Mycobacterial Interspersed Repetitive Unit Typing of *Mycobacterium tuberculosis* Compared to IS6110-Based Restriction Fragment Length Polymorphism Analysis for Investigation of Apparently Clustered Cases of Tuberculosis

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An evaluation of the utility of IS6110-based restriction fragment length polymorphism (RFLP) typing compared to a combination of variable number tandem repeat (VNTR) typing and mycobacterial interspersed repetitive unit (MIRU) typing was undertaken. A total of 53 patient isolates of *Mycobacterium tuberculosis* from four presumed episodes of cross-infection were examined. Genomic DNA was extracted from the isolates by a cetyl trimethylammonium bromide method. The number of copies of tandem repeats of the five loci ETR_A to ETR_E and 12 MIRU loci was determined by PCR amplification and agarose gel electrophoresis of the amplicons. VNTR typing identified the major clusters of strains in the three investigations in which they occurred (each representing a different evolutionary clade: 32333, 42235, and 32433). The majority of unrelated isolates (by epidemiology and RFLP typing) were also identified by VNTR typing. The concordance between the RFLP and MIRU typing was complete, with the exception of two isolates with RFLP patterns that differed by one band each from the rest of the major epidemiologically linked groups of isolates in investigation A. All of these isolates had identical MIRU and VNTR types. A further pair of isolates differed in the number of tandem repeat copies at two MIRU alleles but had identical RFLP patterns. The speed of the combined VNTR and MIRU typing approach enabled results for some of the investigations to be supplied in “real time,” influencing choices in contact tracing. The ease of comparison of results of MIRU and VNTR typing, which are recorded as single multidigit numbers, was also found to greatly facilitate investigation management and the communication of results to health care professionals.

It is widely accepted that tuberculosis is one of the most important threats to human health on a global scale. Infection with *Mycobacterium tuberculosis* complex causes the greatest number of deaths by a single infectious agent, and it has been estimated that approximately one-third of the world population has latent infections (9). While conventional contact tracing and treatment of contacts remain important components of control, the ability to ascribe a subtype to infecting strains is a powerful adjunct in recognizing unsuspected sources and chains of transmission. There is therefore an undoubted need for a reproducible, discriminatory, epidemiologically relevant, easy, and portable typing method. The relative merits and drawbacks of the range of molecular typing methods have recently been reviewed (6, 32), and the problems with the most established typing method, IS6110-based restriction fragment length polymorphism (RFLP), have been discussed. Although this method has been widely used and is referred to as a “gold standard” (18), it is labor-intensive, requires culture, and cannot type strains with low copy numbers (≥ 5) of IS6110. Possibly the biggest drawback to the method is that the data are

generated as an analogue band pattern. This presents difficulties for reproducible analysis of bands and exchange of data between different centers. In order to try to circumvent this drawback, the IS6110 RFLP method has been carefully standardized (31). The problems of sizing DNA fragments with potentially infinitely variable sizes still remain. Deciding on confidence intervals for size calling to distinguish truly different sized fragments is always, therefore, a compromise. Additionally, recording of the fragment pattern is cumbersome and poorly transportable between centers. IS6110 RFLP has been applied in many settings such as regional epidemiological studies (3, 19), point source outbreaks related to recreational activities in the community (35), nosocomial transmission (7), and prison outbreaks (5), among others. The method has also been used to identify and track drug-resistant strains such as “strain W” (2) and to uncover laboratory cross-contamination, often occurring inadvertently as part of a wider epidemiological study (25). In consequence, there is a large body of information showing the close correlation of IS6110 RFLP typing and the transmission of *M. tuberculosis* (6). The method, therefore, is the most useful comparator method for any newer typing methods.

Some solutions for the problems of IS6110-RFLP have focused on PCR-based methodology for characterizing IS6110

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polymorphism, such as mixed linker typing (4). Although this method is attractive, reproducibility is sometimes a problem, and the fundamental problems outlined above are not addressed. Multilocus sequence typing has been used to produce a discriminatory, digital, PCR-based typing method allowing much typing information to be gathered about a range of bacteria, notably methicillin-resistant *Staphylococcus aureus* and *Neisseria meningitidis* (20). Unfortunately, polymorphism in the genome sequence of *M. tuberculosis* is limited, so the method cannot be applied (27). Spoligotyping analyzes the direct repeat (DR) locus in the genome of *M. tuberculosis*, which is composed of a cluster of 36-bp repeat sequences interspersed with unique spacers of 35 to 41 bp. By using a reverse Southern blotting technique, the variability in the spacer sequences can be interrogated and recorded in a digital code (17). Although this method provides digital typing data, it is only measuring variability in a single locus and does not generally provide sufficient discrimination for outbreak investigation (18). A recent study has suggested that there is sufficient variation in sporadic isolates to distinguish relapse from reinfection (33).

