Russian ‘successful’ clone В0/W148 of *Mycobacterium tuberculosis* Beijing genotype: multiplex PCR assay for rapid detection and global screening

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We describe a multiplex PCR assay to detect *Mycobacterium tuberculosis* Beijing genotype variant 27B0/W148 that is considered a ‘successful’ clone of *M. tuberculosis*, widespread in Russia. Specificity and sensitivity of the assay were 100% as based on the analysis of a collection of 516 *M. tuberculosis* isolates of different genotypes and origins. The developed assay may be used for accurate and simple detection and surveillance of this clinically and epidemiologically important variant of *M. tuberculosis*. 
Tuberculosis caused by *Mycobacterium tuberculosis* is one of the most devastating infectious diseases but some of its lineages and strains are more prone to disseminate and cause active disease. A clonal group designated B0 (8, 9) or W148 (1) has been described in one forth of the Beijing genotype isolates in different parts of the former Soviet Union as well as in isolates recovered from Russian immigrants (1, 2). The Beijing B0/W148 variant was initially identified using IS6110-RFLP typing (1, 8). The B0/W148 and similar profiles sharing the same characteristic double band in the upper part of the profile were more recently defined as B0/W148-cluster (6).

The B0/W148 isolates, compared to other Beijing genotype isolates, demonstrated an increased virulence in the macrophage model (3), a stronger association with multidrug resistance (9) and an increased transmissibility (10). It has been suggested that the Beijing B0/W148-cluster represents a ‘successful’ clone of *M. tuberculosis* in Russia (6). We sought to develop and evaluate a method for simple and rapid detection of the Beijing B0/W148-cluster isolates.

Retrospective collections of *M. tuberculosis* DNA used in this study were partly described previously (3, 4, 7, 12) and have been characterized by IS6110-RFLP and spoligotyping (Table S1).

An *in silico* genome analysis and identification of the B0/W148-specific IS6110 insertion in contig 1.39 of strain W-148 (GenBank ACSX01000039) are described in detail below. A multiplex PCR targeting the B0/W148-specific IS6110 insertion was performed using primers INS1 (5’-CGTGAGGGCATCGAGGTGGC) (11), Rv2665R (5’-CTCGGCCGTACGGACGACGATC) and W139F2 (5’-GCGTTCCAACGGTTCGGGCC) in a C1000 Thermal Cycler (Bio-Rad) in 25 μl (1 unit of *Taq* polymerase (Sileks, Russia), 2 mM MgCl₂, 20 pmol of Rv2665R and W139F2 each and 25 pmol of INS1) under the following conditions: 95°C, 5 min, 6 cycles 94.5°C, 45 s, 66°C, 45 s, 72°C, 1 min 20 s, 34 cycles 94.5°C, 45 s, 67°C, 45 s, 72°C, 1 min 20 s, 72°C, 8 min. The amplified fragments were electrophoresed in 1.2% agarose gels.

The digoxigenin (DIG)-labeled PCR fragments amplified with either primers INS1 and INS2 (5’-GCGTAGGCGTACGGTGACAAA) (11) or primers W139F2 and W139R (5’-TCAGGAGACGGGGTGTCGC) were hybridized separately to the same blots with immobilized *Pvu*II-digested *M. tuberculosis* DNA. The hybridization signals were revealed using Anti-DIG-
antibodies conjugated to alkaline phosphatase, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The resulting fragments were compared to the IS6110-RFLP profiles of the same isolates.

**Results and Discussion.** An *in silico* analysis of the complete genome sequence of *M. tuberculosis* strain W-148 (GenBank ACSX00000000.1) identified an inverted IS6110 in contig 1.39 (positions from 19418 to 20772) located within the 9162-bp *Pvu*II digest (Fig. 1). Theoretically, it corresponded to the largest ~9.2 kb band in the IS6110-RFLP profile of the B0/W148 strain (Fig. S1). This insertion corresponds to positions 2982598/9 in the complete genome sequence of strain H37Rv (GenBank NC_000962.2) located in a short intergenic region between genes Rv2664 and Rv2665.

The specificity of this particular IS6110 insertion for clinical isolates with the Beijing B0/W148 profile was experimentally confirmed by Southern hybridization of the *Pvu*II digested DNA of the same isolates with probes: (a) INS1/INS2 flanked the IS6110 fragment and (b) a fragment of the genome region flanked with primers W139F2 and W139R and located upstream of the aforementioned IS6110 in strain W-148 contig 1.39 (Fig. 1). A comparison of the hybridization profiles confirmed that the 9.2-kb fragment in the IS6110-RFLP profile of the Beijing B0/W148 isolates spanned indeed the above mentioned IS6110 insertion in the Rv2664/Rv2665 intergenic region. Strain H37Rv presented a 8.4-kb band, thus confirming the absence of IS6110 in its Rv2664/Rv2665 intergenic region (Fig. 2b).

