Characterization of Clonal Complexity in Tuberculosis by Mycobacterial Interspersed Repetitive Unit–Variable-Number Tandem Repeat Typing

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In recent years, the application of molecular tools has shown us that clonal complexity in infection by Mycobacterium tuberculosis is not anecdotal. Exogenous reinfections, mixed infections, compartmentalization, and microevolution are different aspects of this issue. The detection and characterization of clonal variants of M. tuberculosis by standard genotyping methods is laborious and frequently requires expertise. Our aim was to evaluate a new genotyping PCR-based method for M. tuberculosis, mycobacterial interspersed repetitive unit–variable-number tandem repeat typing (MIRU-VNTR), as a potential tool to simplify and optimize the clonal analysis of tuberculosis. MIRU-VNTR was able to detect mixed clonal variants in vitro, even for clones at low ratios (1:99). This technique was prospectively applied to search for cases infected by more than one clone. Clonal variants within the same host were detected in 3 out of 115 cases (2.6%), including cases with clones which were indistinguishable by restriction fragment length polymorphism or spoligotyping. In one case, coinfected clonal variants differed in antibiotic susceptibilities. MIRU-VNTR was applied to cases with proven polyclonal infection, and it succeeded in detecting the coinfesting strains and proved useful in confirming cases of compartmentalized infection. MIRU-VNTR is a simple, rapid, and sensitive method which could facilitate and optimize the identification and characterization of clonal complexity in M. tuberculosis infection.

MATERIALS AND METHODS

Clinical isolates. Clinical specimens were processed according to standard methods and inoculated in Lowenstein-Jensen slants. Susceptibility testing with isoniazid, rifampin, streptomycin, and ethambutol was performed for all the strains using the mycobacterial growth indicator tube–streptomycin-isoniazid-rifampin-ethambutol system (Becton Dickinson). A total of 115 M. tuberculosis cultures were available for study. These cultures corresponded to those received in our laboratory between January 2004 and June 2004 for genotyping purposes.

M. tuberculosis strains from patients with polyclonal infection corresponded to those reported elsewhere (9).

Genotyping procedure. MIRU-VNTR is a PCR-based typing method which assigns the number of tandem repeats in 12 independent loci (MIRUs).

MIRU-VNTR was performed directly from the primary culture. DNA was extracted as described previously (8) and used as a template for primers published elsewhere (16). Amplified products were run in MS-8, with 2% agarose (Promadisa, Madrid, Spain).

RFLP. The procedure followed international standardization guidelines (18). For restriction fragment length polymorphism (RFLP) analysis, single colonies were obtained after subculturing the primary culture on 7H11 agar plates. Single colonies were picked and inoculated in mycobacterial growth indicator tube liquid media. After three weeks, DNA was purified and RFLP analysis was performed.

Spoligotyping. Spoligotyping was performed using primers of a spoligotyping kit (Isogen, The Netherlands) and following the manufacturer’s instructions. Spoligotyping was performed on single colonies as has been explained for RFLP.

RESULTS

In vitro evaluation of VNTR-MIRU. As an initial approach to evaluating VNTR-MIRU in the context of clonally complex
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*M. tuberculosis* infections, we tested its efficiency at detecting a mixture of two different, arbitrarily selected strains which had different MIRU types (strain 1, 223222153323, and strain 2, 124326153220; MIRU loci in which the strains differed are in bold type). We amplified the loci showing allelic differences by using a set of mixtures with different ratios (from 1:99 to 50:50) of purified DNA from strains 1 and 2 as a template, and the alleles of both DNAs were detected, even in conditions in which one of them was clearly underrepresented (1% of the total). Equivalent results were obtained when directly mixing the two strains in different proportions before extracting the DNA; VNTR-MIRU simultaneously detected the alleles of each strain, even in the proportions in which one strain was clearly underrepresented in the sample (1%).

**Clinical evaluation of MIRU-VNTR.** (i) MIRU-VNTR as a tool for identifying cases with clonally complex infection. MIRU-VNTR was applied to prospectively identify candidates with clonally complex *M. tuberculosis* infections among 115 respiratory specimens from independent cases. In three cases (2.6%), two or more amplification products (i.e., the presence of different alleles) were obtained for certain loci after genotyping directly from the *M. tuberculosis* culture (loci 4, 20, and 31) (Fig. 1), which suggested infection by two genetically different *M. tuberculosis* strains. The presence of more than one clone in these cultures was confirmed by genotyping 50 single colonies and observing the different alleles (Fig. 1).

