DNA Fingerprinting and Phenotyping of Mycobacterium tuberculosis Isolates from Human Immunodeficiency Virus (HIV)-Seropositive and HIV-Seronegative Patients in Tanzania


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With the purpose of determining whether the risk of infection with a particular clone of Mycobacterium tuberculosis is influenced by the human immunodeficiency virus (HIV) status of the host, we analyzed and compared 68 mycobacterial isolates obtained from HIV-seropositive patients with tuberculosis (TB) in Dar es Salaam, Tanzania, with 66 mycobacterial isolates obtained from HIV-seronegative patients with TB in the same geographical region by using both DNA fingerprinting and classical phenotyping methods. One hundred one different IS6110 fingerprinting patterns were observed in the 134 isolates. The level of diversity of the DNA fingerprints observed in the HIV-seropositive group was comparable to the level of the diversity observed in the HIV-seronegative group. Resistance to a single anti-TB drug was found in 8.8% of the tested isolates, and 3.2% of the isolates were resistant to more than one anti-TB drug. The drug susceptibility profiles were not significantly different between the two groups of isolates compared in the present study. Phenotypic characteristics which classify M. tuberculosis strains as belonging to the Asian subgroup correlated with a low IS6110 copy number per isolate. However, the occurrence of Asian subgroup strains was not associated with the HIV status of the patients. The results of the study suggested an equal risk of infection with a defined M. tuberculosis clone for HIV-seropositive and HIV-seronegative individuals.

The emergence of the AIDS pandemic has caused profound changes in the epidemiological aspects of mycobacterial diseases both in developing and in industrialized countries (3–5, 15, 16, 21). A number of studies based on clinical observations (10, 12, 18–20, 27) and on epidemiological surveys (9, 17, 25) of tuberculosis (TB) and human immunodeficiency virus (HIV) seroprevalence have provided evidence that HIV infection is a risk factor for the development of active and often lethal TB. This might be explained by increased reactivation of previously acquired, dormant mycobacteria and enhanced susceptibility to both reinfection and primary infection. It was previously suggested (22) that reactivation is responsible for the major part of HIV-associated TB, especially in countries with a high level of TB transmission. However, study of the DNA fingerprints of Mycobacterium tuberculosis isolates obtained from AIDS patients by the technique of restriction fragment length polymorphism (RFLP) analysis showed that reinfection and new infection also occurs in AIDS patients (8, 14, 24).

The development of DNA fingerprinting techniques for M. tuberculosis has permitted detailed studies of the transmission of TB. The method can effectively be used to distinguish between a newly acquired infection and a relapse (2, 5, 8, 11, 13, 14). It could be hypothesized that the HIV-infected population comprises an ecological niche for M. tuberculosis, also allowing less virulent strains to replicate freely without the selection pressure normally provided by an active cellular immune sys-
obtained in parallel from both the laboratory in Tanzania and the laboratory at the Statens Serum Institut in Copenhagen.

The *M. tuberculosis* complex strains were, in addition, typed according to the criteria suggested by Collins and colleagues (7) on the basis of their resistance to cycloserine and pyrazinamide, ability to grow on solid medium containing thiopheno-2-carboxylic acid hydrazide, oxygen preference, and ability to reduce nitrate in a standard in vitro assay. According to these analyses the isolates were divided into subgroups designated classical human, Asian human, African I, African II, and classical bovine.

**DNA fingerprinting**. DNA fingerprinting was performed as described previously (28). In brief, the isolates were cultivated on Löwenstein-Jensen slopes at 37°C for 3 to 4 weeks. Chromosomal DNA was extracted from the bacteria with chloroform-isoamyl alcohol (24:1); vol/vol). The restriction endonuclease *Pvu*II was used for digestion of the genomic DNA. Restriction fragments were separated by electrophoresis in a 0.8% agarose gel in 1X TBE (Tris-borate-EDTA) buffer overnight at 0.8 V/cm and were transferred onto a nylon membrane (Hybond N-plus; Amersham International plc, Buckinghamshire, United Kingdom) with a vacuum transfer device (Milliblot TM-v; Millipore Corp., Bedford, Mass.). The probe used in the study was a 245-bp PCR product of the right arm of IS6110 (29), which was purified by agarose electrophoresis and which was nonradioactively labelled with an enhanced chemiluminescence kit (Amersham International plc). Hybridization and detection were performed according to the instructions provided by the manufacturer of the enhanced chemiluminescence kit. The external marker for monitoring the separation of the restriction fragments during electrophoresis was a mixture of HindIII-digested bacteriophage lambda DNA and *Hae*III-digested *XbaI* DNA. The internal marker included to facilitate the computer-assisted determination of the size of each IS6110-hybridizing fragment for comparison of DNA fingerprints was a mixture of *Pvu*II-digested supercoiled DNA ladder and *Hae*III-digested *XbaI* DNA. The reference DNA included in all experiments as a control was the chromosomal DNA extracted from *M. tuberculosis* Mt 14323 (Strain Collection, Laboratory of Bacteriology and Antimicrobial Agents, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands).