The use of polymorphism in the number of copies of tandem repeat sequences is an invaluable tool for the genotyping of higher eukaryotes. Short repeated sequences (1 to 5 bp) are referred to as microsatellites, and longer repeated sequences (5 to 100 bp) are referred to as minisatellites. Although these sequences have a limited distribution in bacteria (30), approximately 40 minisatellite-like structures have been identified in *M. tuberculosis* (28). A limited number of these structures (five in total) were described by Frothingham and Meeker-O'Connell in 1998, referred to as variable number tandem repeats (VNTR), and used to type *M. tuberculosis* and, notably, *Mycobacterium bovis* BCG (11). However, the discrimination of clones was inadequate for the full description of all episodes of cross-infection (35). The discriminatory power of tandem repeat typing can be substantially increased by examining a larger number of loci. Twelve such loci, mycobacterial interspersed repetitive units (MIRU), have been suggested. This technique has the advantage that type identification is expressed by a 12-digit numerical code (almost all MIRU loci have up to 9 repeats) and can be fully automated (29). The technique has recently been applied to a collection of 72 isolates of *M. tuberculosis* from transmission, relapses, and laboratory cross-contamination events in France (22). This study showed perfect clustering for epidemiologically related isolates (as defined by a range of other typing techniques including IS6110-based RFLP), suggesting that this method may have considerable promise and potential for creating large-scale databases. To date, MIRU typing has been shown to be useful for global and molecular epidemiological studies (21, 29). It has not yet been applied to the direct field investigation of outbreaks in real time, which would enable sampling and epidemiological investigative decisions to be influenced as isolates became available from cultures. We have applied MIRU, with the inclusion of ETR_A to ETR_E types to further expand discrimination, to isolates from four geographically different, epidemiologically related outbreaks of *M. tuberculosis* cross-infection defined by using IS6110-based RFLP fingerprinting in order to test the field applicability of a combination of MIRU and VNTR tandem repeat typing.

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA. Single *M. tuberculosis* isolates were selected from each patient. A total of 53 isolates from the four separate investigations were typed. Identification to species level was confirmed using the AccuProbe *M. tuberculosis* complex culture identification test (BioMérieux s.a., Marcy L'Etoile, France) (23). DNA hybridization assay strains recovered were maintained on Löwenstein-Jensen (LJ) slopes at 37°C for a minimum of 4 weeks and in mycobacterial growth indicator tubes (MGIT) (Becton Dickinson Biosciences, Oxford, United Kingdom) until a positive growth index was obtained.

DNA extraction. Mycobacterial cells from MGIT tubes indicating a positive growth index and from LJ slopes showing visible positive growth were extracted by using the cetyltrimethylammonium bromide method (31). When necessary, the DNA was stored at -70°C until required.

VNTR analysis. VNTR analysis was performed by using the oligonucleotides for the five loci ETR_A to ETR_E as originally described by Frothingham and Meeker-O'Connell (11). A total PCR volume of 25 µl was used for each reaction. This contained 400 nM each primer, 2.5 µl of PCR Gold Buffer (Applied Biosystems, Warrington, United Kingdom), 1.5 mM MgCl₂, 200 µM each of the four deoxynucleoside triphosphates, 4% (vol/vol) dimethyl sulfoxide, 0.5 U of AmpliTaq Gold (Applied Biosystems), and 2 ng of DNA. Amplification was performed in a Multi-Block System (MBS) Thermal Cycler (ThermoHybaid, Ashford, United Kingdom). An initial denaturation of 7 min at 95°C was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A final extension step of 5 min at 72°C concluded the reaction program. The amplicons were analyzed on a 2% (wt/vol) agarose-1000 gel (Invitrogen, Paisley, United Kingdom) for 2.5 h at 150 V with 50-bp ladder size standards.