(a) Case A. Two clones with different alleles in MIRU locus 20 were identified (A1, 214325143322, and A2, 214351543322; MIRU loci in which the strains differed are in bold type) (Fig. 1a). To measure the relative proportions of these clones, 50 single colonies from the culture were MIRU typed for locus 20 and the distribution of clones was 65% A1 and 35% A2. The two clones with different MIRU types were indistinguishable by spoligotyping and RFLP (Fig. 1a). The susceptibilities of the two clones differed; both were resistant to isoniazid and rifampin, and A2 was also resistant to streptomycin.

(b) Case B. Two clones with different alleles in MIRU locus 4 were detected (B1, 2453251534323, and B2, 225325154323; MIRU loci in which the strains differed are in bold type). The proportions of the two clones were 50% B1 and 50% B2. The two clones with different MIRU types were indistinguishable by spoligotyping but showed subtle differences in their RFLP types (Fig. 1b).

(c) Case C. Two clones with different alleles in MIRU locus 31 were detected (C1, 224226153314, and C2, 224226153321; MIRU loci in which the strains differed are in bold type). The proportions of the two clones were 88% C1 and 12% C2. When assaying single colonies representing each of the MIRU types, we observed that most of the C1 and C2 clones were indistinguishable by spoligotyping but for a low proportion of C2 clones (2%), where a spoligotype differing in one spacer was obtained (Fig. 1c). RFLP also showed subtle differences between representatives of C1 and C2 MIRU types (Fig. 1c). It is worth mentioning that, in another specimen obtained the same day from the same patient, it was possible to identify an additional clone (C3, 224226153414) together with the C1 and C2 clones. In this last specimen, the relative proportions of the C1 and C2 clones were different than that found in the previous sample (C1, 34%; C2, 48%; and C3, 18%). C3 was undistinguishable by spoligotyping but showed subtle differences in RFLP (Fig. 1c).

(ii) MIRU-VNTR as a tool to confirm polyclonal cases. We had previously identified cases of polyclonal tuberculosis (9) with infection by more than one *M. tuberculosis* strain (with clear differences in RFLP type and spoligotype). In the present study, we applied MIRU-VNTR to evaluate whether these cases, which had been detected by RFLP and spoligotyping, would have also been detected if MIRU-VNTR had been applied. In the three cases (D, E, F), two different clones with differences at several MIRU loci were obtained (D1, 224326153324, and D2, 22422516332; E1, 126325163323, and E2, 223425153322; and F1, 323125153325, and F2, 224326153321; MIRU loci in which the strains differed are in bold type).

(iii) MIRU-VNTR as a tool to confirm compartmentalization. We had also previously reported cases of polyclonal infection where compartmentalization of the infection was observed, with one *M. tuberculosis* strain infecting the lungs and a different clone infecting the extrapulmonary sites of these patients (9). Considering the high sensitivity of MIRU-VNTR when detecting mixed populations, even in conditions in which one strain was underrepresented (1%), we applied this technique to test whether the compartmentalization in these patients was strict. MIRU was performed on the respiratory and extrapulmonary cultures, and no double bands were found for any of the MIRU loci in which the coinfecting clones had different alleles. These data confirmed clonal homogeneity at each infected site (a single strain at respiratory and extrapulmonary sites) and ruled out the existence of mixed infections in each compartment.

**DISCUSSION**

Analysis of *M. tuberculosis* infection using molecular tools has shown us that tuberculosis is more complex than expected. Exogenous reinfection (2, 4, 5, 7, 19) and mixed infections with more than one strain (3, 5, 8, 13, 20) have been reported in different circumstances, and in some cases the coinfecting strains are not equally distributed in the different infected tissues or sites, leading to a compartmentalization of the infection (3, 9, 12). In general, these clonally complex situations have been explored by the technique most frequently applied to genotyping *M. tuberculosis*, IS6110 RFLP (18). The existence of more than one clone is usually suggested by the detection of low-intensity bands (6). These bands require one of the two coinfecting clones to be underrepresented in the mixture, which does not always occur. High sensitivity and expertise are required to detect and interpret low-intensity bands. Spoligotyping, another widely used genotyping tool based on hybridization with a set of spacers of the direct repeat region (11), cannot detect polyclonality because clones harboring spacers mask other clones which lack spacers. A more certain approach to identifying clonal complexity involves analyzing multiple independent colonies to detect the presence of different clones in the culture, which is very laborious.