**Analysis of RFLP patterns**. The DNA fingerprint patterns of the mycobacterial isolates were analyzed and compared by a computer-assisted analysis as described previously (30). The software used for the analysis was BioImage Whole Band Analyzer, version 3 (Millipore Corporation, Ann Arbor, Mich.).

**RESULTS**

**Phenotypic characteristics of the strains**. A total of 134 isolates obtained from patients with newly diagnosed cases of TB at a major referral center in Dar es Salaam were included in the present study. Of these 134 isolates, 1 was identified as *Mycobacterium avium* and the rest were all confirmed to be *M. tuberculosis*. One hundred twenty-five isolates were tested for their susceptibilities to conventional anti-TB drugs like ethambutol, isoniazid, streptomycin, rifampin, and pyrazinamide. The isolates were, in addition, tested for their susceptibilities to fusidic acid, which is known to be potent against other gram-positive bacteria. The resistance profiles of the isolates are given in Table 1. Eleven isolates (8.8%) were observed to be resistant to only a single drug. Four isolates (3.2%) were found to be resistant to two or more of the drugs. No relationship between HIV status and anti-TB drug susceptibilities was observed.

Apart from phage typing, one of the previously described methods available for subtyping strains belonging to the TB complex is based on phenotypic markers like oxygen preference and the ability of the strains to reduce nitrate in an in vitro assay. These results were combined with susceptibility to pyrazinamide and cycloserine and the ability to grow in the presence of thiopheno-2-carboxylic acid hydrazide, whereby the isolates were assigned to subgroups, as summarized in Table 2. The occurrence of each of the variants was not associated with the HIV status of the TB patient.

**RFLP patterns**. The RFLP patterns of the 134 isolates investigated are given in Fig. 1 by two-lane maps (HIV seropositive versus HIV seronegative). One hundred five different IS6110 fingerprinting patterns were observed in the 134 isolates, and 45 of the isolates showed RFLP patterns identical to each other. The IS6110 copy number of the isolates ranged from 1 to 20. Thirty-five isolates (26.1%) carried one to five copies of the IS6110 element. The isolates carrying only a single copy of the IS6110 element accounted for about 8% of the total number investigated. A clear correlation between the occurrence of a low number of IS6110 elements per chromosome and classification into the Asian subgroup was observed. A total of 16 isolates were classified as belonging to the Asian subgroup. Fourteen of these isolates carried from one to five IS6110 copies, and two carried seven or eight IS6110 copies. The Asian subgroup made up 40% of the total number of isolates carrying only a low IS6110 copy number. The single IS6110 copy was, as shown in Fig. 1, integrated into two different chromosomal locations. One of the sites was the so-called hot spot for integration of IS6110 located on a 1.5-kb *Pvu*II restriction fragment, and the other site was on a 5-kb *Pvu*II restriction fragment.

Despite the great diversity of the patterns among the isolates, a computer-assisted similarity analysis demonstrated a relatively high level of relatedness among a large number of different patterns. About 45% of the patterns were included in three big clusters. The similarity among the individual non-identical patterns within each of the three clusters ranged from 70 to 96 percent (Fig. 2). This relatively high level of similarity is also demonstrated by the lane map.

**TABLE 2. Variants of *M. tuberculosis* complex strains from Dar es Salaam**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>No. (%) of isolates from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV-seropositive patients</td>
</tr>
<tr>
<td>Classical bovine</td>
<td>15 (11.9)</td>
</tr>
<tr>
<td>African I</td>
<td>0 (0)</td>
</tr>
<tr>
<td>African II</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
</tr>
</tbody>
</table>

**TABLE 1. Drug resistance profiles of *M. tuberculosis* isolates from Dar es Salaam in relation to HIV status of the patients**

