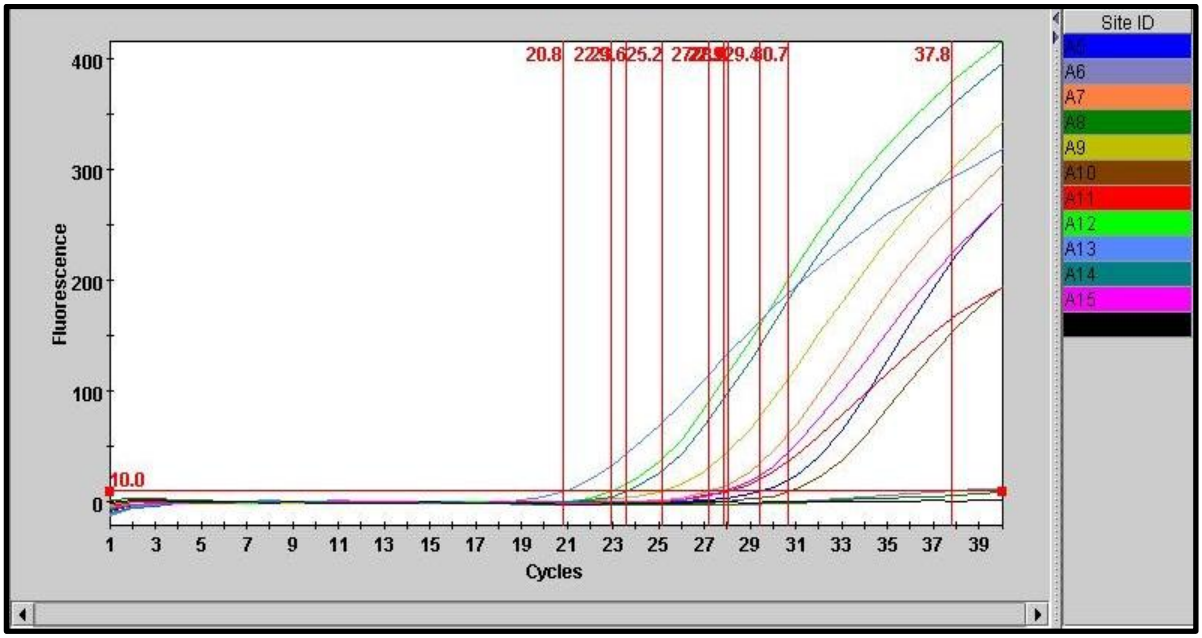

Figure 1- The FAM channel of Real Time PCR run for the nine specimens for detection *M. tuberculosis* and three controls (Negative Control of Amplification, Positive Control of Amplification, and Negative Control of Extraction), for each specimen specific color to recognize it from the other specimens, all these specimens and positive control appeared as colored amplification curves above the threshold line (positive result), negative control was showed in the colored line under threshold line.

References:

