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Original Article

Impact of 63-bp deletion and single-base mutation in mpt64 gene on *M.tb* diagnosis

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Abstract: After previous research, which selected 180 clinical isolates of the Mycobacterium tuberculosis complex (MTC) from patients in China and performed comparative sequence analysis of the mpt64 gene after amplification and found the polymorphisms of the mpt64 gene in MTC, in order to further investigate the impact of polymorphism of antigen MPT64 on the diagnostic accuracy of MPT64-based test kit, testing on 180 strains by MPT64-based immunochromatographic test (ICT) was conducted. As a result, 180 strains were detected positive except 8 isolates were negative. First, 8 strains harbored 63-bp deletion had a major impact on the biological function of mpt64 and led to the negative results; however, 4 isolates had nonsynonymous nucleotide mutation which led to rare changes in protein structure, did not led to functional change as those 4 strains detected positive by ICT; one strain with a single-base insertion, as the insertion occurred in the last amino acid codon that did not affect T-cell epitopes in MPT64, detected positive by this method. These demonstrate that different mutations in mpt64 gene had impact on diagnostic test kit inconsistently, this was different with some previous studies. And the performance of the mpt64-based diagnostic test kit was still very well with a sensitivity of 95.6% (172/180).

Keywords: *Mycobacterium tuberculosis*, Mpt64, mutation, diagnosis

Introduction

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease [1]. The spread of drug resistant TB and emergence of multidrug resistant, extensively drug resistant and totally drug resistant TB poses a real threat to global TB control [2, 3]. Thus, improving diagnosis and effective vaccines are significant for TB control problem worldwide.

Recently, a novel immunochromatographic test (ICT) based on MPT64 detection has been developed for rapid identification of MTC and has shown promise [4]. The M.tuberculosis Diagnostic Kit (Colloidal Gold) (GENESIS, Kaibili, China) (TB14201) is a commercial MPT64-based ICT kit, ICT is a rapid and low technology method for identify MTBC, which use a monoclonal antibody to detect MPB64 in liquid or solid media and can performed without additional equipment or laboratory bench space. Test results are available after 15 mins with the color band in test zone indicates the existence of MPT64 and the growth of MTC. Thus strains with positive results are identified as MTC finally.

MPT64 (Rv1980c), which is described as MPB64 for Mycobacterium bovis, is a 24 kDa protein secreted by MTC during bacterial growth. It is highly specific for MTC, including M. tuberculosis (MTB), Mycobacterium africanum, M.bovis and some substrains of M.bovis bacilli Calmette-Guerin (BCG) [5-9].

Previous studies which showed that the MPT64 antigen is highly conserved [10], while Dr. Jiang reported the polymorphism of antigen MPT64 in *M.tb* strains [11]. Besides, numerous studies reported the variability in the diagnostic accuracy of MPT64, depending on the recombinant antigen used in assays [12, 13]. In order to further investigate the impact of different muta-

Table 1. Spoligotypes and ICT results of 13 variant strains

Strains	Spoligotype	mutation	ICT results
AH03031	Beijing family	ACG-GCG*	+
ShanXi05105	Beijing family	ACA-CCA*	+
HeN06035	Beijing family	TCC-GCC*	+
XJ06177	U	CCG-CAG*	+
XJ06112	MANU	a single-base insertion	+
AH03009	T	63-bp deletion	–
ShanXi05290	U	63-bp deletion	–
FJ05395	T	63-bp deletion	–
GX06043	U	63-bp deletion	–
GX06130	U	63-bp deletion	–
HuN06004	MANU	63-bp deletion	–
HuN06026	T	63-bp deletion	–
HuN06101	new	63-bp deletion	–

*Nonsynonymous nucleotide mutation.

tions of MPT64 on diagnostic accuracy of commercial test kits, research on previous strains was conducted using the M.tuberculosis Diagnostic Kit (Colloidal Gold).

Materials and methods

Using the same sets as previous, we selected those 180 strains for research, including 8 strains harbored a 63-bp deletion and 4 strains showed a single-base nonsynonymous mutation and one strain had single-base insertion [11]. As those 180 clinical isolates were selected from a very large geographical area and contained different spoligotyping patterns in China, the data from these strains are representative of the genetic diversity of China. First, reconstituting a lyophilized vial of BBL MGIT PANTA Antibiotic Mixture with 15 ml of BACTEC MGIT Growth Supplement; Then label the MGIT tube with the specimen number; unscrew the cap and aseptically add 0.8 ml of the Growth Supplement/MGIT PANTA Antibiotic Mixture; add 0.5 ml of the bacterial suspension to inoculate Mycobacterium growth indicator tube (MGIT) (BACTEC MGIT 960, Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) for each isolates; tightly recap the tube and mix well; incubated in the BD Bactec MGIT system (Becton Dickinson Microbiology System, Cockeysville, MD) [14]; tubes entered into the MGIT 960 instrument will be automatically tested for the duration of the recommended 42 day testing protocol. Once a positive signal was detected of these tubes by the system, it will be removed from the system and stained for the presence of Acid-fast bacilli (AFB) using Ziehl-Neelsen method to dismiss contamination [15]. Processing a positive MGIT Tube - All steps should be performed in a biological safety cabinet. Remove the MGIT tube from the instrument; using a sterile transfer pipet, remove an aliquot from the bottom of the tube (approx.0.1 ml) for stain preparations (AFB and Gram stains). All instrument-positive samples is determined by the BACTEC MGIT instrument and confirmed by an acid-fast smear. After confirmed as positive for AFB, it will tested by *M.tuberculosis* Diagnosis kit (Colloidal Gold), a MPT64-based commercial test kit.

