Use of PCR-Based *Mycobacterium tuberculosis* Genotyping To Prioritize Tuberculosis Outbreak Control Activities

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Received 7 June 2007/Returned for modification 4 August 2007/Accepted 23 December 2007

Genotypic analysis of *Mycobacterium tuberculosis* isolates is increasingly applied in direct support of tuberculosis outbreak control activities. This is facilitated by PCR-based strain typing methods that enable the genotypic characterization of samples containing small numbers of *M. tuberculosis* cells. By using DNA extracted directly from primary diagnostic cultures, PCR-based methods were applied to a tuberculosis outbreak investigation and to surveillance in King County, Washington. In the outbreak investigation, five epidemiologically linked *M. tuberculosis* isolates had a unique pattern at mycobacterial interspersed repeating unit (MIRU) loci 10 and 23 when the pattern was compared to the patterns in a local MIRU locus database. In order to quickly identify new cases involving this strain (termed SBRI10), targeted genotyping at these two loci was performed with cultures from epidemiologically associated tuberculosis cases. Isolates with the characteristic genotypes at loci 10 and 23 were further analyzed by use of a 12-locus MIRU panel and by repetitive-unit-sequence-based PCR (rep-PCR). Between May 2004 and January 2005, 82 cases were screened, of which 14 were identified for further analysis and 13 were confirmed to be infected with SBRI10. Between September 2005 and August 2006, surveillance universal genotyping was performed by using the 12-locus MIRU panel with DNA from primary diagnostic enrichment cultures. A total of 161 samples were submitted for analysis, and 156 were successfully typed. Fifty-one cases formed 18 presumptive clusters by MIRU locus typing. Of these, 30 cases were confirmed to be members of 11 clusters by rep-PCR. Presumptive genotypic data were available rapidly, sometimes within 2 weeks of diagnosis. In this fashion, PCR-based genotyping provided data that can be used to prioritize disease control activities.

Tuberculosis infects one-third of the world’s population (32). Although resource-poor countries carry the greatest burden of the disease, outbreaks occur in low-incidence countries as well, especially among homeless persons and immigrants. Genotypic fingerprinting techniques have been used to study the phylogeny and epidemiology of tuberculosis both in areas of high endemicity and in areas of low endemicity.

Several investigators have carried *Mycobacterium tuberculosis* strain fingerprinting into public health practice as well. Freeman et al. (8), Rajakumar et al. (24), Mardassi et al. (17), and Schmid et al. (25) reported the use of molecular fingerprinting techniques in the control of tuberculosis outbreaks. Schmid and colleagues (25) employed the “gold standard” fingerprinting technique for tuberculosis, IS6110-based restriction fragment length polymorphism (RFLP) analysis. This method is labor-intensive and requires large amounts of culture material, a characteristic that extends the time required to obtain results. Mardassi and colleagues (17) employed a slightly less discriminatory approach, IS6110 ligation-mediated PCR, to more quickly identify the outbreak strain among many isolates in a high-incidence area. Both Freeman et al. (8) and Rajakumar et al. (24) circumvented these limitations of RFLP analysis by means of deligotyping. They utilized microarrays to detect characteristic deletion sequences in the outbreak strains of interest and then employed PCR with primers flanking the deletions to quickly confirm or refute the presence of the outbreak genotype in isolates from new cases. This PCR-based approach yielded rapid results because it could be applied to isolates for which only small amounts of culture material were available. In the case of Freeman et al. (8), it was applied to DNA extracted directly from positive clinical laboratory enrichment cultures, yielding genotypic results within days of the confirmed diagnosis. This facilitated the prioritization of tuberculosis control resources.

