Analysis of Clonal Composition of *Mycobacterium tuberculosis* Isolates in Primary Infections in Children

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The assumption that *Mycobacterium tuberculosis* infections should be considered clonally homogeneous has been weakened in the last few years. Recent studies have shown (i) the isolation of different *M. tuberculosis* strains from sequential episodes, (ii) mixed infections by two *M. tuberculosis* strains, and (iii) genetic variations in *M. tuberculosis* subpopulations due to microevolution events. Nevertheless, it is unknown whether clonal heterogeneity could be found in the initial steps of *M. tuberculosis* infection, i.e., the primary infection. In the present study we analyzed the clonal composition of the *M. tuberculosis* isolates causing primary infections in children. Cultures were clonally homogeneous in most cases (11 of 12). In 1 of the 12 cases (8.3%), clonal heterogeneity among the *M. tuberculosis* isolates was found by spoligotyping and IS6110-based restriction fragment length polymorphism analysis. This case occurred in a 2-year-old child in whom microevolution events were unlikely and who had no risk factors for overexposure to *M. tuberculosis*. Clonal heterogeneity should also be considered in primary *M. tuberculosis* infections, including circumstances in which it is usually unexpected.

In recent years, clonal heterogeneity in the *Mycobacterium tuberculosis* populations causing infections has been found more frequently than was initially expected. Different studies have shown that a relevant proportion of tuberculosis (TB) recurrences are caused by strains other than those involved in the first episode (1, 3, 4, 7, 9, 11, 13). Furthermore, mixed infections by more than one *M. tuberculosis* strain during the same episode have been reported (2, 4, 10, 14, 15), and we have found (8) that in some cases this polyclonality leads to compartmentalization of the infection, with different clones infecting respiratory and extrarespiratory sites. In addition, clonal heterogeneity has also been proposed to be the result of the acquisition of subtle genetic changes in an *M. tuberculosis* subpopulation due to microevolution events during the course of the infection (6).

In general, clonal heterogeneity in an *M. tuberculosis* isolate is assumed to be the result of a superinfection in a previous TB episode by a new *M. tuberculosis* strain. This would lead to the simultaneous or sequential presence of two different strains in the same patient. In some cases, a simultaneous coinfection with more than one strain has been proved, but only in high-risk circumstances (2, 4). As far as the microevolution events causing clonal heterogeneity in the *M. tuberculosis* isolates are concerned, it has been proposed that a minimum time of progression of the infection is required to allow mutations and rearrangements to occur (5).

Therefore, the circumstances in which clonal heterogeneity has been explored in TB are mainly restricted to (i) recurrences, (ii) superinfections of uncured cases, and (iii) cases in high-incidence settings. Our study aimed to analyze the clonal composition of *M. tuberculosis* isolates in a circumstance, primarily infection, that has received little attention from a molecular point of view.


**MATERIALS AND METHODS**

**Patients.** Over a 2-year period, 12 children with TB (Table 1) were selected as candidates for primary *M. tuberculosis* infection. Cultures of respiratory samples taken from all patients close to the time of appearance of clinical symptoms were positive for *M. tuberculosis*.

**Microbiological methods.** Clinical specimens were processed by standard methods and inoculated on Lowenstein-Jensen slants and in mycobacterial growth indicator tube (MGIT; Becton Dickinson, Sparks, Md.) liquid medium. Testing for susceptibility to isoniazid, rifampin, streptomycin, and ethambutol was performed for all the strains with the MB/BacT system (Organon Teknika, Durham, N.C.).

The MTB cultures from the clinical samples were totally harvested from the solid media (in order to guarantee that the different *M. tuberculosis* clones potentially present in the culture were selected for analysis) and were stored frozen at −70°C until analysis.

**Molecular typing.** To analyze the clonal composition of the *M. tuberculosis* isolates, molecular typing was performed with a random selection of single colonies from the *M. tuberculosis* cultures. These single colonies were obtained by plating the positive cultures onto Middlebrook 7H11 agar plates to obtain single colonies. Thirty independent colonies from each plate were picked and subcultured in MGIT medium for molecular analysis. All the cultures that grew from these single colonies were fingerprinted by spoligotyping. When different spoligotypes were observed for colonies from the same clinical culture, spoligotyping was repeated to confirm the results. Molecular typing by IS6110-based restriction fragment length polymorphism analysis (RFLP) was also performed as a second-line assay to confirm polyclonality.

Laboratory cross-contamination was ruled out as a cause of misassignment of the typing patterns by checking that the typing patterns were not shared by any other sample handled on the same day.

