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GENOTYPING OF *MYCOBACTERIUM TUBERCULOSIS* FROM IRAQIS PATIENTS

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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). SNP genotyping of 50 *M. tuberculosis* DNA samples revealed the presence of 69 strains which mean some of patients were infected with two or more strains (mixed infection), and the main phylogenetic lineages of *M. tuberculosis* present in Baghdad city were 12 (17.4%) of Lineage 1,3 (4.3%) of Lineage 2 which was the less incidence, 26 (37.7%) of Lineage 3 which was the most frequent, 18 (26.1%) of Lineage 4, the remaining 10 (14.5%) samples could not be assigned to any known Lineages therefore considered unknown genotypes.

KEY WORDS: *M. tuberculosis* genotypes.

INTRODUTION

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 (Kethireddy *et al.*, 2010; Reddington *et al.*, 2012; WHO, 2013). According to the World Health Organization (WHO), TB infection is the second highest mortality causing infectious disease worldwide (WHO, 2011). Iraq is considered among eight high TB burden countries in Eastern Mediterranean Region (EMR) (MOH-NTP, 2013). Although TB is still a public health problem in Iraq, there is little information about the genetic characteristics of the bacteria. A better knowledge of the molecular characteristics of *M. tuberculosis* will contribute to understand the transmission dynamics of the disease in our country. The objectives of this study are to use rapid molecular technique to estimation the prevalence of *M .tuberculosis* strains by TaqMan Single Nucleotide Polymorphisms (SNPs) genotyping assays and determination of predominating genotypes in Baghdad.

PATIENTS & METHODS

Dating from August to October 2014 the Institute of Chest and Respiratory Diseases/Baghdad medical city in Baghdad was received 629 patients with suspected pulmonary tuberculosis lesions. Two samples were collected from each patient. First, one was taken from patient when he just reached the institute; second sample collected at early morning before breakfast, collected specimens were stored at –20°C until use (IUATLD, 2000; Ssengooba *et al.*, 2012). Ziehl-Neelsen Stain sputum smears were examined for the presence of pulmonary acid fast bacilli. Of them, 56 were acid fast bacilli smear positive pulmonary tuberculosis. These 56 specimens were selected for evaluation of the assay. 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 *M. tuberculosis*

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.

Genotyping of *Mycobacterium tuberculosis* by real time PCR

All DNA samples that make sure the presence of *M. tuberculosis* by MTB Diff Real-TM kit (Sacace, Italy) with real time PCR which have undergone to SNP-genotyping by TaqMan real-time PCR assays for the objective of classification to main lineages and strains to investigate epidemiology of MTB (Table 1).

TABLE 1: Sequence information of probes and primers used in this study to detect main phylogenetic lineages of *Mycobacterium tuberculosis* isolates by single nucleotide polymorphisms genotyping (Asante-Poku *et al*., 2014); Primers and Probes sequences were confirmed by the Real Time qPCR Assay Design software, Biosearch Technologies, USA.

MTB Lineage[1]	LSP name[2]	Spoligotype name[3]	Gene name[4]	SNP[5]	Primer and probe sequences[6] 5 ⟶ 3′
1	Indo-oceanic	EAI , MANU1	TB 7.3	Rv3597682CT	Forward primer: TGTCAACGAAGGCGATCAGA Reverse primer: GACCGTTCCGGCAGCTT Wild type probe: FAM-ACAAGGGCGACGTC-BHQ1 Lineage specific probe: VIC-ACAAGGGCGACATC-BHQ1
2	East Asian	Beijing	Methylase	Rv3304966CT	Forward primer: CCTTCGATGTTGTGCTCAATGT Reverse primer: CATGCGGCGATCTCATTGT Wild type probe: FAM-CCCAGGAGGGTAC-BHQ1 Lineage specific probe: VIC-CCCAGGAAGGTACT-BHQ1
3	East-African-Indian	CAS/Delhi	fbpD	Rv4266647AG	Forward primer: GCATGGATGCGTTGAGATGA Reverse primer: CGAGTCGACGCGACATACC Wild type probe: VIC-AAGAATGCAGCTTGTTGA-BHQ1 Lineage specific probe: FAM-AAGAATGCAGCTTGTCGA-BHQ1
4	Euro-American	X, Haarlem, LAM, Uganda	KatG	Rv2154724AC	Forward primer: CCGAGATTGCCAGCCTTAAG Reverse primer: GAAACTAGCTGTGAGACAGTCAATCC Wild type probe: FAM-CAGATCCGGGCATC-BHQ1 Lineage specific probe: VIC-CCAGATCCTGGCATC-BHQ1

[1] *Mycobacterium tuberculosis* (MTB) Nomenclature according to Coscolla and Gagneux (2010).

[2] Large Sequence Polymorphism (LSP) Nomenclature according to Gagneux *et al.* (2006).

[3] Nomenclature according to Filliol *et al.* (2003).