MIRU analysis. MIRU analysis was performed using the primers for the 12 MIRU loci as originally described by Supply et al. (29). Four different concentrations of MgCl₂ were used. MgCl₂ was added to the reaction mixture at 1.5 mM for MIRU loci 20, 24, and 27. For MIRU loci 10, 16, and 31, 2 mM MgCl₂ was used. For MIRU loci 2, 23, and 39, 2.5 mM MgCl₂ was used. For MIRU loci 4, 26, and 40, 3 mM MgCl₂ was used. Each PCR mixture contained 4% (vol/vol) dimethyl sulfoxide, 200 µM each of the four deoxynucleoside triphosphates, 2.5 µl of PCR Gold buffer (Applied Biosystems), 400 nM each primer, 0.25 U of AmpliTaq Gold (Applied Biosystems), 2 ng of template DNA, and H₂O to a final volume of 25 µl. The reaction was performed on an MBS Thermal Cycler (ThermoHybaid) by using the following program: an initial denaturation for 7 min at 95°C and 40 cycles of denaturation for 7 min at 95°C, annealing at 59°C for 1 min, and extension for 1 min, 30 s, at 72°C. A finishing extension step of 10 min at 72°C terminated the program. Amplicons were analyzed as previously described for VNTR analysis.

IS6110 RFLP typing. Genomic DNA was digested with *Pvu*II restriction endonuclease and separated by agarose gel electrophoresis and Southern hybridization according to the International Standard Typing method for *M. tuberculosis* (31). Hybridization was performed overnight at 60°C. The banding patterns were detected using an Enhanced Chemiluminescence detection kit (Amersham Biosciences, Chalfont St. Giles, United Kingdom), and images were captured using the Image Master video documentation system (Amersham Biosciences).

Cluster analysis. IS6110 RFLP results were entered into BioNumerics (Applied Maths, St-Martin-Latem, Belgium), analyzed by using the Dice coefficient, and displayed via the unweighted pair group method using arithmetic averages (UPGMA). A cluster was defined as a series of isolates that had 100% IS6110 fingerprint identity. Band tolerance levels were set at 1%.

RESULTS

Molecular typing. The clustering of isolates by IS6110 RFLP is shown in Fig. 1. The VNTR and MIRU codes are shown adjacent to each isolate number. Inspection of the data set reveals a very strong correlation between RFLP type and both the VNTR and the MIRU code. The VNTR codes identified the major clusters in investigations B and C and identified almost all of the unrelated (by RFLP analysis) isolates. In investigation A, the predominant group of indistinguishable isolates (by RFLP analysis) belonged to VNTR type 42235, and another six unrelated isolates showed the same VNTR code. Isolates 09 and 92 were less than 100% related by IS6110-based RFLP (each had an additional band) but had identical MIRU codes. These patients were considered to be

FIG. 1. UPMGA dendrograms based on IS6110 RFLP patterns of isolates from four distinct geographical locations in the West Midlands area of the United Kingdom (investigations A to D). VNTR and MIRU typing results are shown for each isolate. Dots represent alleles with the same numbers of repeat units, indicating isolates with identical types.

Investigation C **Investigation D**

FIG. 1—Continued.

epidemiologically part of the main cluster. The same was true to a lesser extent of investigation D, in which isolates 17, 130, and 221, from family members, were all VNTR type 42235 but one (isolate 221) had a different subtype by *IS6110* RFLP. The other two patient isolates had indistinguishable RFLP patterns but differing MIRU codes (two different alleles). In investigations A and D, the patients were predominantly South Asian in ethnic origin and VNTR type 42235 was extremely common. VNTR type 42235 has recently been reported to be highly associated with that ethnic group, both in the United Kingdom

and in Pakistan (24). MIRU typing split VNTR type 42235 into subtypes which correlated strongly with *IS6110*-based RFLP and epidemiological findings.

Epidemiological data. Information from contact tracing and screening was collated by the Consultant in Communicable Disease Control (CCDC) at each of the four locations of the clusters. This information was then considered together with the results of molecular typing by the microbiologists in order to confirm or refute the clinical hypothesis of source and secondary cases, together with linkage.

Epidemiological backgrounds of clusters. (i) Investigation

A. A large point source outbreak of tuberculosis occurred in an area of high incidence following delayed diagnosis in the index case. Molecular typing allowed hypotheses about the source and extent of the outbreak to be examined, differentiating outbreak cases from background activity, and helped to direct measures to control the outbreak and prevent further spread. Molecular typing was applied to later isolates in the same population to validate the control measures.