**Spoligotyping.** Spoligotyping, which explores polymorphisms in the direct repeat (DR) locus of *M. tuberculosis*, was performed as follows. For chromosome extractions, 1 ml of the bacteria cultured in MGIT (BACTEC; Becton Dickinson) was centrifuged. The cells were resuspended in a dilution of lysis reagent

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from the Accuprobe kit (Gene Probe) and boiled for 5 min. Glass beads (Sigma) were added, and the suspension was sonicated for 5 min. The PCR for amplification of the DR region was performed with the primers in the spoligotyping kit (Isogen, Maarsen, The Netherlands) and according to the instructions of the manufacturer. All spoligotypes were confirmed by repeating the assay. When a spoligotype showed ambiguities in any of the boxes, the assay was repeated until definite hybridization signals were obtained. Spoligotypes were considered the same when all spacers showed identical hybridization patterns between strains. IS6110-based RFLP typing. RFLP was used as a confirmatory typing method when different spoligotyping patterns were obtained. The procedure was performed according to international standardization guidelines (12). In the RFLP assay, apart from the standard assay in which the DNA was digested with PvuII, an independent assay in which the DNA was digested with Smal was also performed to confirm clonal heterogeneity. A marker strain (M. tuberculosis H37Rv clonal heterogeneity. A marker strain (M. tuberculosis H37Rv) was included as a reference, and the molecular sizes of the bands were calculated with the ChemiDoc system (Bio-Rad, Hercules, Calif.) and Diversity 14323) was included as a reference, and the molecular sizes of the bands were calculated with the ChemiDoc system (Bio-Rad, Hercules, Calif.) and Diversity database software (Bio-Rad).

RESULTS

The M. tuberculosis isolates from the respiratory samples of 12 children were available for study (Table 1). Spoligotyping was performed with 360 independent colonies (30 colonies per patient).

For 11 patients, all 30 colonies analyzed shared the same spoligotype (Fig. 1), indicating that a single M. tuberculosis clone was causing the primary infection. For the remaining patient (patient 8), two different spoligotypes (genotypes I and J) were obtained (proportion, 6:24) (Fig. 1). Molecular typing by IS6110-based RFLP analysis was also performed with all single colonies corresponding to spoligotypes I and J (Fig. 2a, lanes 1 and 2), and the differences between the genetic profiles detected by spoligotyping were confirmed, with RFLP types differing at three of nine bands (Fig. 2b, lanes 1 and 2). The sizes of the bands which differed between the patterns were 4.94, 1.83, and 1.07 kb, thus ruling out the possibility that the two small bands were derived from the long band by loss of a restriction site. RFLP analysis was also performed directly with a sample from the culture of a respiratory sample from this patient, and the typing pattern corresponded to the addition of the two profiles observed in the assay with single colonies (Fig. 2a, lane 3). Lastly, RFLP analysis was performed after digestion of the DNA with a different enzyme (Smal) (Fig. 2b, lanes 1 and 2), and clonal differences were again observed. These data together confirm the presence of clonal heterogeneity during the primary infection.

The child infected with isolates with clonal heterogeneity during primary infection (patient 8) was a 2-year-old girl with tuberculous meningitis (Table 1) and no risk factors for TB. She was human immunodeficiency virus (HIV) negative, and high-risk, socioepidemiological circumstances to justify exposure to TB were not present. The epidemiological research covered her household and school background and did not find an index case.

TABLE 1. Clinical and microbiological features of the study patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Type of TB at diagnosis</th>
<th>HIV infection status</th>
<th>Sample tested</th>
<th>Predisposing risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16 yr/M</td>
<td>Lymphatic</td>
<td>Unknown</td>
<td>Sputum</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>13 yr/F</td>
<td>Pulmonary</td>
<td>Unknown</td>
<td>Sputum</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>10 mo/M</td>
<td>Pulmonary</td>
<td>–</td>
<td>Gastric aspirate</td>
<td>Parent(s) IVDU</td>
</tr>
<tr>
<td>4</td>
<td>13 mo/M</td>
<td>Pulmonary</td>
<td>–</td>
<td>Gastric aspirate</td>
<td>Parent(s) IVDU</td>
</tr>
<tr>
<td>5</td>
<td>3 yr/M</td>
<td>Meningitis</td>
<td>–</td>
<td>Sputum</td>
<td>Unknown</td>
</tr>
<tr>
<td>6</td>
<td>13 yr/F</td>
<td>Pulmonary</td>
<td>–</td>
<td>Gastric aspirate</td>
<td>Unknown</td>
</tr>
<tr>
<td>7</td>
<td>15 yr/F</td>
<td>Pulmonary</td>
<td>Unknown</td>
<td>Bronchial aspirate</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>2 yr/F</td>
<td>Meningitis</td>
<td>–</td>
<td>Gastric aspirate</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>5 mo/F</td>
<td>Meningitis</td>
<td>+</td>
<td>Gastric aspirate</td>
<td>HIV infection</td>
</tr>
<tr>
<td>10</td>
<td>14 yr/M</td>
<td>Pulmonary</td>
<td>–</td>
<td>Sputum</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>4 yr/M</td>
<td>Pulmonary</td>
<td>–</td>
<td>Gastric aspirate</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>7 mo/M</td>
<td>Lymphatic</td>
<td>–</td>
<td>Gastric aspirate</td>
<td>Parent(s) IVDU</td>
</tr>
</tbody>
</table>

*None of the isolates was drug resistant.

a M. male; f, female.

IVDU, intravenous drug user.

