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Characterization of *Mycobacterium tuberculosis* Complex Direct Repeat Sequence for Use in Cycling Probe Reaction

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Cycling probe technology (CPT) is a unique and simple method for the detection of specific target sequences. CPT utilizes a chimeric DNA-RNA-DNA probe providing an RNase H-sensitive scissile linkage when bound to a complementary target sequence. For this study, a diagnostic assay based on CPT was developed for the detection of the 36-bp direct repeat (DR) region in *Mycobacterium tuberculosis*. To determine the feasibility of using the DR for detecting *M. tuberculosis* by CPT, a wide variety of mycobacteria were tested by Southern blot hybridization with three DR probes to verify their specificity. The entire DR region of *Mycobacterium bovis* 401 was sequenced, and the data were used to design a PCR assay that would allow us to estimate the number of DRs present in a variety of strains. A CPT assay which uses a probe complementary to the DR region was developed and evaluated with synthetic targets and genomic DNA from mycobacteria. In summary, the 36-bp DR provides an attractive target for detecting *M. tuberculosis* because the sequence is present in high copy numbers in the genome, is specific for the *M. tuberculosis* complex, and is found in strains that lack IS*6110*.

Cycling probe technology (CPT) is a unique and simple method for the detection of specific target sequences (3). For this study, a diagnostic assay based on CPT was developed for the detection of the 36-bp direct repeat (DR) region in *Mycobacterium tuberculosis*. The DR region contains a large number of 36-bp repeats, which are separated from one another by unique spacer sequences that vary in size from 35 to 41 bps. The majority of strains contain a copy of IS*6110* integrated into the DR region (6). Part of the DR sequence was selected as a target for CPT since it occurs multiple times within the genomes of members of the *M. tuberculosis* complex and has several characteristics favorable for the performance of a cycling probe. Typically, CPT probes are less than 30 nucleotides in length, with four contiguous internal purine ribonucleotides, and designed so that there is minimal potential for the formation of inter- and intraprobe interactions.

CPT utilizes a chimeric DNA-RNA-DNA probe providing an RNase H-sensitive scissile linkage when bound to a complementary target sequence (Fig. 1) (3). CPT reactions are performed at a constant specific temperature which allows hybridization of the chimeric probe with its complementary single-stranded target DNA. Within the resulting target-probe duplex, RNase H recognizes the DNA-RNA hybrid and specifically cleaves the RNA portion of the probe. The cleaved fragments are not stable at the reaction temperature and disassociate from the target. The target is then free to hybridize with another probe molecule, and the cycle is repeated. The probe fragments accumulate, serving as a basis for the detection of target. Over time, the accumulation of cleaved probe fragments follows linear kinetics and therefore the amount of target can be quantified.

CPT is simple, using only one probe and one enzyme at a constant temperature. Another advantage of CPT compared with target-based amplification technologies, e.g., PCR, is that target sequences are not amplified, thereby minimizing the risk of carryover contamination.

To determine the feasibility of using the DR for detecting *M. tuberculosis* by CPT, a wide variety of mycobacteria were tested by Southern blot hybridization with three DR probes to verify their specificity. The entire DR region of *Mycobacterium bovis* 401 was sequenced, and the data were used to design a PCR assay that would allow us to estimate the number of DRs present in a variety of strains. A CPT assay which uses a probe complementary to the DR region was developed and evaluated with genomic DNA from mycobacteria.

MATERIALS AND METHODS

Cells and growth media. The mycobacterial strains used in this study are presented in Table 1. All mycobacterial cells were cultivated in Dubos broth containing 0.1% Tween and albumin enrichment (Difco Laboratories, Detroit, Mich.) at the appropriate incubation temperature.

Preparation of genomic DNA from mycobacteria. For large-scale DNA preparation, cells (50 to 100 ml) were incubated to a Klett reading of 150 to 200, which represents log phase growth, and treated with D-cycloserine (1 μg/ml) for 24 h. After centrifugation, the cell pellets were heat killed and resuspended in 3 ml of sucrose buffer (15% sucrose, 50 mM Tris [pH 8.5], 50 mM EDTA) containing 3 mg of lysozyme and incubated at 37°C overnight. Cells were then incubated with proteinase K (20 mg/ml) and 1.5% sodium dodecyl sulfate (SDS) at 60°C for 2 h. The lysate was allowed to cool to room temperature and aspirated 10 to 20 times with a 20-gauge needle and syringe. Debris was pelleted by centrifugation at 19,800 × *g* for 45 min at 4°C. Cesium chloride (final density of 1.55 g/ml) and ethidium bromide (5 mg/ml) were added to the supernatant. DNA was separated by ultracentrifugation at 192,000 × *g* for 40 h at 20°C. After dialysis in Tris-EDTA buffer overnight, the amount of DNA was determined by UV spectrophotometry.