1. Kethireddy, S., Nalgonda, D., and Pradesh, A. **2010**. Tuberculosis-A Review of Clinical Features, Differential Diagnosis and Treatments available. *IJPT*. 2 (2):206-244.
2. Reddington, K., Zumla, A., Bates, M., van Soolingen, D., Niemann, S., Barry, T., and O'Grady, J. **2012**. SeekTB, a Two-Stage Multiplex Real-Time-PCR-Based Method for Differentiation of the *Mycobacterium tuberculosis* Complex. *Journal of Clinical Microbiology*. 50(7):2203–2206.
3. WHO. **2013**. Global Tuberculosis Report 2013. World Health Organization. Geneva, Switzerland.
4. WHO. **2011**. Global tuberculosis control 2011.World Health Organization, Geneva, Switzerland.
5. MOH-NTP. **2013**. Epidemiological Situation in Iraq, Fifth National Conference of tuberculosis/Al- Basra. Ministry of health of Iraq-Notional TB programme TB.
6. Miller, N., Cleary, T., Kraus, G., Young, A., Spruill, G., and Hnatyszyn, H**. 2002**. Rapid and Specific Detection of *Mycobacterium tuberculosis* from Acid-Fast Bacillus Smear-Positive Respiratory Specimens and BacT/ALERT MP Culture Bottles by Using Fluorogenic Probes and Real-Time PCR. *J. Clin. Microbiol*. 40 (11):4143–4147.
7. Nakajima, C., Rahim, Z., Fukushima, Y., Sugawara, I., van der Zanden, A., Tamaru, A., and Suzuki, Y. **2010**. Identification of *Mycobacterium tuberculosis* clinical isolates in Bangladesh by a species distinguishable multiplex PCR. *BMC Infectious Diseases*.10:118.
8. Ruiz, M., Torres, M., Llanos, A., Arroyo, A., Palomares, J., and Aznar, J. **2004**. Direct Detection of Rifampin- and Isoniazid-Resistant *Mycobacterium tuberculosis* in Auramine-Rhodamine-Positive Sputum Specimens by Real-Time PCR. *J. Clin. Microbiol*. 42 (4):1585-1589.
9. IUATLD. **2000**. *Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries*. Technical guide. Fifth edition. International Union Against Tuberculosis and Lung Disease. Paris, France.
10. Ssengooba, W., Kateete, D., Wajja, A., Bugumirwa, E., Mboowa, G., Namaganda, C., Nakayita, G., Nassolo, M., Mumbowa, F., Asiimwe, B., Waako, J., Verver, S., Musoke, P., Mayanja-Kizza, H., and Joloba, M. **2012**. An Early Morning Sputum Sample Is Necessary for the Diagnosis of Pulmonary Tuberculosis, Even with More Sensitive Techniques: A Prospective Cohort Study among Adolescent TB-Suspects in Uganda. *Tuberculosis Research and Treatment*. vol. 2012, Article ID 970203, 6 pages. doi:10.1155/2012/970203.
11. Binjomah, A. **2013**. Studies on the Phenotypes of *Mycobacterium tuberculosis* in Sputum. Ph.D. Thesis, College of Medicine, University of Leicester.

12. Parsons, L., Somoskövi, A., Gutierrez, C., Lee, E., Paramasivan, C., Abimiku, A., Spector, S., Roscingno, G., and Nkengasong, J. **2011**. Laboratory Diagnosis of Tuberculosis in Resource-Poor Countries: Challenges and Opportunities. *Clin. Microbiol. Rev*. 24 (2): 314–350.

13. Datta, S., Sherman, J., Bravard, M., Valencia, T., Gilman, R., and Evans, C. **2015**. Clinical Evaluation of Tuberculosis Viability Microscopy for Assessing Treatment Response. *Clin Infect Dis*. doi: 10.1093/cid/ciu1153.

14. Chan, C., Zhao, B., Cazenave-Gassiot, A., **2013**. Novel phage display-derived mycolic acid-specific antibodies with potential for tuberculosis diagnosis. *J. Lipid Res*.

15. Warren, R., van Pittius, N.., Barnard, M., Hesseling, A., Engelke, E., de Kock, M., Gutierrez, M., Chege, G., Victor, T., Hoal, E., and van Helden, P. **2006**. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. *Int J Tuberc Lung Dis*. 10 (7):818–822.

16. Vasconcellos, S., Huard, R., Niemann, S., Kremer, K., Santos, A., Suffys, P., and Ho, J. **2010**. Distinct genotypic profiles of the two major clades of *Mycobacterium africanum. BMC Infectious Diseases*. 10:80.

17. Elhassan, M., Eltikeina, A., Saleh, R., Almekki, M., and Hamid, M. **2011**. Molecular Detection of Mycobacterium Other Than Tuberculosis among Patients with Pulmonary Infection. *Bahrain Med Bull*. 33 (2).

18. Abbadi, S., El-Hadidy, G., Gomaa, N., and Cooksey, R. **2009**. Strain differentiation of Mycobacterium tuberculosis complex isolated from sputum of pulmonary tuberculosis patients. *International Journal of Infectious Diseases*. 13: 236-242.

19. Ali, R. **2013**. Molecular study and Genotyping of *Mycobacterium tuberculosis* complex isolated in Respiratory Center in Baghdad. Ph.D. Thesis. Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad.

20. Al-Saadi, M., Naher, H., and Shalan, A. **2014**. Evaluation of Commercial Real Time PCR and Immunochromatography Techniques in Laboratory Diagnosis of Tuberculosis. *Medical Journal of Babylon*. 11 (1): 58-69.