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Prevalence of *Mycobacterium tuberculosis* Beijing strains in Punjab Pakistan

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A B S T R A C T

Beijing strains possess unique properties such as, multi-drug resistance, evasion of BCG vaccination and disseminating at a greater rate than non-Beijing strains. Various methods exist for the detection of Beijing strains such as, RFLP or spoligotyping; however, these methods require established cultures and DNA isolated in High concentration. Relatively newer methods such as, MIRU VNTR, are more expensive and require complex software for analysis. Warren and his coworkers in 2004 illustrated a simple, one step single tube multiplex PCR assay which proved to be a cost effective and easier method with eliminating the need for culturing. In our study, we collected 56 cultures from various districts of Punjab at Institute of Public Health, Lahore and analyzed them by method illustrated by Warren and coworkers, using primers to identify the Beijing strains by the presence of a characteristic deletion which occurs in all such strains. From our study, we found the prevalence of Beijing strains in Punjab is 8.9% of the TB infections.

Introduction

Tuberculosis is one of the most widely occurring infectious diseases. According to WHO one third of the world's population is infected by latent TB (WHO Tuberculosis, 2013). Globally at least 3 million people die of the disease each year (Tortora *et al*., 2011). In United States alone 10 to 12 million people are infected by TB and on a global scale, number of TB cases rising per year is at a rate of 2% (Tortora *et al*., 2011).

The causative agent of TB is *Mycobacterium tuberculosis,* which is a slow growing intracellular bacillus and requires prolonged antibiotic treatments (Prescott *et al.,* 2002). The prolonged, taxing treatment and the immergence of multidrug resistant (MDR) strains are few of the reasons why tuberculosis still stands a threat in this world.

In early 1990, the causative agent for many institutional outbreaks of tuberculosis in New York was identified as a multidrug-resistant strain of *Mycobacterium tuberculosis* designated as "W" (Hewlett *et al*., 1993). Similarly in 1995, *Mycobacterium* isolated from patients in China reported to have similar multi-banded IS6110 RFLP patterns and were designated as "Beijing" (Van Soolingen *et al*., 1995). Molecular markers used to identify W strains can be used to identify the closely related Beijing strains (Bifani *et al*., 2002). Both strains are identical to each other in regards of having an IS6110 insertion sequence in origin of replication (A1 insertion) (Kurepina *et al*., 1998) and having same spoligotype pattern (S00034) (Bifani *et al*., 1999) What distinguishes the Beijing from the MDR NYC strain W and its descendants is the possession of single IS6110 insertion in NTF region by Beijing while the others have two copies (Bifani *et al*., 2002). The current molecular data suggests that the Beijing and W strains are descendants of a common ancestral strain forming part of the W-Beijing family (Kurepina *et al*., 1998) which evolved in different geographical regions (Bifani *et al*., 2002).

Through comparative genomics it has been shown that Beijing strains have evolved through a variety of mechanisms including IS6110 transposition (Beggs *et al.,* 2000), Single Nucleotide Polymorphisms in the mismatch repair genes (Rad *et al., 2003*) and synonymous Single Nucleotide Polymorphisms (Filliol *et al.,* 2006), as well as deletion of chromosomal domains (Tsolaki *et al.,* 2005). Mutations in the mismatch repair genes mutT2, mutT4 and ogt genes are absent in ancestral strains and further mutations in mutT2 and ogt produced two independently evolving subgroups (Rad *et al., 2003*). Mapping of the Region of Difference (RD) domains showed that Beijing strains family is a monophyletic clade with four separate evolutionary branches showing RD181, RD150, RD 105, and RD142 deletion (Tsolaki *et al.,* 2005).

The Beijing strains are most prevalent in China accounting for 92% of strains (Van Soolingen *et al.,* 1995). Its prevalence is considerably high in Vietnam 53% (Anh *et al.,* 2000), Hong Kong (Das *et al.,* 1993), Indonesia 34% and South Korea 43% (Bifani *et al.,* 2002), Thailand 44% (Palittapongarnpim *et al.,* 1997) and Taiwan (Lin *et al.,* 1996) and former Soviet Union (Portaels *et al.,*1999). The prevalence of Beijing Strains in South Asian region is summarized in Table 1.