DISCUSSION

During recent years, different studies have led us to reconsider the assumption that infection by M. tuberculosis is clonally homogeneous, and we are beginning to understand that the clonal composition of M. tuberculosis infection is much more complex than was initially thought.

It has been proved that recurrences are caused, at a greater rate than expected, by strains other than those involved in the first episode. This has been demonstrated not only in high-prevalence settings (13) but also in contexts with moderate incidences of the disease (1, 3, 7). In addition to sequential episodes of TB, the simultaneous isolation of different M. tuberculosis strains from single episodes has been explored (4, 10, 14, 15), and we have found that polyclonality could lead to compartmentalization of the infection, with one of the strains being selected to disseminate, whereas the other remains at the respiratory level (8).

In general, infections in patients infected with M. tuberculosis isolates with clonal heterogeneity are considered to correspond to polyclonality caused by a superinfection of an uncured previous episode. Clonal heterogeneity due to polyclonality has also been thought to result from simultaneous coinfection by multiple strains (2, 4, 9), and other investigators consider clonal heterogeneity in M. tuberculosis isolates to be due to microevolution events during the course of the infection.

Recently, an ambitious study performed in The Netherlands (6) examined the presence of clonal heterogeneity in 1,277 cases of TB by analyzing the presence of low-intensity bands
(LIBs) by RFLP analysis. The investigators ruled out the presence of multiple infections and assumed that the proportion of cases in which LIBs are detected is a consequence of genetic variations caused by microevolution events, probably as a result of adaptation of the bacterial population to latency or reactivation status. This hypothesis is consistent with the observation that LIBs are more frequently observed in older patients (6).

Our study analyzes the clonal composition of M. tuberculosis isolates in a context in which clonal heterogeneity is usually unexpected: primary infections in a general setting. The main limitation of studying primary infection is that it is difficult to be certain that in adults, cases are not reactivations of latent infections. Therefore, we selected children (median age, 3.5 years) with TB as candidates for primary infection because in this group the possibility of reactivation of latent infections is highly minimized. Unfortunately, it is not easy to compile cases of TB in children with an M. tuberculosis-positive culture, a requirement for isolate typing, and we were able to study only 12 patients over a 2-year period.

Our data indicate that the clinical isolates from most patients analyzed showed clonal homogeneity. Nevertheless, in 1 of our 12 patients (8.3%) we detected the coexistence of two different typing patterns when independent colonies from the clinical culture were analyzed. The potential role of laboratory cross-contamination in misassignment of a typing pattern was ruled out after analysis of all the typing profiles for the strains handled in the laboratory at that time and by checking that they were not shared by the analysis group.

As explained above, the clonal heterogeneity of strains in patients with TB can be due to (i) genetic variations in the bacterial population due to intrapatient microevolution or (ii) sequential or simultaneous dual infections by two different strains.

Microevolution events were unlikely in our study, when it is considered that the course of the infection in our patients was not long enough to allow microevolution to occur. In children, the time between exposure and the appearance of clinical symptoms is very short. In addition, it has been reported that the rate of change in RFLP patterns as a result of microevolution is 3.2 years (5). These aspects, together with the age of our patient (2 years), minimize the possibility that the bacterial population could have acquired genetic variations either randomly or as a result of adaptations to dormancy and reactivation states. Finally, in our patient whose isolates showed clonal heterogeneity, the RFLP patterns between the isolates differed by three bands (one large band disappeared and two new smaller bands appeared), and it can be considered that these differences were due to the loss of a restriction site. Nevertheless, the sum of the sizes of the two smaller bands is not
equivalent to the size of the large band. This is not consistent with the hypothesis that subtle genetic changes due to microevolution are responsible for the clonal heterogeneity detected.

With regard to the alternative explanation for clonal heterogeneity, i.e., dual infection by two different strains, the profile of the case did not fit with a risk for overexposure. The patient in whom clonal heterogeneity was detected during primary infection did not have risk factors for TB, was HIV negative, and did not live under high-risk socioepidemiological circumstances associated with overexposure. On the contrary, in our study other patients who had risk factors for TB or who came from environments with conflicting high-risk socioepidemiological circumstances for TB (Table 1) were infected with clonally homogeneous populations. Therefore, in the analysis group, there did not seem to be a direct correlation between clonal heterogeneity and the socioepidemiological circumstances usually associated with overexposure. The possibility that the patient in whom clonal heterogeneity was detected was infected by two variants of a single parental clone which had microevolved in the source patient should also be considered.

It is difficult to understand the real cause of clonal heterogeneity in the primary infection found in our study, and the limitations of our sample size make it difficult to draw definitive conclusions. Additional studies with patients with primary infections are therefore needed to further evaluate clonality. Nevertheless, we believe that the relevant issue is that clonal heterogeneity can also be detected in circumstances in which neither microevolution nor overexposure is expected.

Our data suggest that the factors involved in the clonal composition of TB isolates are much more complex than generally expected. Our study extends the contexts in which clonal heterogeneity in TB can be found and looks closely at cases in which it is usually disregarded.

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