The deligotyping approaches used by Freeman et al. (8) and Rajakumar et al. (24) were limited by their focus on specific outbreak strains. Numerous investigators have suggested the application of molecular fingerprinting techniques to tuberculosis surveillance. This form of surveillance could lead to the earlier detection of new outbreaks and to the identification of transmission events in the absence of known epidemiologic links. At least two groups of investigators have previously reported on the application of universal *M. tuberculosis* genotyping to such programs (4, 16). As with outbreak investigations, the results must be made available rapidly and must be specific if the full benefits of universal genotyping are to be realized. PCR-based fingerprinting approaches may make this possible. These include mycobacterial interspersed-repetitive-unit (MIRU) typing (30), spoligotyping (12), IS6110 ligation-mediated PCR typing (23), and repetitive-unit-sequence-based...
PCR (rep-PCR) (3). Like deligotyping, these approaches require small amounts of M. tuberculosis culture material. In contrast to deligotyping, however, they yield high-resolution DNA fingerprints of different strains rather than a binary indication of the presence or the absence of deletions associated with a specific strain.

This paper reports on the use of two PCR-based pathogen genotyping approaches, MIRU typing and rep-PCR, to facilitate the control of a tuberculosis outbreak and to monitor the population for new outbreaks in King County, Washington, during 2005 and 2006. In both operations, most isolates were genotyped by using DNA extracted directly from primary isolation cultures, yielding results in a rapid and useful fashion.

MATERIALS AND METHODS

Samples. Public Health-Seattle & King County serves a metropolitan area with approximately 1,777,000 people in the state of Washington. All isolates of M. tuberculosis from cases residing in King County taken at the Harborview Medical Center laboratory and the Seattle-King County laboratory were submitted to the Seattle Biomedical Research Institute (SBRI). Isolates were submitted as 1- to 2-ml aliquots of primary diagnostic cultures from the state of Washington. All isolates of SBRI131 and SBRI341 were included in all rep-PCR runs and chips as positive controls representing SBRI9 and SBRI10, respectively. The DiversiLab system was previously shown to exhibit a resolving power equivalent to that of IS6110-based RFLP analysis when ≥93% similarity was used as the threshold value to establish strain identity (3).

Characterization of outbreak strain. In 2004, the Seattle-King County Tuberculosis Control Program identified an epidemiologically linked cluster of five tuberculosis cases. Isolates from the index cases were characterized by spoligotyping, MIRU typing, and rep-PCR with the DiversiLab system (Bacterial Barcodes). Strain identity was determined by ≥93% similarity by rep-PCR, as described previously (3). The MIRU typing results for the outbreak strain, named SBRI10, were compared to the types in the SBRI MIRU database to find highly discriminatory loci for use in targeted screening of subsequent submissions.

Outbreak investigation. Between May 2004 and August 2005, tuberculosis cases suspected of being associated with ongoing transmission were submitted for genotyping. These cases were screened by using the most highly discriminatory MIRU loci. Those with the characteristic genotype were analyzed by rep-PCR, and of these, those that matched SBRI341, the outbreak isolate used as the positive control, by ≥93% were considered SBRI10. Between January and August 2005, the continued outbreak investigation overlapped with a pilot study of universal genotyping approaches (Universal Genotyping Pilot; Fig. 1). During this period, samples from all tuberculosis cases were submitted for analysis. All isolates showing ≥90% similarity to the outbreak strain by rep-PCR were analyzed by use of the 12-locus MIRU panel. In addition, targeted MIRU analysis was performed with all isolates that were not clustered by rep-PCR, and those with the characteristic SBRI10 genotype at the discriminatory loci were analyzed by use of the 12-locus MIRU panel, in accordance with the ongoing outbreak investigation strategy. Genotypic confirmation of the outbreak strain triggered a repeat interview of clustered cases and prioritized the use of public health resources.

Universal genotyping with rep-PCR as the initial screening tool (universal genotyping phase 1). Between September 2005 and August 2006, isolates from all tuberculosis cases were submitted for genotyping. In the first phase of this program, conducted from September 2005 to March 2006 (Fig. 1), isolates were first analyzed by rep-PCR, and all those displaying ≥92% similarity to one or more other isolates were typed at all 12 MIRU loci. The 92% cutoff was used instead of the 93% cutoff reported previously (3) in order to increase sensitivity.