[4] As reported in (http://www.xbase.ac.uk/genome/mycobacterium-tuberculosis-h37rv).

[5] Position of Single Nucleotide Polymorphism (SNP) in reference to the H37Rv genome.

[6] Primer and probe sequences supplied by Alpha DNA, Montreal, Quebec.

Required quantity of 25µl Smart Cycler tubes were prepared for samples and controls. TaqMan Real Time PCR was optimized and performed as follows: Briefly, in a 25µl SmartCycler reaction tube, 5 µl of DNA was added to 10 µl GoTaq Probe qPCR Master Mix; and 1 µl nuclease free water, 1µl from 5 pmol/µl for each forward and reverse primers for the targeted regions, probe A for ancestral allele (wild type) and probe B for mutant allele (lineage specific) (each labeled with different dyes).

Three controls were prepared as mentioned in (CDRH, 2014) for every run:

● Nuclease free water (6 µl) was added to10 µl GoTaq Probe qPCR Master Mix; and 1µl from 5 pmol/µl for each forward and reverse primers, probe A for ancestral allele and probe B for mutant allele was added. The tube was labeled Negative Control of Amplification (NCA).

● DNA (5µl) contain ancestral and mutant alleles (specific ancestral and mutant alleles was used for each MTBC lineage) was added to 10 µl GoTaq Probe qPCR Master Mix; and 1 µl nuclease free water, 1µl from 5 pmol/µl for each forward and reverse primers, probe A for ancestral allele and probe B for mutant allele. The tube was labeled Positive Control of Amplification (C+).

● Negative Control of Extraction (NCE) (5 µl) was added to 10 µl GoTaq Probe qPCR Master Mix; and 1 µl nuclease free water, 1µl from 5 pmol/µl for each forward and reverse primers, probe A for ancestral allele and probe B for mutant allele. The tube was labeled NCE.

All the tubes were centrifuged and inserted in the Real Time thermocycler. A temperature profile on Cepheid SmartCycler was created as follows: 60°C 30 seconds; 95°C 10 minutes; 95°C 15 seconds and 60°C 1 minute for 40 cycles; 60 °C 30 seconds (Asante-Poku *et al*., 2014).

RESULTS & DISCUSSION

During the study interval (August to October 2014), a total of 629 sputum samples of suspected pulmonary tuberculosis (PTB) cases were examined in the National Reference Laboratory (NRL) at the Institute of Chest and Respiratory diseases / Baghdad. The results revealed among total 629 specimens, 56 (8.9%) of the specimens were positive by direct examination (acid fast bacilli smear microscopy) and 573 (91.1%) negative specimens by smear microscopy. Fifty six DNA samples were extracted from positive ZN smears of sputum specimens were subjected to molecular diagnosis by real time PCR to confirmation presence of *Mycobacterium tuberculosis*, using MTB Diff Real-TM kit (Sacace, Italy). The results were clarified that the 50 samples (89.29%) were *M. tuberculosis* and 6 (10.71%) were negative and then excluded from this study.

