مجلة جامعة تشرين للبحوث والدراسات العلمية _ سلسلة العلوم البيولوجية المجلد (33) العدد (3) 2011
Tishreen University Journal for Research and Scientific Studies - Biological Sciences Series Vol. (33) No. (3) 2011

Testing for the Presence of *Mycobacterium tuberculosis* Beijing Genotype Strains Using Real Time PCR and Amplicon Melting Temperature Profile

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(Received 6 / 3 / 2011. Accepted 27 / 6 /2011)

□ ABSTRACT □

The Beijing family of *Mycobacterium tuberculosis* has been reported to have exceptional capacity to spread tuberculosis and induce drug multi-resistance. A method has been developed to distinguish this family from the rest of *Mycobacterium tuberculosis* families through real time DNA amplification and subsequent analysis of amplicon's melting point. Measuring the derivative of fluorescence emission with respect to temperature and plotting it against temperature unveiled the presence of a peak reflecting the amplicon's melting temperature that is diagnostic of the Beijing family (T_m: 87.23°C), and another one specific for the non Beijing families of strains (T_m: 82.67°C). This research presents an effective differentiation of bacterial strains, by applying the analysis of differential amplicon melting points.

Key words: Beijing strain – *Myccobacterium tuberculosis* – real time PCR – DNA melting.

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مجلة جامعة تشرين للبحوث والدراسات العلمية _ سلسلة العلوم البيولوجية المجلد (33) العدد (3) 2011
Tishreen University Journal for Research and Scientific Studies - Biological Sciences Series Vol. (33) No. (3) 2011

الكشف عن سلالات النمط الوراثي بيجينغ للمتفطرات السلية باستخدام تضخيم الدنا بالزمن الحقيقي ودرجة حرارة انفصال شريطيها

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(تاريخ الإيداع 6 / 3 / 2011. قبل للنشر في 27 / 6 / 2011)

□ ملخّص □

تبين أن لعائلة سلالات البيجينغ التابعة للمتفطرات السلية قدرة مميزة على نشر مرض السل وإحداث مقاومة دوائية متعددة. تم تطوير تقنية هدفها تمييز هذه العائلة من باقي عوائل المتفطرات السلية؛ من خلال تضخيم الدنا بالزمن الحقيقي Real time PCR ودراسة درجة حرارة انصهار Melting point temperature الدنا المضخمة Amplicon. وأظهر تتبع انصهار الدنا بقياس اشتقاق الفلورة المنبعثة بالنسبة إلى متغير درجة الحرارة وجود قمة تعكس درجة حرارة انصهار الدنا المضخمة؛ التي تشخص وجود سلالة تنتمي إلى عائلة بيجينغ (درجة حرارة انصهار 87.23 م)؛ وقمة أخرى خاصة بالسلالات التي لا تنتمي إلى عائلة بيجينغ (درجة حرارة انصهار 82.67 م). ويمثل هذا البحث تطبيقاً فعالاً لتقنية تمييز السلالات البكتيرية الممرضة انطلاقا من تباين درجة حرارة انصهار الدنا المضخمة.

الكلمات المفتاحية: سلالة بيجينغ – المتفطرات السلية – تضخيم الدنا بالزمن الحقيقي – انصهار الدنا

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Introduction:

Tuberculosis (TB) is one of the main infectious causes of death worldwide, with more than 9 million new cases of active disease every year and nearly 2 million deaths [1]. TB is an endemic disease in Syria and accounts for thousands of notified cases (new and relapse incidence rate: 21/100000 pop/year)[2]. *Mycobacterium tuberculosis* (MTB) is the causative agent of most TB cases; its ability to spread and the outcome of infection depend on epidemiological, host, and bacterial factors [1]. Cases of drug resistant (DR) and multi-drug resistant (MDR) tuberculosis (i.e. at least resistant to rifampicin (RIF) and isoniazid (INH)) are increasing worldwide. In some parts of the world 10% or more of new TB cases are MDR [3]. In the Baltic and Russia a large increase in DR-TB has occurred during the last two decades [4]. During this period many Russians of Jewish decent have immigrated to the Middle East (Palestine).