The Rv2664/Rv2665 intergenic region is both intact and identical in all other published complete genomes of *M. tuberculosis* complex strains of different genotypes available in GenBank (as of 31.07.2012) except for *M. bovis* and *M. bovis* BCG strains with an A>G substitution (position 2935950, GenBank NC_008769.1) and *M. canetti* (no similarity found).

Accordingly, a multiplex PCR procedure was designed for detection of the Beijing B0/W148-cluster isolates (Fig. 1, Fig. 3). *M. tuberculosis* isolates with an intact Rv2664/Rv2665 region are characterized by a 410-bp band amplified with primers W139F2 and Rv2665R. *M. tuberculosis* isolates with the Beijing B0/W148-specific IS6110 insertion in this genome region are characterized by a 1018-bp band amplified with primers W139F2 and INS1 (Fig. 1). The assay was optimized with reference strains H37Rv, BCG, Mt14323, and a limited number of the Beijing genotype isolates previously...
characterized by IS6110-RFLP typing (by definition, the ‘gold standard’ method to detect a B0/W148 strain) and spoligotyping (Fig. 3).

The specificity and sensitivity of the method were evaluated with an enlarged collection of 227 Beijing and 164 non-Beijing isolates from different locations, characterized by spoligotyping (all isolates) and IS6110-RFLP typing (Beijing genotype isolates) (Table S1). All 62 isolates of the B0/W148-cluster, i.e., B0/W148 profile and similar profiles (gray shade in Fig. S1), were correctly identified by the multiplex PCR assay. All non-Beijing isolates (n=164) and all non-B0/W148 Beijing isolates (n=165) were characterized by amplification of the 410-bp band correctly indicating at their non-B0/W148 status. Thus the specificity and sensitivity of the multiplex PCR assay to detect the Beijing B0/W148 variant were 100%.

The method was additionally applied to the available DNA collections previously characterized by spoligotyping only. This permitted to detect the B0/W148 genotype in 18 of 49 Beijing isolates from St. Petersburg, Russia, in 7 of 39 Beijing isolates from Pskov, Russia, and in none of the isolates from Vietnam (Table S1).

In total, the B0/W148-cluster in this study was found only in different post-Soviet settings and its rate (of all Beijing isolates) varied from 18.0% in Pskov and 23.3% in Karelia to a higher rate of 28.8% in St. Petersburg and even 52.5% in Belorussia. However, the latter collection included only drug resistant isolates and was thus biased. In contrast, no B0/W148-cluster strain was identified among isolates from China, Vietnam and Brazil (Table S1). Since neither of these countries received a significant influx of immigrants from the former Soviet Union (5), this finding is not unexpected.

Indeed, the major direction of the Russian (ex-Soviet) emigration has been towards regions in West Europe and North America (5) where B0/W148 isolates have been described (1, 2).

In conclusion, we have developed a multiplex PCR-based procedure for fast, simple, and reliable detection of the epidemiologically and clinically important genetic variant of M. tuberculosis, the Beijing B0/W148-cluster. We propose this assay for screening and surveillance of this genotype in the areas of its epidemic circulation, such as the countries of the former Soviet Union, and in the areas receiving immigrants from these countries.
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REFERENCES

Legends to Figures

FIG. 1. Schematic view of the genome region containing the IS6110 insertion specific of strain W-148 (not to scale). Short arrows indicate the primers. PvuII-flanked fragments are shown by dashed lines.

FIG. 2. Southern hybridization of PvuII-digested DNA of M. tuberculosis isolates with probes: a) internal IS6110 fragment (primers INS1/INS2); b) Rv2664/Rv2665 fragment (primers W139F2/W139R). Lanes: 1-5 – Beijing B0/W148 isolates, 6 – strain H37Rv, M – strain Mt14323 used as molecular weights marker. Double-headed arrow indicates particular associated fragments of IS6110 –RFLP profiles and PvuII digest spanning the Rv2664/Rv2665 region in the Beijing B0/W148 isolates.

FIG. 3. Agarose gel-electrophoresis of the multiplex PCR products of M. tuberculosis isolates. Lanes: 3-5, 7, 9 - Beijing B0/W148-cluster isolates; 1, 2 - isolates of other Beijing variants; 6 - H37Rv, 7 - BCG. M – ‘100 base-pair DNA ladder’ (GE Healthcare). Arrows show specific bands for Beijing B0/W148 (1018 bp) and other genotypes (410 bp).