(ii) Investigation B. Molecular typing was used to examine isolates from a community with a previously low incidence of tuberculosis where a sharp increase in the number of cases over the preceding 12 to 18 months had been noted. Typing was unable to distinguish between strains from two apparently distinct epidemiological clusters, prompting further investigation, which revealed unsuspected social links between the two clusters. The information was used to plan the extent of contact examination.

(iii) Investigation C. An increase in the number of strains of tuberculosis with resistance to isoniazid in patients over a period of 5 years from a conurbation was investigated by examination of strains by molecular typing. Preliminary epidemiological investigation demonstrated geographical clustering of some cases with indistinguishable strains, prompting a more detailed investigation.

(iv) Investigation D. Molecular typing was applied to a group of strains from an area of high incidence, from patients with some epidemiological links. Strain types in two members of one family cluster of three were indistinguishable by IS6110-based RFLP. Although the strains were isolated 3 years apart, these showed differences in two MIRU alleles, suggesting evolution. Unexpectedly, it was found that a household contact did not share the same type. Three other patients with epidemiological links were shown not to have types identical with each other or with other members of the investigation group.

DISCUSSION

MIRU accurately identified the clusters in investigations A, B, and C and confirmed the lack of a suspected cluster in the case of investigation D. In addition, five pairs of strains from patients were identified by IS6110-based RFLP, and four of these were confirmed by MIRU. The two strains of the fifth pair in investigation D were isolated 3 years apart and were differentiated by a single-copy-number change at two MIRU alleles, suggesting *in vivo* evolution. Our experience is in accord with the report of Supply and colleagues (29), who applied MIRU typing to a blinded set of 90 strains from 38 countries, demonstrating that it is 100% reproducible, sensitive, and specific for *M. tuberculosis*. Mazars and colleagues (21) also applied MIRU typing to a laboratory collection of 72 isolates, including some unrelated isolates and 10 isolates from four transmission events in Paris, France. MIRU identified the four clusters of two to three isolates which were also defined by IS6110-based RFLP. Our study has characterized 53 field isolates, and MIRU typing identified the 30 linked isolates of those groups of isolates involved in cross-infection among more than two people. Two of these isolates in one cluster had IS6110-based RFLP patterns differing by one band; otherwise, the concordance was 100%. In addition, MIRU identified four

of the five pairs of isolates identified by IS6110-based RFLP; the pair not identified by MIRU were isolates from a husband and wife and were separated by 3 years, suggesting a higher rate of evolution at loci 16 and 26 compared to that of IS6110-based RFLP patterns. Both these loci have been reported to exhibit the highest degree of diversity in strains (21). The stability of MIRU has recently been investigated with pairs of isolates from patients separated by as many as 6 years (22). Only in a single case were isolates found with identical IS6110 patterns and a single change in a MIRU locus. Our finding for a strain with a high copy number of IS6110 is in contrast to the observation that the "molecular clock" for MIRU runs at a lower rate than that for IS6110-based RFLP (29). It is possible that once greater experience with MIRU typing is acquired, the rate may be higher than first thought. In investigation A, IS6110 RFLP failed to identify a pair of epidemiologically related strains identified by MIRU; these strains also had a rare VNTR type, confirming their close relationship. In the case of *M. bovis*, recent work has defined an extremely powerful set of tandem repeats for typing of veterinary isolates (24). Although substantial experience needs to be obtained with the current set of alleles for typing *M. tuberculosis*, selection of a limited number of other alleles in apparently identical but unrelated or closely related strains may provide valuable insight into the evolutionary relationships of those strains, as well as further molecular epidemiological information.

Investigation A concerned a complex, large-scale outbreak centered around a single index case in a school. However, the high background level of tuberculosis in the community and in pupils in the school not directly related to the index case required the use of molecular typing in order to fully understand routes of transmission. Pairs of cases (e.g., 67–68, 08–51, and 78–263) which were not caused by the outbreak strain but were subsequently linked epidemiologically were identified. A number of subsequent cases, which could have represented failure of control measures, could be quickly shown (2 to 3 days) not to be due to the original outbreak strain. It is this ability to rapidly and conclusively exclude strains (and therefore cases) from large-scale outbreaks which can be most useful. Two isolates, 092 and 922, showed differences in the IS6110 pattern (single additional bands), in each case together with "slippage" during electrophoresis, but had identical MIRU codes. Epidemiologically, these isolates were indisputably part of the main outbreak, a case where the greater stability of MIRU enhanced the clarity of typing. We found, as others have, that relying on absolute identity of IS6110 RFLP patterns leads to erroneous exclusion of a significant proportion of isolates (36). MIRU typing in our hands resulted in the potential exclusion of only 2 of 56 isolates, a finding similar to a recent report (22). A large number of isolates in this investigation belonged to VNTR profile 42235, which is very common in the United Kingdom and Pakistan among people of South Asian origin (12). Most of the patients in this outbreak belonged to that ethnic group.