The method used as gold standard for discrimination in *Mycobacteria* is IS6110 Restriction Fragment Length Polymorphism (RFLP) (Van Embden *et al.,* 1993). However, this method requires previous cultures before isolating DNA and it is less discriminatory with isolates having low IS6110 copy number (Cowan *et al.,* 2005). A relatively better method, Spoligotyping, is a PCR based technique, thus can be applied directly to clinical samples (Hermans *et al.,* 1991). Spoligotyping involves amplifying Direct Repeats (DR region); the DR locus consists of 36 bp DR elements interspersed with varying spacer regions which hybridize to covalently attached oligonucleotides on membrane corresponding to spacer regions; the pattern of positive and negative hybridization signals produced is unique to each strain (Goyal *et al.,* 1997).

Another PCR based approach is the analysis of Variable Number Tandem Repeats based on Mycobacterial Interspersed Repetitive Units (MIRU VNTR) (Frothingham & Meeker-O'Connell., 1998).

Table.1 Prevalence of Beijing strains in South Asian region

Country	Prevalence Beijing strains %	Year	Reference
Afghanistan	3.9% (1/26)	2003	(Lillebaek *et al.*, 2003)
Bangladesh	44% (15/34)	2004	(Banu *et al., 2004)*
China	92% (45/49)	1992-1994	(Glynn *et al.*, 2002)
India	10% (9/91)	2006	(Gutierrez *et al.*, 2006)
Iran	10% (10/97)	1995-1996	(Glynn *et al.*, 2002)
Pakistan	6% (18/314)	2006	(Hassan *et al.*, 2006)
Sri Lanka	8.8% (3/34)	2003	(Lillebaek *et al.*, 2003)

Table.2 Primers sequences used for identification of Beijing strains

Primer (Name)	Sequence(5' to 3')	Product Size (bp)	Melting Temperature (°C)
Beijing forward (BjF)	CTCGGCAGCTTCCTCGAT	129bp	58.9
Beijing reverse (BjR)	CGAACTCGAGGCTGCCTACTAC		60.9
Non-Beijing forward (nBjF)	AAGCATTCCCTTGACAGTCGAA	95bp	57.3
Non-Beijing reverse (nBjR)	GGCGCATGACTCGAAAGAAG		57.8