Strains with changed biological properties belonging to the family of strains: Beijing, have been implicated in many outbreaks of TB; suggesting more efficient transmission or enhanced ability to progress to active disease compared to other TB strains [5]. Beijing strains, in vitro and in vivo models, show more virulent phenotypes than other MTB lineages. They are often strongly associated with primary drug resistance, and cause in some regions' clusters ten times as big as "traditional" strains [6]. Beijing strains appear better adapted to intracellular growth, and drug resistance. They have specific properties in terms of protein and lipid structures, which allow the immune-modulation of host response to the pathogen [7].

Beijing strains represent about 50% of strains in East Asia and at least 13% of strains worldwide [8]. They appear to spread further and establish themselves in new regions. In Cape Town during 2000–2003, the Beijing clade spread and became the cause of children's disease; number of cases became 33% instead of earlier values of 13% [9]. It has been suggested that emergence of the Beijing genotype family represents an evolutionary response of *M. tuberculosis* to BCG vaccination or/and antibiotic treatment, resulting in a significant impact on tuberculosis control [10].

Active disease caused by Beijing strains was associated, in some populations, with exceptional clinical phenotypes. In a study in Russia, Beijing strains were associated with more severe radiographic manifestations of pulmonary TB. Other studies associated the Beijing family with extra-pulmonary TB, a more likely poor outcome in elderly patients, and in many areas an increasing risk for the relapses [5].

The MTB genome is highly conserved, but several large sequence polymorphisms defining different, genetically related lineages, have been identified. The Beijing family has been identified using the "gold standard" method IS6110 RFLP, but it was found that it can be also identified reliably and more rapidly by targeting other definitive genetic features. These include a characteristic deletion of spacers 1-34 (the so-called RD207 deletion), an intact open readings frame in the pks15/1 gene, and a deletion of the genomic region RD105 [7]. Beside the mostly applied spoligotyping method, several other methods, based on targeted genome morphisms and chosen techniques, have been proposed; these include: deletion-Targeted Multiplex PCR (DTM-PCR) method [11], PCR-SSCP method [12], and real time PCR using specific probes [13]. The use of real time PCR provides accurate quantification of strains. Furthermore, no post PCR detection steps, that can cause cross-contamination, are required for amplicon identification.

Aim and importance of the research:

Hillerman et al [13] has developed a multiplex real time PCR assay which allows identification of Beijing and non Beijing family of strains in one tube. The assay proved to be highly sensitive; detecting 5/10 copies of chromosomal DNA, and very specific; grouping 103 isolates based on spoligotyping and IS6110 DNA fingerprinting accurately. The assay requires however the presence of two flourogenic probes [13]. The real time PCR method presented by the author's study, targeted the same TB genome locies; but detection was based on SYBR Green amplicon , followed by profiling of amplicon's characteristic melting point. This research provides quantitatively efficient, qualitatively unequivocal and sensitive essay of a virulent Beijing strain. The research has been entirely performed at the National Commission for Biotechnology in Damascus/Syria.

Materials and methods:

***Mycobacterium tuberculosis* samples**: Beijing and non Beijing strain's control DNA were obtained from Azm center for biotechnology at the Lebanese university [13]. Association to the family Beijing strains has been established earlier through the standard method of spoligotyping.

Real time PCR assay: real time PCR experiments were done using the Stratagene Mx3005®. Primers were synthesized according to the sequence published in [14]. Synthesis was performed by VBC Biotech (HPLC grade). PCR polymerase mix: DyNAmo® flash SYBR Green qPCR kit, was purchased from Finnzymes. Primer concentrations used were 0.5µM. Final reaction volume was 50µl, and MTB genome DNA amount added, was 5µl after 1000x dilution. The thermal profile was: 95° for 10 min, and 40-50 two steps cycle; consisting of a denaturation step: 92°C for 15 s, and an annealing/extension step: 60°C/62°C/64°C for one min.

Amplicon Melting point temperature: determination of dissociation curve, and the melting point temperature (Tm) which is based on maximal fluorescence emission change at varying temperature multiplied by -1; (-dF/dT), were performed at the temperature range [60 – 94 °C], using the Fam filter (λ_{ex} : 492nm, λ_{em} : 516nm) suitable for the SYBR green flourophore. The applied software was: MxPro from stratagene.

Statistical calculations: standard error (SE) was calculated using the excel office software from Microsoft.

Results:

Based on the analysis of BLASTN of TB genome sequence, the expected non Beijing and Beijing family of strains amplicon length produced, should be 129 bp for the Beijing strains, and 95 bp for the non Beijing ones [14].

Testing non Beijing and Beijing primers on non Beijing and Beijing samples resulted in T_m peaks and associated emission as described in table (1).