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>HIV-seropositive patients</th>
<th>HIV-seronegative patients</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Isoniazid</td>
<td>1 (0.8)</td>
<td>1 (0.8)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>1 (0.8)</td>
<td>1 (0.8)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td></td>
<td>Pyrazinamide</td>
<td>2 (1.6)</td>
<td>2 (1.6)</td>
<td>4 (3.1)</td>
</tr>
<tr>
<td></td>
<td>Fusidic acid</td>
<td>3 (2.4)</td>
<td>3 (2.4)</td>
<td>6 (4.5)</td>
</tr>
<tr>
<td>II</td>
<td>Isoniazid</td>
<td>1 (0.8)</td>
<td>1 (0.8)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td></td>
<td>Rifampin</td>
<td>1 (0.8)</td>
<td>1 (0.8)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>1 (0.8)</td>
<td>1 (0.8)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td></td>
<td>Pyrazinamide</td>
<td>1 (0.8)</td>
<td>1 (0.8)</td>
<td>2 (1.6)</td>
</tr>
</tbody>
</table>

**a** A total of 125 isolates were tested.  
**b** Group I includes isolates resistant to one drug, and group II includes isolates resistant to two or more drugs.
FIG. 1. Computer-generated lane maps displaying all of the DNA fingerprints of the analyzed isolates. (A) Lane map showing the 68 isolates obtained from HIV-seropositive patients. (B) Lane map showing the 66 isolates obtained from HIV-seronegative patients except for the first lane, which holds the same isolate as that in lane 1 in the lane map in panel A. Use of the same isolate as a matching reference in generating the lane maps facilitates comparison of the two sets of data. The sizes of the standard DNA fragments (in kilobase pairs) are indicated on the left.
Fifteen patterns were shared by two or more isolates. No correlation was found between drug susceptibility and the RFLP patterns of the isolates.

**Geographical distribution of the M. tuberculosis clones.** Geographically, the city of Dar es Salaam is divided into three districts: Kinondoni, Ilala, and Temeke. These three districts cover approximately 1,800 km². A clear correlation between the geographical origin and the genetically defined clusters of the isolates was observed. It appeared that certain clusters of strains were predominant in particular regions or in two neighboring districts (data not shown).

**Comparison of the RFLP patterns between HIV-seropositive and HIV-seronegative TB patients.** The level of the diversity of the DNA fingerprints observed in the HIV-seropositive TB group was comparable to the level of diversity observed in the HIV-seronegative TB group. As exemplified in Fig. 1, 60 DNA fingerprint patterns were observed among 68 isolates obtained from HIV-seropositive TB patients, and 56 patterns were found among 66 isolates derived from HIV-seronegative TB patients. The risk of belonging to a group of patients with identical RFLP patterns (to be understood as the risk of belonging to a chain of recent TB transmission) was 36.8% for the HIV-seropositive group and 30.3% for the HIV-seronegative group ($P > 0.10$). When these patterns were assigned to the predominant clusters, the isolates segregated randomly and were not associated with the HIV status of the patients. This was also reflected in the number of IS6110 copies per isolate, which ranged from 1 to 18 for the HIV-seropositive group and from 1 to 20 for the HIV-seronegative group. As demonstrated in Fig. 3, the frequency of occurrence of IS6110 in the investigated isolates appeared to be rather similar between the two groups being compared. The three relatively prevalent patterns observed in the study, which each contained five or seven isolates, included isolates obtained from both HIV-seropositive and HIV-seronegative patients.

**DISCUSSION**

The pathogenesis of HIV-associated TB is as yet far from clear. The sequence of events may be either a primary or a secondary exogenous infection of a highly susceptible HIV-infected host or reactivation of previously acquired dormant bacteria. However, it is obvious that patients infected by both HIV and *M. tuberculosis* have an accelerated progression to overt tuberculous disease. Tanzania is one of the countries in Africa in which a very extensive anti-TB campaign has been implemented. A national tuberculosis program was initiated in 1978 in which short-course anti-TB therapy was introduced in all regions of the country. Initially, this led to a significant decrease in the number of new cases of TB up to 1982. Despite these efforts, since 1983, when the first case of AIDS was officially reported in Tanzania, this positive trend was reversed into a tragic increase in the incidence of TB, especially during the last 2 or 3 years. The HIV seroprevalence in 1992 was reported to be as high as 7% for adult female blood donors and 5.4% for adult male blood donors (16a).

The purpose of the present study was to study the influence of the HIV status of the host on the mycobacterial infection. In particular, we wanted to assess whether certain mycobacterial strains characterized by either RFLP pattern or any phenotypic characteristics could be identified. The correlation between these two methods, genotyping (IS6110 RFLP pattern) and phenotyping, was also examined.

