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Isolation, identification and DNA fingerprinting of Mycobacterial Isolates from AIDS patients

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ABSTRACT

Tuberculosis (TB) is one of the most important AIDS associated infectious diseases worldwide. It is a leading cause of illness and death among people with HIV/AIDS in resource-poor areas of the world. The annual incidence of TB among indigenous Iranians stands at 14 cases per 100,000 inhabitants. This study aimed to identify *Mycobacterium* infection among Iranian HIV positive patients. Two sputum specimens were collected from smear positive AIDS patients. Samples were cultured on Lowenstein-Jensen media for three weeks. DNA was extracted from two samples based on van Embden protocol. To identify *Mycobacterium tuberculosis* complex, a PCR was conducted to amplify a 245 bp fragment of IS*6110* element, followed by RD12 method to confirm PCR result. Whole DNA-RFLP with *Pvu*II restriction enzyme was employed to genotype the cultured isolates. The results obtained by colonial morphology, PCR, and RD12 methods showed that both isolates were belonging to *M. tuberculosis*, and Genotyping of the isolates by RFLP technique displayed that two isolates were belonging to different strains of *M. tuberculosis*.

1. Introduction

Tuberculosis (TB), the "White Plague" as known in the past, is an ancient and often neglected disease. Recent genetic evidences suggest that it is most probable that the remote hominid ancestors, living three million years ago, might had suffered from TB (Gutierrez et al., 2005). TB is one of the most important AIDS associated infectious diseases, worldwide. The clinical presentations of TB in HIV/AIDS patients are clearly related to the degree of immunodepression of patients, which is measured as the blood level of CD_4^+ T lymphocytes (Sutherland et al., 2006). A level of 200 CD_4^+ T cells per µl represents an

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approximate threshold for severe immunodepression (Jones et al., 1993).

Several opportunistic infections and noninfectious agents coexist in addition to the HIV itself, which implement their own pathogenic mechanisms among all opportunistic diseases associated with HIV/AIDS (Shaffer et al., 1992).

The distinctive feature of TB lies mainly in its airborne dissemination to other patients, as well as entire community (Pitche et al., 2005). TB develops in HIV-infected hosts by either of the two pathogenic mechanisms, either endogenous reactivation or exogenous reinfection eventually, while both mechanisms can coexist (van Rie et al., 1999).

Molecular techniques such as RFLP have been employed to trace source of infection and route of transmission in case of Mycobacterial infections (Collins et al., 1997). Since the annual incidence of TB among indigenous Iranians is high with 14 cases per 100,000 inhabitants (World TB day, 2009), this study was conducted to characterize and identify *Mycobacterium* infection among Iranian HIV positive patients.

2. Material and methods

Two sputum specimens were collected from smear positive AIDS patients during October 2009 up to March 2010. The samples were culture on two slopes of Lowenstein-Jensen (L-J) medium each with either pyruvate or glycerin for 3 weeks. The genomic DNA was extracted from the visible colonies according to the method described by Van Soolingen et al. (1997).

A 245 bp fragment of the IS*6110* element was amplified to identify *M. tuberculosis* complex isolates. Forward and reverse primers and PCR condition were same as those given by Huard et al. (2003). Cycling condition was set up as follows: 94°C for 3 min as initial denaturation, 94 ºC for 1 min, 65 ºC for 1 min, and 72 ºC for 2 min for 25 cycles, and 72 ºC for 4 min as final extension. The PCR products were examined by electrophoresis on 1.5% agarose gel after staining with ethidium bromide. Moreover, RD12 technique was performed to differentiate members of *M. tuberculosis* complex (Parsonsl et al., 2002). The condition used for RD12 was 1 min at 94 °C, followed by 30 cycles of 21 sec at 94 °C, 21sec at 55 °C, and 22 sec at 72 °C.

A RFLP method was carried out by restriction endonuclease *Pvu*II to digest the whole DNA. Restriction fragments were separated by electrophoresis in a 0.8% agarose gel in TBE buffer. The fragments in the gel were transferred onto a nylon membrane with a southern blotting Capillary transfer device. Before, the 245 bp PCR products had been purified from the gel and sent to be labeled by digoxigenin at 3' end by tailing method. These labeled fragments were used as probe. The detection was performed by their Hybridization with Anti-digoxigenin antibody conjugated with HRP and NBT and BCIP. RFLP band patterns were finally analyzed by gel pro-analyzer.

3. Results

Based on the colonial morphology test, both mycobacterial isolates were identified to be *M. tuberculosis* as they both grew on L-J medium with glycerol, while did not growth on L-J medium with pyruvate.

Based on the PCR method, a 245 bp PCR amplicon was achieved by both isolates, which reflected that both isolates were *M. tuberculosis* as IS*6110* element is part of its genome (Figure 1).