Table (1) The T_m peaks for Beijing and non Beijing samples using Beijing and non Beijing specific primers:

N.	Primer	sample	T_{m1}	Emission	T_{m2}	Emission	T_{m3}	Emission	T_{m4}	Emission	T_{m5}	Emission	T_{m6}	Emission	T_a
1	nB	nB			82.78	2885,89									62
2	nB	nB			82.75	3628,22									62
3	nB	nB			82.75	5295,56									62
4	nB	nB			82.25	4399,33									62
5	nB	nB			82.83	2382,78									62
6	nB	B	77.45	3538											62
7	nB	B	77.42	2816,4											62
8	nB	B	77.45	1874,67											62
9	B	nB	77.92	820			85.95	743,44					90.12	930,89	62
10	B	nB	77.38	1035			85.48	819					90.1	1167,33	62
11	B	nB	76.83	1507,08							88.46	4071,08			**64**
12	B	nB	77.97	366							88.67	1275,2			**60**
13	B	nB	78,35	1016,83			85,38	669,5					89.05	3364,17	62
14	B	nB	78.3	2264,33			85,7	846					89,85	479	62
15	B	nB	77.85	1934,83			85,7	444,17					89,45	240,17	62
16	B	nB	78.28	1289,83							88.58	1230,83	91.45	2603,5	62
17	B	nB	76,33	753,5							88.46	793,58	90,99	1116	**64**
18	B	B	78.24	1348,67					87.22	3486,27					62
19	B	B	77.67	506,33					87.22	1324					62
20	B	B	78.2	1834,5					87.35	2874					62
21	B	B	77.33	748,33					86.96	1253,75					**64**
22	B	B	77.93	469,2					87.03	822					60
23	nB	NTC	77.33	1802,93											**60**
24	nB	H DNA	**79.73**	824,93									91.63	753,6	**60**

B: Beijing, nB: non Beijing, Tm: DNA melting point temperature, Ta: PCR annealing temperature, NTC: no template control, H DNA: Human DNA control

Testing non Beijing primers on non Beijing samples resulted in one T_m peaks (~82.67 °C). Using the non Beijing primers with a Beijing sample resulted in only one T_m peak (~77°C). Therefore the T_m of ~82.67°C was considered the diagnostic peak for the non Beijing strains. Annealing temperature has been modified from 60°C as described in [14] to 62°C which improved the peak absorbance ratio of diagnostic T_m peak at ~82.67°C (SE: 0.11) compared to the T_m peak ~77°C figure (1).