By use of IS6110 DNA fingerprinting the degree of DNA polymorphism of *M. tuberculosis* strains isolated in Dar es Salaam was found to be similar to that recently observed in New York and San Francisco (1, 23) because the average number of isolates per pattern was 1.33 for the present study, whereas for the study in New York it was 1.35 and for the study in San Francisco it was 1.5. It might be expected that regions suffering from a high level of TB transmission and insufficient
hospital facilities with a high risk of nosocomial infections would be characterized by a relatively limited variety of mycobacterial clones. The diversity of the patterns observed in the present study may be explained by the fact that Dar es Salaam is a large city inhabited by people originating from many parts of Tanzania as well as a large number of immigrants, especially from Asia but also from the Arab world. Furthermore, Dar es Salaam is one of the major trade centers of eastern Africa.

In contrast to recent observations from a large-scale RFLP study of *M. tuberculosis* isolates in Tunisia (6) and an ongoing study in Denmark (31), the number of IS6110 copies observed in the isolates investigated in the present study was rather evenly distributed within a range of from 1 to 20. In the study in Tunisia, about 75% of the investigated isolates of *M. tuberculosis* were found to carry from 6 to 10 copies of the IS6110 element. In the study in Denmark, about half of the isolates analyzed carried from 11 to 15 copies of the insertion element. The proportion of isolates with a low copy number or only one copy of the IS6110 element was much larger in the present study (26.1%) than in any of the two studies mentioned above (8.6% in the Danish study and 5.5% in the Tunisian study). Despite the observation of a large number of unique patterns, more than 40% of the patterns could be assigned to three big clusters, each of which revealed a high degree of genetic similarity among the isolates. This observation is in accordance with the fact that TB has haunted this part of the world for generations. Although the stability of the IS6110 element is high, it is by nature transposable, and the highly related but nonidentical patterns reflect the result of evolution through generations of TB infection in this region. Although the diversity of the patterns, as mentioned above, was great, the relatedness of the DNA fingerprint patterns tended to be in accordance with the geographical origins of the isolates, as also reported previously by others (14, 30).

By comparing the RFLP patterns of HIV-related *M. tuberculosis* isolates with the RFLP patterns of non-HIV-related isolates, we found that the DNA fingerprints were equally polymorphic in the two groups. The patterns shared by two or more isolates were, in most cases, observed in both HIV-related TB and non-HIV-related TB groups.

It might be expected that HIV-related TB could be associated with special clones of mycobacteria with low levels of virulence which would be able to infect the immunocompromised host. However, from the present study we found no indication that any particular clones of *M. tuberculosis* have played any dominant role in the HIV-related TB patients. This was also reflected in the observation that infection with strains of *M. tuberculosis* of the Asian subgroup, which mainly contained a low number of IS6110 copies, was not associated with the HIV status of the patient. In industrialized countries HIV-associated TB is characterized by a higher incidence of extrapulmonary TB, and therefore probably has fewer infectious manifestations. However, in a country with a high level of TB transmission like Tanzania, more than 50% of the HIV-positive patients have pulmonary TB, as in the present study, in which all of the HIV-positive patients had pulmonary, smear-positive TB.

Resistance to anti-TB drugs can develop during the initial infection, but it may also occur as a result of re-infection with drug-resistant strains of *M. tuberculosis*. Exogenous re-infection with multidrug-resistant *M. tuberculosis* may happen either during therapy of the original infection or after therapy has been completed (24). In the present study, drug resistance to one or more anti-TB drugs was found in 12% of the tested isolates. Since all of the isolates were primary isolates, the drug resistance found in the present study should be considered primary drug resistance. The drug susceptibility profiles obtained from the present study showed that there was no significant difference between the two groups of isolates being compared. The low rate of primary drug resistance observed in the present

![FIG. 3. Number of IS6110 copies per strain. Closed bars, strains isolated from HIV-seropositive patients; open bars, strains isolated from HIV-seronegative patients.](http://jcm.asm.org/1068.png)
study showed that, despite the substantial difficulties in Tanzania, as in all other developing countries, the national TB control program in Tanzania has proved to be quite efficient. In accordance with previous findings (6, 26), there was no correlation between DNA fingerprint patterns and drug resistance. However, phenotypic characteristics which classify M. tuberculosis strains as being of the Asian subgroup correlated with a low IS6110 copy number per isolate.

The relationship between the IS6110-associated RFLP and other phenotypic characteristics like the virulence of the bacteria, with all of the inherent problems of defining and assaying this trait, remains to be studied further. The data presented in this paper have been entered into the centralized, international database which is being established at the National Institute of Public Health and Environmental Protection (Rijksinstituut voor Volksgezondheid en Milieuhygiëne) in Bilthoven, The Netherlands, and may thereby contribute to future studies on the molecular epidemiology of TB.

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