Universal genotyping with MIRU as the initial screening tool (universal genotyping phase 2). To the higher quality of DNA required for rep-PCR than for MIRU analysis, it was determined that preliminary results could be obtained more rapidly and inexpensively if a 12-locus MIRU panel was used in place of rep-PCR as the first typing method. This strategy was applied in the second phase of the universal genotyping program, conducted from March 2006 to August 2006 (Fig. 1). During this phase, all isolates were first analyzed by use of the 12-locus MIRU panel, and those that matched at all 12 loci were analyzed by rep-PCR to confirm the strain identity. Preliminary (MIRU typing) results were used by the health department to rule out epidemiologically suspected clusters. Final (MIRU typing and rep-PCR) results prompted greater scrutiny where needed.

Human subjects. This research was approved by the Institutional Review Boards of Seattle Biomedical Research Institute and the University of Washington in the minimal risk category.

RESULTS

Characterization of outbreak strain. Isolates from the initial five suspected outbreak cases (coded as isolates SBRI298, SBRI307, SBRI312, SBRI316, and SBRI341) underwent MIRU genotyping, with all yielding the MIRU genotype...
The 2' signifies a product length consistent with two 77-bp repeats and a 53-bp truncated repeat; this truncated repeat is seen in most clinical *M. tuberculosis* isolates but is absent in H37Rv, H37Ra, and *M. bovis* BCG strains (14, 15, 29). The “n” indicates that during the investigation there were difficulties in amplifying MIRU locus 20. Due to these difficulties and the low degree of heterogeneity at that locus, the isolates used to characterize strain SBR110 were temporarily assumed to have the same number of repeats at locus 20, and this assumption was confirmed retrospectively. The outbreak genotype was compared to the genotypes in the SBRI MIRU database (*n* = 341 isolates), and unusual repeat numbers were noted at locus 10 (five repeats) and locus 23 (three repeats). The strain identity of the five initial outbreak isolates was confirmed by using rep-PCR (Fig. 2) and spoligotyping. These isolates, as well as three additional isolates identified later by targeted MIRU analysis (isolates SBR1342, SBR1345, and SBR1355), shared the sequence type 134 spoligotype (2) and 12-locus MIRU type 22’5313153323.

**Outbreak investigation.** Targeted genotyping at MIRU loci 10 and 23 formed the basis of an outbreak investigation that spanned a 15-month period beginning in May 2004. Between May and December 2004, 82 cultures were submitted for targeted genotyping at MIRU loci 10 and 23. Fourteen samples were identified with five repeats at MIRU locus 10 and with three repeats at MIRU locus 23. By rep-PCR, 13 of the 14 isolates with the targeted genotype were confirmed to be part of the SBRI10 outbreak. Isolate SBRI378 was a complete match to the outbreak strain by 12-locus MIRU genotyping (22’5313153323) but was only 60% related by rep-PCR (Fig. 2).

During the targeted MIRU genotyping, a single outbreak strain was missed. On initial targeted genotyping, sample 368 amplified three repeats for MIRU locus 10 and therefore did not immediately go on to confirmatory genotyping. The public health authorities requested reexamination of this case based on contact tracing. By 12-locus MIRU analysis and rep-PCR, isolate SBR1368 was shown to match the outbreak strain with
greater than 93% similarity and to share the 12-locus MIRU genotype 22/ST1353323 (Fig. 2).

The predictive value of targeted MIRU genotyping for identifying SBRI10 in our population was calculated against a combined criterion of 12-locus MIRU analysis, rep-PCR, and classical epidemiology. Among the isolates submitted on the basis of minimal epidemiologic suspicion, MIRU genotyping at loci 10 and 23 had a positive predictive value of 93% (13/14 isolates).

Between January 2005 and August 2005, the outbreak investigation continued while the universal genotyping approaches were pilot tested (Fig. 1). During this period, cultures of samples from 91 unique cases were submitted for universal genotyping by rep-PCR and MIRU analysis. One isolate did not yield a product at MIRU locus 10, and another did not give an interpretable rep-PCR result. Of the 90 isolates successfully typed at MIRU loci 10 and 23, none were found to match SBRI10 at those loci. Of the 90 isolates successfully typed by rep-PCR, two isolates (isolates SBRI447 and SBRI481) were found to be more than 93% related to SBRI1341, the representative SBRI10 strain used as a positive control in rep-PCR. It was determined by classical descriptive epidemiology that these isolates were unlikely to be outbreak related.