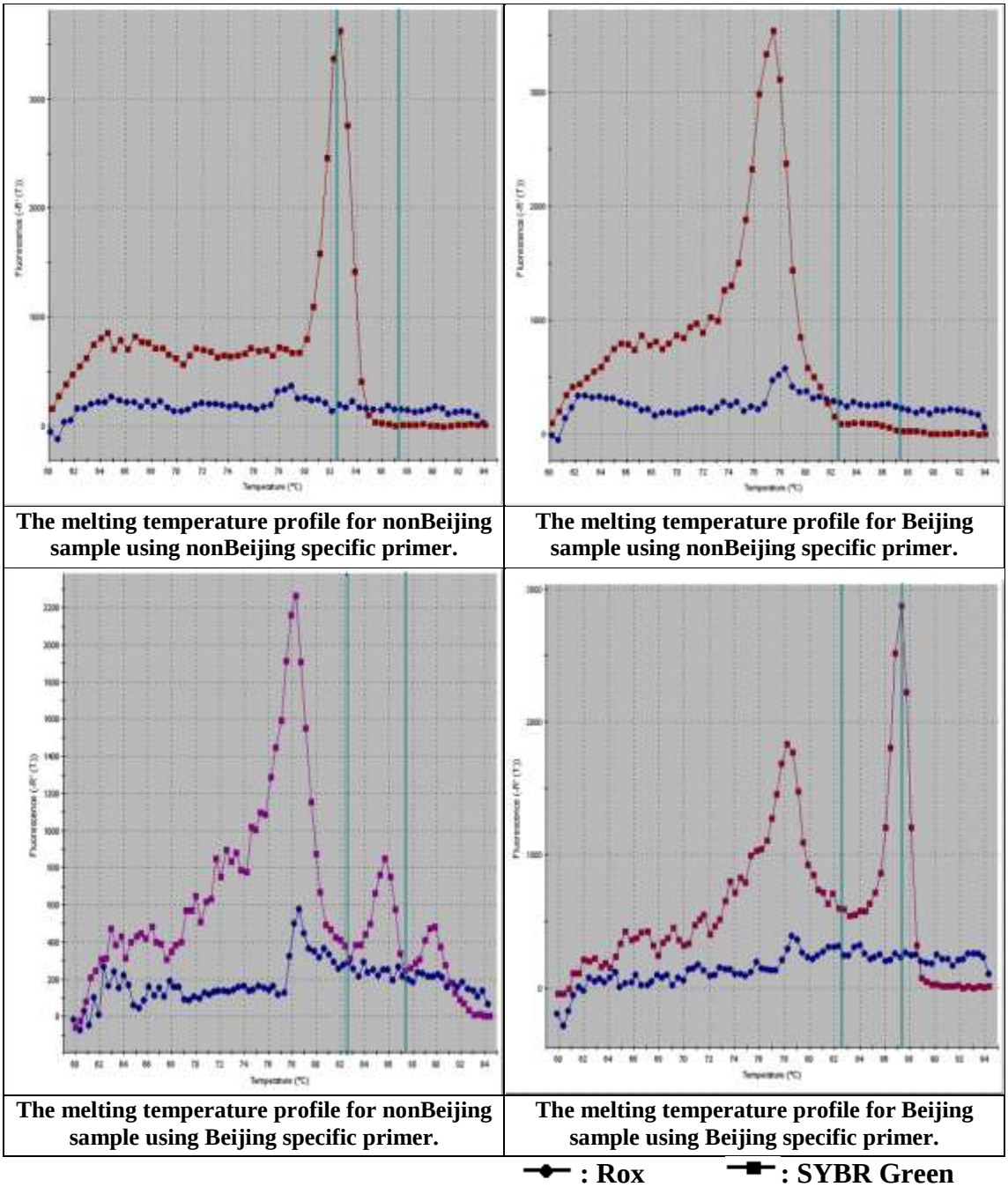
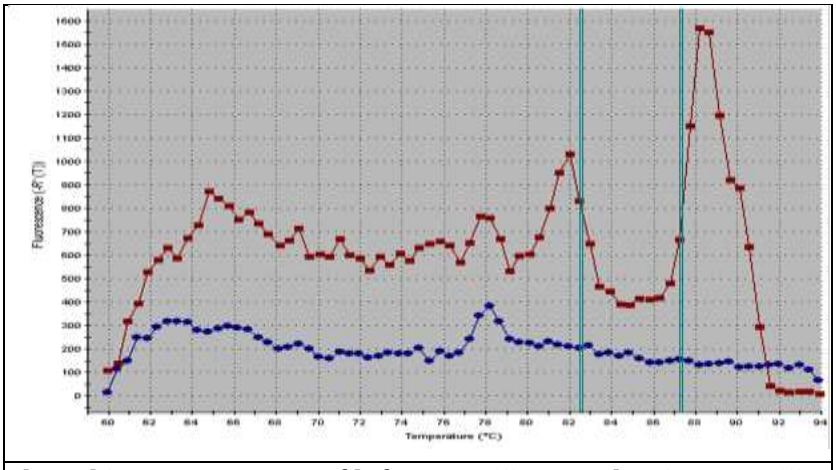


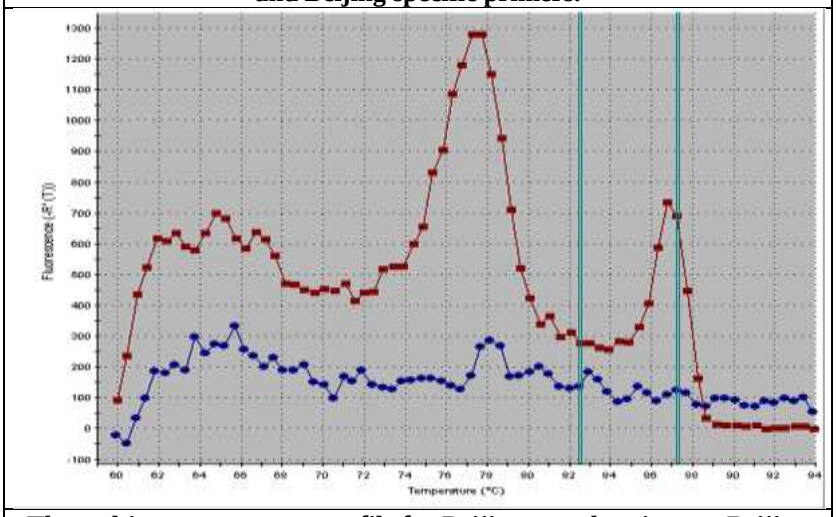
Figure (1): the melting temperature profile for nonBeijing/Beijing samples using the specific designated primers.