In the case of investigation B, MIRU typing linked four family members (isolates 134, 03, 32, and 79), as might be expected, but also linked these with other cases in a close geographical location which were indistinguishable by typing and led to the discovery of an unsuspected social link. Two other isolates, 121 and 239, which might have been linked were

shown to be clearly unrelated. The outbreak strain carried only 2 copies of IS6110, which can easily result in the false identification of clusters (14); however, distinct and unique MIRU codes were found. We have shown the value of 5-allele-based VNTR typing to subdivide such clusters; in principle, MIRU will be more discriminatory (1).

Investigation C was triggered by the recognition of an increase in the number of isoniazid-resistant strains and the concern that transmission of a resistant clone was occurring. MIRU typing confirmed this for 11 isolates which also had indistinguishable IS6110 RFLP patterns. These isolates belonged to the Haarlem clade, which is common in Caucasians in the United Kingdom, and only two patients had South Asian ethnicity as judged by family name. This was in contrast to the other six isolates, all of which were from patients with South Asian family names and were not of the Haarlem VNTR type. The investigation is ongoing, and previously unsuspected geographical conjunctions have been identified for some patients in the cluster.

Investigation D concerned a small group of strains that were thought to be all clustered, but IS6110 RFLP typing showed them to be all unrelated with the exception of isolates 17 and 130, which were from a husband and wife. The close family contact (isolate 221) had a different type, as did all the other contacts. MIRU typing showed differences at two loci (see the discussion above), although the isolates were separated by 3 years.

In all of the investigations we have applied molecular typing to isolates of *M. tuberculosis* from four geographically separated locations from which an increased incidence of isolation of *M. tuberculosis* was noted by epidemiologists; in one investigation (investigation C) an increase in isolates resistant to isoniazid was noted. A very close correlation was found between the results of IS6110-based RFLP typing and the MIRU code.

Although VNTR typing using ETR_A to ETR_E exhibits a relatively low level of discrimination, it does enable isolates to be grouped into phylogenetic groups (10, 26). We found this useful in first-line typing before proceeding to characterize the additional 10 loci to generate the MIRU code. In many cases the extra 3 ETR loci not used in the 12-locus MIRU type yielded confirmatory polymorphisms supporting associations for both the IS6110 RFLP and MIRU types. In the "real-time" laboratory situation, when typing from automated liquid culture systems which had given positive signals the previous day, the very rapid availability of the 5-digit type highlighted potential clusters and, more importantly, sometimes excluded strains possibly linked by epidemiology. We have previously found VNTR typing useful in identifying laboratory contamination (13).

This study demonstrates that the combination of VNTR and MIRU typing is as valuable as IS6110 RFLP in the investigation of apparent outbreaks of *M. tuberculosis*. The use of a double-typing strategy using a method of lower discriminatory power to confirm highly suspected clusters—or exclude unasociated isolates if a type completely different from that of the main cluster is assigned to that isolate—has been evaluated by others for population-screening studies (34). The greater technical reliability, ease of automation and storage, and analysis of typing data make this method, in our opinion, superior to

IS6110 RFLP. In The Netherlands all isolates have been typed using IS6110 RFLP and spoligotyping, and in the first 2 years a steep rise in the number of clustered cases was detected, which leveled off at a rate of approximately 50% (32). A similar genotyping network using seven sentinel sites in the United States in which genotyping of all new isolates of *M. tuberculosis* has been undertaken was recently described (8). The number of apparently clustered cases in such national programs may be influenced by a number of factors such as length of study, recent immigration, and discriminatory power of the typing method (16). In addition, sample size (not all true cases can ever be ascertained and typed) has a profound effect on clustering when mathematically modeled in stochastic simulation (15). With the recent introduction of continuously monitored liquid culture systems which have a higher detection rate than solid media, the proportion of culture-positive cases should be greater. The ease with which the VNTR and MIRU typing system can be automated and the facility of data storage and analysis make it attractive for outbreak investigation, as we have shown in this paper, and as a method for compiling a comprehensive national database.

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