Modified MIRU reactions (7, 28) were abandoned during the universal genotyping pilot period, due to difficulties in amplifying some loci, and thereafter the reaction mixtures of Supply and colleagues were used exclusively (28).

Universal genotyping. Two strategies were employed sequentially for the universal genotyping of M. tuberculosis DNA extracted from MGIT cultures. The first strategy (phase 1) was applied between September 2005 and March 2006 (Fig. 1). MGIT cultures of samples from unique cases were genotyped first by rep-PCR, and suspected clusters were identified on the basis of ≥92% similarity. Suspected clusters were then typed by the use of the 12-locus MIRU panel for confirmation. On the basis of the rep-PCR results, 29 of the 60 isolates submitted during this period formed 11 clusters, none of which were confirmed by use of the 12-locus MIRU panel. The amount of mycobacteria in MGIT cultures varied and was not known. When DNA was extracted directly from the MGIT cultures, the DNA often did not meet the purity and quantity requirements for rep-PCR analysis. As a result, multiple rep-PCR runs were required for only two isolates, and the average time from sample submission to the reporting of presumptive results was 42 days (range, 12 to 87 days). This approach reflects the fact that the extracted DNA was typed in batches for cost efficiency; samples of particular concern to the public health authorities were prioritized for analysis within 2 weeks of receipt by the genotyping laboratory.

In the interest of summarizing all clusters in King County for a single year, we retrospectively analyzed all cases from both phases against the second genotyping strategy. This required the generation of new MIRU or rep-PCR data from archived cultures for some strains. Fifty-one of 156 typed isolates formed 18 clusters by use of the 12-locus MIRU panel, and 30 of these isolates were confirmed to form 11 clusters by ≥92% similarity by rep-PCR. In real time, across both phases of the universal genotyping program, the public health authorities were able to rule out six epidemiologically suspected instances of recent transmission on the basis of the genotypic information. In 16 cases that were initially deemed to be unrelated on the basis of classical descriptive epidemiology, public health authorities reevaluated possible transmission on the basis of genotypic information; in 9 of these cases, one or more epidemiologic links that previously went undetected were uncovered.

DISCUSSION

The studies reported here further demonstrate the utility of using genotypic fingerprints to enhance tuberculosis outbreak investigation and surveillance. The use of a two-locus MIRU screen allowed potential strain SBRI10 outbreak cases to be identified quickly. The speed of screening was afforded by the ability to genotype DNA extracted directly from MGIT cultures. This is in contrast to the outbreak control methods employed by Schmid and colleagues (25), who utilized RFLP analysis, which requires subculturing of samples. Both Freeman et al. (8) and Rajakumar et al. (24) were able to speed genotyping by using small amounts of culture material and delotyping. Those investigators, however, invested time and resources into the microarray characterization of index outbreak isolates, in order to identify the appropriate deletion sequences and primers to be used. In contrast, by using standard MIRU primers and a local MIRU database, the present study was able to quickly identify the most informative loci for the outbreak strain.

Although it is an important tuberculosis control activity in North America, contact investigation has limitations that can be overcome with universal genotyping. When several cases of tuberculosis are identified coincidently among high-risk persons, it is difficult to know whether these cases coincided ran-
domly, were reactivation disease brought about by shared risk factors, or were the result of recent transmission. Conversely, several reports have found that tuberculosis transmission can occur via more casual or distant social contacts than are identified by traditional contact tracing (6, 21, 22). Moreover, some vulnerable individuals are reticent to share information about close contacts. The universal application of an affordable, fast genotyping protocol to all cases of tuberculosis in a region can help rule out ongoing transmission as the cause of coincident cases. It can also help identify ongoing transmission among networks of casual or undisclosed contacts that would be missed in a traditional interview.