Testing Beijing primers on Beijing sample at 62°C annealing temperature resulted in two T_m peaks (~78°C, ~87.23°C). Using Beijing primers on non Beijing samples resulted, at 62°C annealing temperature, in several T_m peaks (~78°C,~86°C,~90°C). Therefore the T_m peak at ~87.23°C (SE: 0.01) was considered the diagnostic peak for Beijing strains. The increase of the annealing temperature from 60°C to 62°C enhanced the diagnostic T_m peak ~87.23°C, a further increase to 64°C made the non diagnostic T_m peak appeared at ~88°C by apparently shifting the non diagnostic peak from ~90°C to ~88°C.

Both primer pairs can be used simultaneously to diagnose the presence of Beijing or non Beijing strains figure (2). The diagnostic peaks appear to be slightly shifted to lower temperature (~- 0.6°C) figure (2).



The melting temperature profile for nonBeijing sample using nonBeijing and Beijing specific primers.



The melting temperature profile for Beijing sample using nonBeijing and Beijing specific primers.

: Rox **: SYBR Green**

Figure (2): the melting temperature profile for nonBeijing/Beijing samples using the combined specific primers.

Discussion:

The results of the applied method demonstrate clearly its ability to discriminate between the Beijing strain family and the rest of TB strain families. The distinction is based on the amplified region near RV 2820 uniquely present in Beijing strains; providing the specific fragment of 129 bp, and the amplified region in Rv 2819 present solely in non Beijing strains; providing the specific fragment of 95 bp.

The difference in diagnostic melting temperature (T_m values) obtained for Beijing and non Beijing families (~87.23°C, ~82.67°C resp.) is consistent with the specific amplicon size of Beijing strains; being 36% larger than that of non Beijing strains.

Beijing primers used with non Beijing strains produced three non diagnostic T_m peaks (~78°C,~86°C, ~90°C); while non Beijing primers with Beijing strains produced a mere one non diagnostic T_m peak (~78°C). This appears to be a consequence of deletion of the region causing in Beijing strains the two non diagnostic T_m peaks; the genomic region between IS 6110 and Rv 2820 (that include Rv 2816, Rv 2817, Rv 2818, Rv 2819). The first non diagnostic T_m peak (~78°C), present in both cases, occurring in almost all PCR reactions, appears also in absence of any template DNA. Its low melting temperature suggests that it is, most likely, a primer dimer product.

The major advantage of this method is its rapidity, simplicity and sensitivity. The strains are quantifiable. The modified method presented does not require further any specific probes or modified primers. The detection is done, unlike the method by Hillerman et. al [14], based on just SYBR green selective binding to double stranded DNA. The use of amplicon melting analysis is an established technique in differential detection of microorganisms [15].

Conclusion and recommendation

In contrast to the presented method, the "gold standard" method: IS6110 DNA fingerprinting requires several major steps, a large amount of strains (culture), a complex non robust set up, and analysis of a complex restriction enzyme fragment pattern using suitable softwares. Other PCR based methods like spoligotyping requires post PCR steps that may inflict amplicon cross-contamination. Using the present method allows for fast assessment of epidemiological distribution and dynamics of Beijing family of strains, this may be of exceptional value in regions where their contribution to tuberculosis is still unknown. Simplifying and economizing the detection of Beijing family, provide fast and widespread real time surveillance of their epidemiologic contribution.

Recent studies have shown associations among M. tuberculosis strains, geographic regions, and human populations. These indicate that specific strains of M. tuberculosis coevolved with human subpopulations [5]. The clinical presentations of patients with tuberculosis caused by a Beijing strain were found to vary among different geographical settings [8]. Currently, the exact origin of observed variability in clinical presentation is still unknown [8]. Determining characterized Beijing strains in the Syrian population using the present method may help in assessing the specific contribution of Tb strain genetics and that of the genetic constitution of human populations to observed variability in clinical symptoms.

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