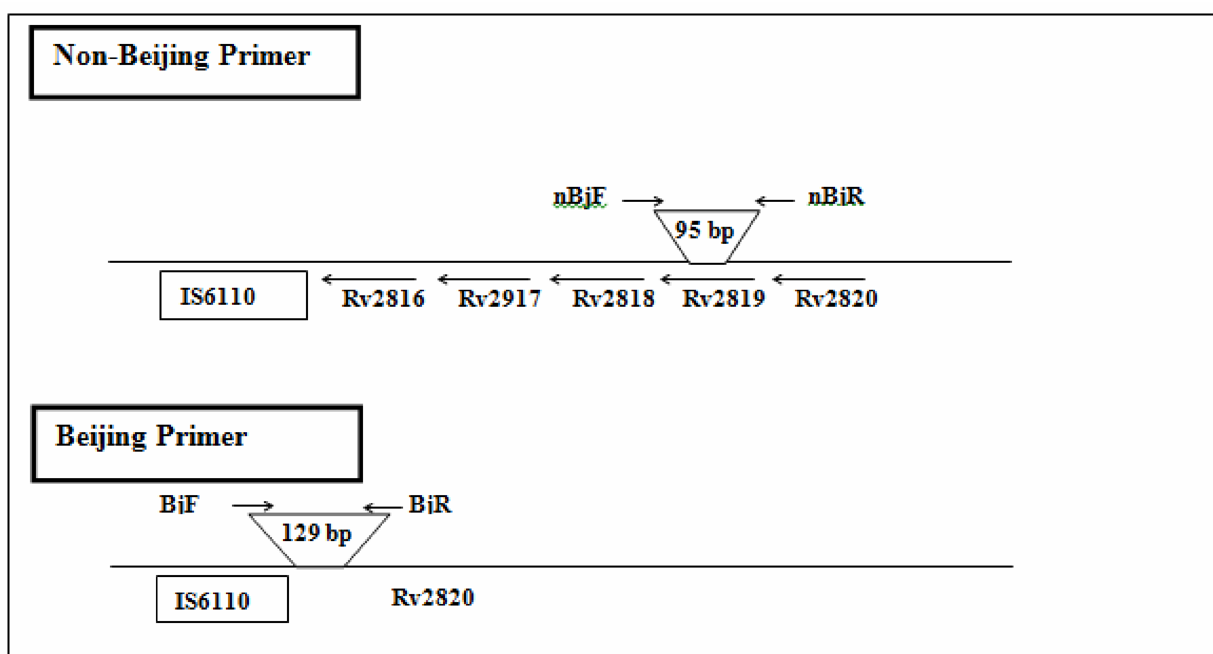

FIG 1. Schematic localization of primers. Adapted from (Hilleman *et al., 2006)*

This Method uses specific primers to amplify regions flanking specific repeat units and the size of amplicons reflects the number of targeted MIRU-VNTR copies with the result expressed as numerical codes, easy to compare and communicate in scientific community (Supply *et al.,* 2006). Number of loci targeted can be 12 (MIRU 12) (Van Embden *et al.,* 1993), 15 (MIRU 15) (Supply *et al.,* 2006) which has a higher discriminatory power (Alonso-Rodríguez *et al.,* 2008). 24 loci MIRU-VNTR was proposed by Supply *et al.,* 2006; however, when tested, MIRU 15 still had more discriminatory power (Alonso-Rodríguez *et al.,* 2008).

A more sensitive PCR based method was introduced by Warren *et al.,* (2004) which target a specific deletion present in Beijing strains. Using multiplex Real Time PCR further improves this method as no gel electrophoresis is required and the results are obtained rapidly; single tube reaction saves valued time and no culture is required as PCR can be performed directly on clinical samples (Hillemann *et al*., 2006). Furthermore, Hillemann *et al.* (2006) used two sets of primers BjF/BjR and nBjF/nBjR for Beijing and non-Beijing strains, respectively (Table 2) (Figure 1). The Beijing primers generated a fragment of 129 bp, while the non-Beijing primers generated a fragment of 95 bp. They tested the efficiency of Real Time PCR by running a mixture of non-Beijing/Beijing DNA with a ratio of 1:1 to 1:100 in a single tube and found clear signals of fluorescence in each case.

Materials and Methods

In our study we collected 56 strains from different districts of Punjab at Institute of Public health, Lahore. DNA was isolated by CTAB method followed by PCR detection as described by Warren *et al.,* (2004).

DNA Isolation: DNA was isolated by CTAB method, as described by Miyata *et al.,* (2011) which was optimized in lab according to our requirements.

PCR: Conventional PCR was performed with each DNA being amplified independently with two set of primers: Beijing (BjF and BjR) and Non-Bejing (nBjF and nBjR). Detection of amplicon with either of the primers gives indication whether the DNA tested was Beijing or Non-Beijing. PCR was done using THERMO SCIENTIFIC ™ kit. The recommended and optimized concentrations are summarized in the Table 3.

The total reaction volume of 25 µL was made and each DNA was tested individually with Beijing and Non-Beijing primers. The reaction mixture was placed in thermo cycler. The initial denaturation was set to 95 °C for 3 minutes followed by 30 cycles of 94 °C for 30 seconds (denaturation), 55 °C for 30 seconds (annealing) 72 °C for 1 minute (polymerization). Final polymerization was done at 72 °C for 7 minutes followed by holding temperature at 4 °C. Amplified product were run and visualized on 2.3% agarose gel.

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Table.3 Recommended and optimized conditions for PCR

Material	Recommended	Optimized
10X buffer	5 µL	2.5 µL
dNTP MIX 2mM each	5 µL (0.2 mM of each)	2.5 µL (0.2 mM of each)
Forward primer	0.1-1.0 µM	0.75 µL (3 µM)
Reverse primer	0.1-1.0 µM	0.75 µL (3 µM)
25mM $MgCl_2$	1-4 mM	2.0 µL (2 µM)
Template DNA	100 ng	100 ng
Taq DNA Polymerase	1.25 u	0.3 u
Water, nuclease-free	Up to 50 µL	Up to 25 µL

Result and Discussion

Out of 56 samples tested, 5 were of Beijing genotype (8.9%) and 51 were of Non-Beijing genotype (91.1%). The primers used for Beijing (BjF & BjR), produced an amplicon of 129 bp (fig 2a) and the primers used to detect non-Beijing primers (nBjF & nBjR) produced an amplicon of 95 bp (fig 2b). The presence of non-Beijing primers confirmed the presence of *Mycobacterium* DNA.