In the present study, six cases suspected of being the result of recent transmission were determined not to have been caused by shared strains. In addition, 16 seemingly isolated cases were found to be caused by strains shared by one or more additional cases. In the 16 cases in which unexpected genotype matches were found, public health authorities reinterviewed the patients and, if needed, intensified their efforts to identify potentially exposed contacts, provide testing, and treat the contacts for latent infection. In 9 of these 16 cases, the reevaluation prompted by genotypic information led to the detection of one or more epidemiologic links.

In the present evaluation, MIRU analysis demonstrated itself to be an excellent first-line tool for universal genotyping in support of tuberculosis control, for several reasons. First, MIRU analysis results could be obtained with DNA extracted from small amounts of primary diagnostic cultures, obviating the need to grow large numbers of cells in order to get preliminary results. It had been hoped that the same would be true of rep-PCR, which exhibits an excellent robustness and resolving power when it is applied to cultured isolates, as recom-

![Clusters identified during phase 2 universal genotyping. Presumptive clusters share 12-locus MIRU genotypes. Shaded sample numbers indicate strains that share the 12-locus MIRU genotype but that were not confirmed to be members of the cluster by rep-PCR.](http://jcm.asm.org/)

FIG. 3. Clusters identified during phase 2 universal genotyping. Presumptive clusters share 12-locus MIRU genotypes. Shaded sample numbers indicate strains that share the 12-locus MIRU genotype but that were not confirmed to be members of the cluster by rep-PCR.
mended by the manufacturer (3, 8, 9). The technology did not, however, yield consistent results when it was applied to DNA extracted directly from primary, diagnostic enrichment cultures, a use not suggested by the manufacturer.

Second, MIRU analysis can also simultaneously serve dual purposes in universal genotyping and outbreak monitoring. Universal genotyping by the MIRU method generates a database that can be used to quickly identify unusual loci in a new outbreak strain. This can be done much faster than the identification of large sequence polymorphisms for deligotyping (8, 24). In the present study these unusual loci were quickly examined on all potential outbreak strains, without slowing the process of universal genotyping or adding the burden of additional genotyping activities to the laboratory.

In addition, MIRU analysis has been shown to be highly discriminatory, both as measured by the discriminative index of Hunter and Gaston and as measured in comparison to the results of IS6110-based RFLP analysis and spoligotyping (1, 5, 7, 13, 18, 20, 26, 27). It has been reported to provide a particular advantage over IS6110-based RFLP analysis by discriminating between low-band-number strains (18) and an advantage over spoligotyping by identifying, but discriminating among, strains of spoligotype-defined families (1, 7, 7, 20, 26, 27). MIRU analysis has been shown to have a low discriminatory power, however, among strains within the Beijing family of strains (11, 31). MIRU approaches involving more than 12 loci have been proposed and evaluated to overcome this shortcoming (10, 28).

The present study identified several advantages to the use of rep-PCR as a confirmatory genotyping approach. Although rep-PCR has more stringent DNA requirements than was originally anticipated, it is still considerably less labor-intensive than IS6110-based RFLP analysis. Additionally, rep-PCR is a flexible technology that can genotype nontuberculous mycobacteria (3, 9) and many other microbial pathogens of interest to clinical microbiologists and infectious disease epidemiologists. These advantages may outweigh its disadvantages. Its disadvantages are that rep-PCR is a proprietary technology supplied by a single manufacturer and that its validity relative to that of the gold standard IS6110-based RFLP method and its reliability have been evaluated in only one study (3). A direct and formal comparison should be made to determine if rep-PCR provides high resolution in situations in which spoligotyping and 12-locus MIRU analysis fail, such as in discriminating members of the Beijing family of strains, as this would constitute another significant advantage.

ACKNOWLEDGMENTS

We thank Justin Nguyen, Jean Pass, Jeanette Frazier, and Paul Swenson of the Public Health—Seattle & King County Laboratory and Carolyn Wallis and the Tuberculosis Laboratory staff of the Clinical Microbiology Laboratory of Harborview Medical Center.

This work was supported by Puget Sound Partners for Global Health, Public Health-Seattle & King County, the Medical Scientist Training Program (grant T32 GM07266), and a National Research Service Award from the National Institute of General Medical Sciences.

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