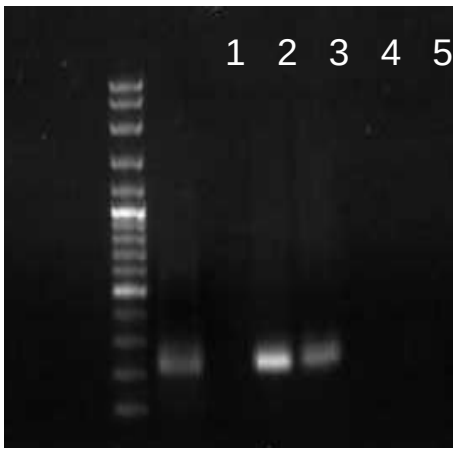

Figure 1. Ethidium bromide-stained agarose gel showing a 245 bp fragment of IS*6110* element amplified by PCR.

Lane 1: Size marker no. 14 Roche lane; 2: Standard strain; lane 3: Negative control; lanes 4-5: samples.

The results obtained by RD12 also confirmed that two Mycobacteria isolates were *M. tuberculosis* (Figure 2).

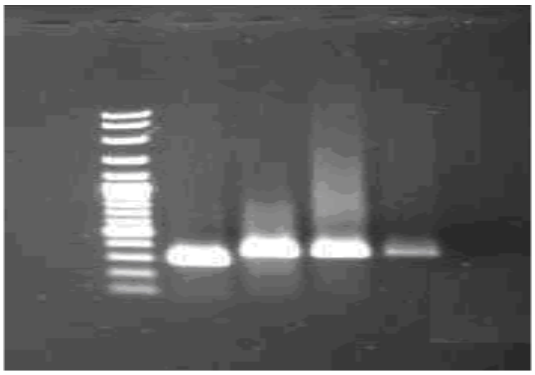

Figure 2. A 370 bp fragment of *M. tuberculosis* genome amplified by RD12. Lane 1: Size marker no. 14 Roche; lane 2: bovine Standard strain; lane 3: human standard strain; lane 4-5: samples; Lane 6: negative control

As shown in figure 3, RFLP Genotyping of IS*6110* elements of two isolates displayed different pattern of restriction, which indicated that *M. tuberculosis* of two samples were belonging to different strains.

Figure 3. DNA fingerprinting of the Isolates by IS*6110*-RFLP with *Pvu*II enzyme. Lane 1: Size marker; lane 2: Standard strain; lanes 3-4: samples.

4. Discussion

A complex biological interplay occurs between *M. tuberculosis* and HIV in the co-infected host that results in the worsening of both pathologies. HIV promotes progression of *M. tuberculosis* latent infection to disease and, in turn, *M. tuberculosis* enhances HIV replication, accelerating the natural evolution of HIV infection (Mariani et al., 2001).

The AIDS pandemic further promoted the emergence of multidrug- resistant TB (MDR-TB). In early 1990s, the first AIDS- associated MDR-TB outbreak was reported in the United States. It was the first alarm signal of the decline of the TB control programs, which was prevalent at that time not only in the US, but also in several other parts of the world. Therefore, identification of sources of infection, routes of transmission and finding the reservoirs were of significance for researchers and physicians in eradication scheme and therapy purposes; all could be achieved by differentiation of *M. tuberculosis* isolates (Frieden et al., 1996). Many different methods have been used in this regard, but now it is believed that employing of molecular strategies can better answer such questions. Molecular techniques such as RFLP have been employed to trace source of infection and route of transmission in cases of *M. tuberculosis* infections.

Mycobacterium avium is also blamed for opportunistic infections in immunocompromised patients such as AIDS patients. Rapid identification of these bacteria is of special importance in these patients (MacGregor et al., 2005). Birkness et al. (1999) and Martin et al. (2000) reported that about 50-60% of AIDS patients were infected with *Mycobacterium avium* complex. However, both isolates collected in our study were belonging to the *Mycobacterium tuberculosis* complex.

It can be concluded that the sequence of events may be either a primary or a secondary exogenous infection of a highly susceptible AIDS patients or reactivation of previously acquired dormant bacteria.

In a study conducted in Tunisia, 75% of the investigated isolates of *M. tuberculosis* were

found to carry 6 to 10 copies of IS*6110* element (Yangi et al., 1995) while the number of IS6110 copies carried by *M. tuberculosis* studied in India was 8 (Sarman et al., 2007). The IS*6110* copy number of the isolates in this study ranged from 8 to 10. The strains of Asian subgroup of *M. tuberculosis* are reported to contain a low numbers of IS*6110* copies (Yangi et al., 1995). Therefore, considering the low copy number of IS*6110* elements in this study, it is more probable that the *M. tuberculosis* isolated from Iranian patients were belonging to Asian subgroup.

The results obtained by this study showed that the IS*6110*-PCR followed by RD12 techniques can be considered as a fast-diagnostic strategy for *M. tuberculosis* complex. Meanwhile, rely on the results obtained in this study, RFLP-genotyping with *Pvu*II and hybridization with IS*6110* probe can be considered as a very useful method to discriminate between *M. tuberculosis* strains.

In conclusion, despite the relatively limited number of isolates in this study, it was the first molecular study on Mycobacterial isolated from AIDS patients, which showed the distribution of different strains at different areas of Iran.

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