In our study, out of 56 samples tested, 5 were of Beijing genotype (8.9%) and 51 were of Non-Beijing genotype (91.1%). A similar study, but countrywide, was done by Hassan *et al* (2006), they found the percentage of Beijing genotype to be 6% (18/314). Although the increase is insignificant and statistically incomparable due to different sample sizes; however, prevalence checks must be made as there are always doubts pertaining to such strains. This has the advantage of not only prevalence checks but also isolation of Beijing strains is achieved for further research.

As seen in Table 1, prevalence in Indian subcontinent and adjacent countries is relatively similar; 10% in India (Gutierrez *et al*., 2006), 10% in Iran (Glynn *et al.,* 2002), 8.8% in Sri Lanka (Lillebaek *et al.,* 2003), and 3.9 % in Afghanistan (Lillebaek *et al.,* 2003). This shows that prevalence of Beijing strains in these countries is relatively lower than China (95%) (Glynn *et al*., 2002) and other Asian and European countries. However, the method of testing and sampling are questionable and may not represent the true general population

The increasing frequency of Beijing strains demands further research. Complete knowledge which governs the proliferating capacity of Beijing strains is yet to be unraveled. Many hypotheses are existent but require supporting evidence to be considered as the actual reason behind the success of the Beijing strains. One of the hypotheses used to explain the spread of Beijing strains in Asian Subcontinent is the resistance against BCG vaccination (Anh *et al*., 2000). However, the fact that BCG vaccination provides a selective pressure for proliferation of the Beijing strains was inconsistent with the capacity of Beijing strains to cause outbreaks in non-vaccinated regions of USA (Bifani *et al*., 2002). During 2000-2003 in Cape Town, the disease incidence in children due to Beijing strains increased from 13% to 33%, suggesting some selective advantage in pathogenesis (Cowley *et al*., 2008).

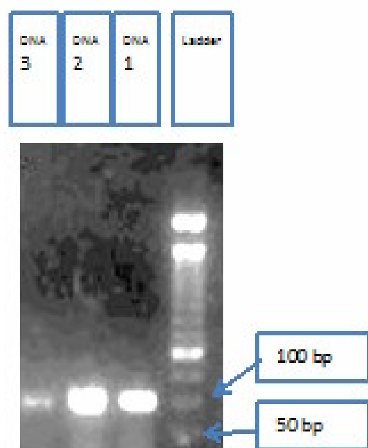

Fig 2a. Typical Non-Beijing DNA (95bp)

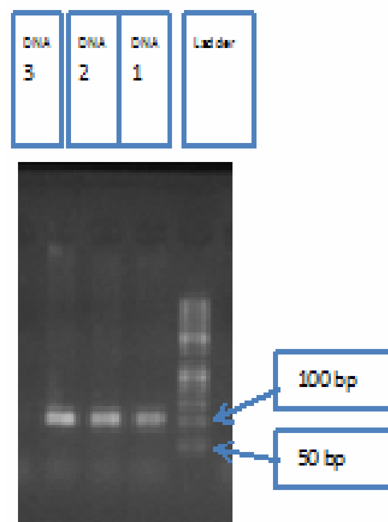

Fig 2b. Typical Beijing DNA (129 bp)

However, there is no apparent evidence that the Beijing strains has high mutation frequency or has some unique drug efflux system which may confer a selective advantage.

In fact, there is little genetic diversity in genes coding for the targets of host immune system, showing limited selective pressure (Musser *et al*., 2000). Despite these facts, the Beijing strains continues to dominate over a wide range which demands the need for further research to unravel the factors behind the propensity of Beijing strains to prevail.

Therefore, keeping in view of the discussion above, it is conclusive that further research needs to be done. Quick, reliable and authentic methods for the detection of Beijing strains should be established which could be adopted universally, statistically accurate data which is a true representative of the population involved should be gathered and kept up to date for proper reasoning as well as extensive studies at molecular level should be carried out to find out the main reason for the success of the Beijing strains and how it can be eradicated.

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