Colloidal Gold test were performed in the Reference Laboratory of Mycobacteriology at the Chinese CDC. The Colloidal Gold assay was performed by placing 0.1 ml from a Bactec cultures onto the specimen placement area of the cartridge according to the manufacturer's recommendations. It allowing a minimum of 15 min incubation at room temperature before the result were visually assessed for positive detection (a visible test line) and reagent function (a visible control line) by observing color change in the test detection line area and quality control line area. It must read the result within one hour. The 13 mutation strains were re-tested by repeating the Colloidal Gold testing from newly inoculated positive MGIT cultures, and yielded the same results as above.

Results

In our study, 180 strains were detected positive except 8 isolates were negative. First, 8 strains harbored 63-bp deletion which had a major impact on the structure of MPT64 led to the negative results; however, 4 isolates had nonsynonymous nucleotide mutation which led to rare changes in protein structure, detected positive by ICT; one strain with a single-base insertion, as the insertion occurred in the last amino acid codon that did not affect T-cell epitopes in MPT64, and detected positive by this method. These demonstrate that different mutations in mpt64 gene yielded results inconsistently. This was different with some previous studies [16]. Besides, the sensitivity of this

Table 2. Summary of negative results of MPB64-based lateral-flow immunochromatographic assays in the detection of Mycobacterium tuberculosis in different studies

Study method	No. of MTB with negative results	Mutation(s) inmpb64 gene (no. of isolates)	Sensitivity (%)	references	Country (ies)
Capilia TB assay	3	63-bp deletion at 196 (3), 1-bp deletion at 266 (1), G->A at 402 (2), IS*6110* insertion at 501 (1), 176-bp deletion at 512 (5)	99.2	[20]	Japan
Capilia TB assay	3	C insertion at 287 (1), A-T at 388 (1), IS*6110* insertion at 177 (1)	92.4	[16]	German
Capilia TB assay	6	63-bp deletion at 196 (5), 2-bp insertion at 436 (1)	97	[18]	Thailand
MGIT TBc ID Test	2	63-bp deletion at 196, downstream 50 A-G (2)	98.9	[22]	Taiwan
M.tb Diagnostic Kit	8	63-bp deletion at 196 (8)	95.6	This study	China

method was 95.6% (172/180), this was consistent with a multi-center study, which has shown that sensitivity of this method was 94.8% [17]. The spoligotypes and test results of those mutation strains are shown in **Table 1**. Mutations in mpt64 gene and sensitivity of ICT method in different studies are shown in **Table 2**.

Discussion

In our study, we validate that the 63-bp deletion which resulted in a drastic change in the overall structural topology and surface exposure of mpt64, had a significant impact on the biological function of this antigen. This, in turn, will influence the diagnostic accuracy of mpt64-based ICT. However, though 4 strains had one nonsynonsymous nucleotide mutation each resulted in rare changes in protein structure, but this did not influence the biological function of this protein as the test results were positive.

Hirano, K., et al., report that in their study all of M.tuberculosis strains with ICT negative results had mutations in the mpb64 gene. Including three strains had a 63-bp deletion from nucleotides 196 to 258 (amino acid positions 43 to 63), two contained a point mutation at position 402 (G to A), which created a stop codon at nucleotides 400 to 402 (TGA). The point mutation in this study led to a negative test result, this was different with our study. The reason for this need further study. Ngamlert, K., et al, reported 5 isolates harbored 63-bp deletion in the mpb64 in their study, which was identical with our study, also led to negative results [18].

The false negative results of ICT using monoclonal antibody to detect MPB64 has been reported due to mutations in MPT64 gene [16, 19-22]. Summary of negative results and sensitivity of MPB64-based ICT in the detection of Mycobacterium tuberculosis in different studies are shown in **Table 2**.

In addition, it is unknown the relationship between spoligotypes and mutation types, as 92 Beijing family strains had positive results in total 180 isolates, maybe Beijing family is more conserved than non-Beijing family strains. Furthermore, the sensitivity of this method was still high even mutations existed in strains in our study. But a systematic review and meta-analysis reported that the sensitivity of the MPT64-based ICTs varied from 76% to 100% [23]. Still, whether the mutations in mpt64 gene will impair the utility of mpt64-based ICTs is unclear. Besides, as the evaluation of ICTs result is qualitative rather than quantitative, a weak positive signal possibly cannot be seeing visually.

In conclusion, mutations in mpt64 gene impact the performance of MPT64-based ICTs variably depend on whether those mutations led to biological function change in mpt64. It is necessary to evaluate the prevalence of genotypic variation in mpt64 before put these assays into routine use since the various mutations may influence test performance.

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Disclosure of conflict of interest

None.

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