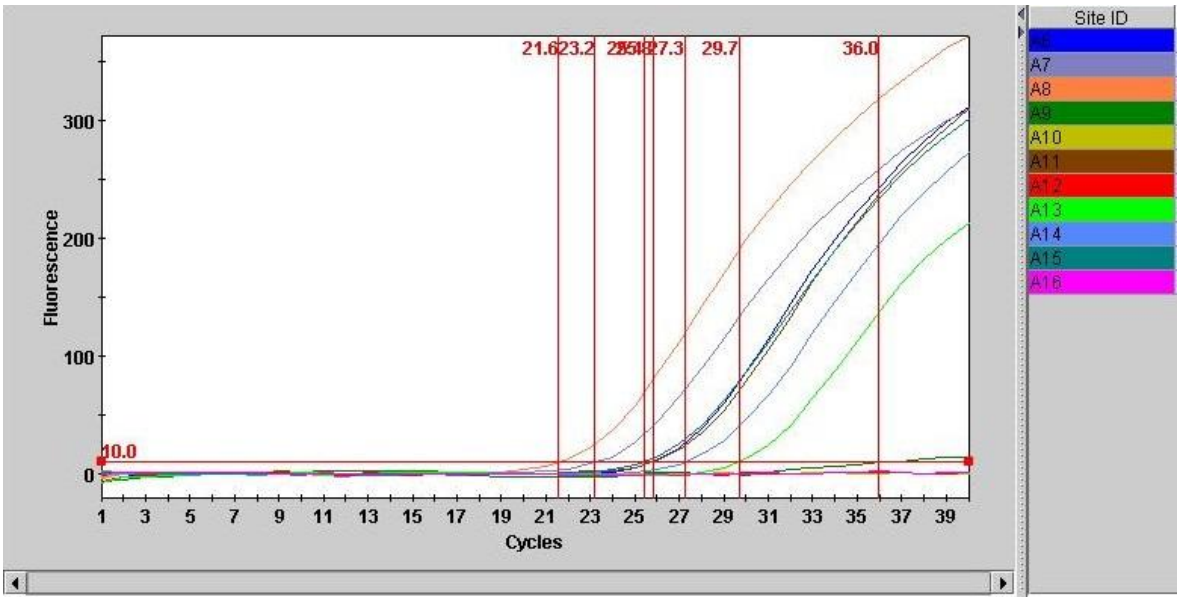

FIGURE 1: The FAM channel of Real Time PCR run for the eight specimens for identification the Central-Asian (CAS) genotype of *M. tuberculosis* and three controls (Negative Control of Amplification, Positive Control of Amplification, and Negative Control of Extraction), from the seven curves (positive results) which appeared as colored amplification curves above the threshold line one was represent positive control and others were CAS genotype. The three lines under threshold line (negative results) were represent one for CAS genotype and other for negative controls.

SNP genotyping of 50 *M. tuberculosis* DNA samples revealed that found 69 strains which mean some of patients were infected with two or more strains (mixed infection), and the main phylogenetic lineages of *M. tuberculosis* present in Baghdad city were 12 (17.4%) of Lineage 1 (Indo-Oceanic Lineage, includes the EAI and MANU1genotypes), 3 (4.3%) of Lineage 2 (East Asian, includes the Beijing genotype) which was the less incidence, 26 (37.7%) of Lineage 3 (East-African-Indian, includes the CAS/Delhi genotype) (Figure 1) which was the most frequent, 18 (26.1%) of Lineage 4 (Euro-American, includes the X, Haarlem, LAM, Uganda genotypes), the remaining 10 (14.5%) samples could not be assigned to any known Lineages therefore considered unknown genotypes. The results was demonstrated that 34 (68%) from fifty genotyped DNA samples of infected patients were contained single Lineage, while 16 (32%) were constituted of two or more genotypes. The combined

of Lineage 3 and Lineage 4 were found in ten patients, Lineage 1 and Lineage 3 considered together were detected in three patients, and each mixes of Lineage 2 and Lineage 3, all lineages, and Lineage 1, Lineage 3, Lineage 4 were determined in one patient. The five from 12 of Lineage 1genotypes, two from 3 of Lineage 2 genotypes, 16 from 26 of Lineage 3 genotypes, and 12 from 18 of Lineages 4 genotypes were detected in one sample with two or more different Lineages. The predominant lineage in this study was the Lineage 3 (37.7%) this was in accordance with recent study in Baghdad by Ali *et al.*, (2014) which found the CAS Lineage strains constitute the biggest (39.6%) group of strains infecting Iraqi patients. The CAS has also been identified as predominant Lineage in Iran (Haeili *et al.*, 2015) and also reported the predominance in Saudi Arabia, the presence of a high proportion of CAS/Delhi strains in this study may either be due to the fact that CAS/Delhi predominates in this region and/or that they are related to the increase in migration, trade and tourism between Iraq and the Middle East (Yimer *et al.*, 2015). The second most frequent Lineage in current study was 18 (26.1%) of Lineage 4 (Euro-American). The high prevalence of Euro-American Lineage strains in the Iraq neighboring countries, Iran and Turkey were reported (Hill *et al.*, 2012). Although there is no geographic link between Iraq and Europe, Americas and Africa, both of the two largest surrounding countries to Iraq, Turkey and Iran are known to share active borders and historic links with European countries; therefore, it may be suggested that the Euro-American Lineage may have first spread in Turkey and/or Iran and then transmitted to Iraq (Ahmed *et al.*, 2014). Indo-Oceanic Lineage 12 (17.4%) was ranked as the third lineage spreading in Iraq. The finding of Lineage 1 in this study was in contrast to some surrounding countries such as Turkey and Iran, where they did not find these lineages. Hence, overall, the identified strains in Baghdad province have been found in many different countries, suggesting that these strains might be circulating worldwide (Merza and Salih, 2012). Unknown genotypes 10 (14.5%) were classified in rank four according to spreading in Iraqi patients, this was in agreement with recent study in Baghdad by Ali *et al*. (2014) which found the percentage of unknown genotype was 5.9%. These genotypes have nucleotides sequence which could not match with any specific probe sequence that use for identification mutant alleles (Lineage specific), so match with all wild type probes. The fewer incidences Lineage was East Asian 3 (4.3%), this finding was in agreement with previous study by CDC, (2003) which reported 7.5% (3/40) of strains isolated from Iraqi patients were Beijing genotype, unlike with several studies that showed the absence of this strain in Iraq (Ali, 2013; Ahmed *et al.*, 2014).

In conclusion, the wide spectrum of the pathogen's population clearly reflects the diversity of geographic origins in the human host population in Iraq. The genotyping of *M. tuberculosis* TB strains is necessary for investigating the transmission dynamics and mechanisms of disease in Iraq and neighboring countries. Relating various strains to strains obtained in other countries using different genotyping procedures is important.

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