In recent years, a new PCR-based genotyping technique for *M. tuberculosis*, MIRU-VNTR, has been developed (16, 17). Our objective was to evaluate its usefulness in searching for and characterizing clonal complexity in tuberculosis. In
FIG. 1. Clinical evaluation of MIRU to detect cases with clonally complex infections. For each case (a, b, or c), the figure shows the following: top panel, amplicons for the 12 MIRU loci and the independent alleles for the loci showing clonal heterogeneity, which were amplified from single colonies; middle panel, MIRU types (with the loci showing different alleles within a box) and spoligotypes for the clones; bottom panel, RFLP types for the clones (asterisks indicate different bands among the clones).
MIRU-VNTR, a single allele is obtained for each locus and it is observed as a single-band PCR pattern for each locus. It is this characteristic of MIRU, i.e., leading to single-band patterns, which gives it the potential to simplify the detection of polyclonality. If two different *M. tuberculosis* strains are present, two different alleles should be observed directly from the *M. tuberculosis* culture, without the need to analyze several colonies.

Recently, MIRU-VNTR has been presented as a useful tool in different clinical mycobacteriology issues, including the detection of mixed infection and genotypic heterogeneity (1, 15). Only one of the studies has applied MIRU-VNTR directly to *M. tuberculosis* culture as a tool to detect clonal complexity, and, unfortunately, only one case of mixed infection was presented. The other report applied MIRU on only single colonies to confirm clonal heterogeneity which was suggested by RFLP or spoligotyping. Our study tried to fill the lack of data about the efficiency of MIRU as a first-line tool to detect clonal complexity directly from *M. tuberculosis* cultures.

The sensitivity of MIRU in detecting clonal variants has not been evaluated. Our data indicate that MIRU is able to detect mixed clonal variants, even in extreme situations where one of the variants is underrepresented in the bacterial population (1%).

MIRU-VNTR also showed high sensitivity in detecting clonal variants which differed only slightly from their accompanying strains. Another study (15) showed that MIRU did not manage to detect genotypic heterogeneity in eight cases with clones showing subtle differences in RFLP types and spoligotypes and succeeded in detecting differences in only two cases with marked genotypic differences. However, our study showed that, in the three cases where MIRU detected clonal heterogeneity, the coinfecting variants either shared identical RFLP types and spoligotypes or had highly similar patterns. Our data mean that MIRU typing would guarantee improved identification of cases infected by clonal variants or different strains that would probably go undetected by the standard typing methods/approaches.

The proportion of cases in which MIRU-VNTR detected clonal heterogeneity was not anecdotal (around 3%). Recently, Glynn et al. (10) analyzed the appearance of clonal variants related to treatment, recurrences, or transmission. In our study, only one case had a previously treated tuberculosis episode and, for the remaining cases, the appearance of genetic variants was not associated with long histories of tuberculosis or selective pressure by antibiotics. Therefore, as elsewhere (8), we now report the presence of clonal variants on the diagnosis of first episodes, which suggests that the circum-

![FIG. 1—Continued.](image_url)
stances in which these variants can be detected should be broadened.

The presence of subtle clonal variants should not be considered to be caused by random variations due to instability of MIRUs, as it has been stated that these markers have higher stabilities than that of RFLP (14). Neither should we consider these findings microbiological rarities, as they depend on highly refined microbiological tools and have no clinical significance. In one of the cases, it is worth noting that, in spite of the high genetic similarity between the infecting clones, phenotypic differences were observed and the variants differed in their susceptibilities to streptomycin. This finding is remarkable from a therapeutic point of view because both strains were multidrug resistant.

It is also interesting to observe the peculiarities of case C, in which two clones were identified in one specimen and an additional third clone was identified in another specimen that was obtained the same day. Also, the relative proportions of each of the variants were different in each specimen. This variable representativeness of clones in independent specimens has also been found by other authors (3), and it has been proposed that different clones could inhabit different lung sites and, therefore, could be contributing separately to each sputum.

The high sensitivity of MIRU in detecting clonal variants was also applied to confirm cases of compartmentalized infection by *M. tuberculosis* strains. It could be argued that compartmentalization is not strict and that the different clones are also present at the different infected sites although underrepresented and, therefore, below the limits of detection. In order to try to answer to this question, we applied MIRU to three cases of compartmentalization and, in all three, we proved clonal homogeneity (a single strain) at each of the infected sites.

Our data illustrate that MIRU-VNTR is a rapid, simple, and sensitive method for exploring clonal complexity in tuberculosis. It can detect situations of unexpected clonal complexity not related to the circumstances in which clonal complexity has been described. It may help us to understand the real proportions of clonal heterogeneity and polyclonality in tuberculosis, and it would provide an in-depth analysis of the microbiological and clinical implications of this phenomenon.

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