DNA from isolates received for IS*6110* fingerprinting was prepared by a small-scale DNA isolation method (8). DNA from isolates used in the cycling probe assay was prepared by a modified bead beating procedure. Cell pellets containing approximately 10^{10} cells were recovered in 200 μl of lysis buffer (10 mM Tris [pH 8.1], 0.1 mM EDTA, 1% Triton X-100) and incubated at 95°C for 30 min. The sample was transferred to a Microspin tube (Pharmacia Biotechnology, Alameda, Calif.) containing 0.1-mm zirconium beads and subjected to bead beating in a Bead-beater unit (Biospec Products, Bartlesville, Okla.) for 5 min. The lysates were collected in a microcentrifuge tube by centrifugation at 16,000 × *g* for 4 min and incubated at 95°C for 10 min. Aliquots of the lysate and

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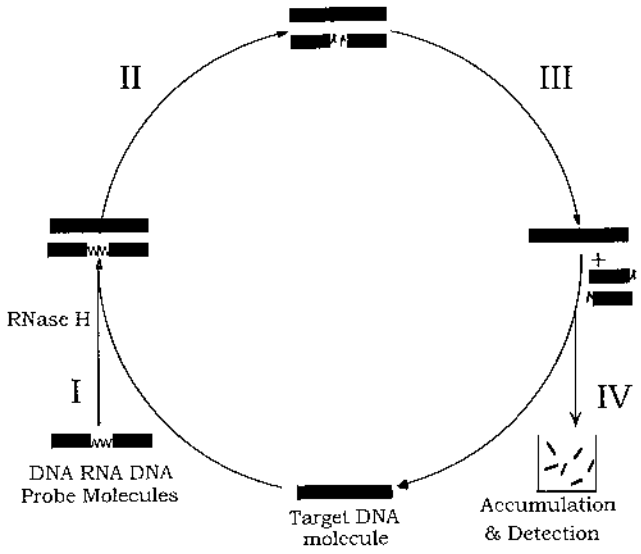


FIG. 1. Diagram of CPT. I, hybridization of scissile-link probe to target DNA; II, cleavage by RNase H of RNA portion of hybridized probe; III, dissociation of probe fragments; IV, accumulation of probe fragments as additional probes are cycled against same target DNA.

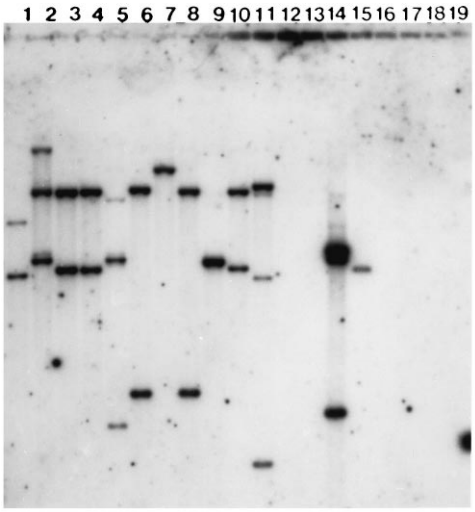


FIG. 2. Southern blot of *Bam*HI restriction digests of genomic DNA probed with ARK2. Lane 1, *M. tuberculosis* H37Rv; lanes 2 to 11, clinical isolates of *M. tuberculosis* from Kampala, Uganda; lane 12, *M. genavense*; lane 13, *M. intracellulare*; lane 14, *M. africanum*; lane 15, *M. tuberculosis* New York strain W; lane 16, *M. triviale*; lane 17, *M. fortuitum*; lane 18, *M. avium*; lane 19, *M. xenopi.*

known concentrations of calf thymus DNA were resolved by electrophoresis on an agarose gel and visualized by ethidium bromide staining. The final DNA concentration in the lysate was estimated to be approximately 200 ng/μl.

Probing for the DR. Approximately 1 μg of genomic DNA from each strain was restricted with *Bam*HI and electrophoresed in a 0.8% agarose gel. DNA was transferred to Hybond nylon membrane following the manufacturer's recommended procedure (Amersham Life Science, Amersham, United Kingdom). The oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and were 5′-labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase (9). The ARK1 DNA probe is a 16-mer, 5′-AGA CCC AAA ACC CCG A-3′; the ARK2 DNA probe is a 27-mer, 5′-GTC GTC AGA CCC AAA ACC CCG AGA GGG-3′; and the ARK3 DNA probe is a 36-mer, 5′-GTC GTC AGA CCC AAA ACC CCG AGA GGG GAC GGA AAC-3′. The labeled probes were column purified on a TE Midi Select-D G-25 column (5 Prime to 3 Prime, Inc., Boulder, Colo.) prior to use. Hybridizations were performed in 1 M NaCl–1% SDS at 48°C for ARK1, 60°C for ARK2, and 65°C for ARK3. Membranes were washed two times for 5 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), two times for 40 min at the hybridization temperature in 2× SSC–1% SDS, and two times for 40 min at room temperature in 0.1× SSC. After exposure to X-ray film, the membranes were stripped of probe and reused.

TABLE 1. Mycobacterial strains screened for the presence of the 36-bp DR

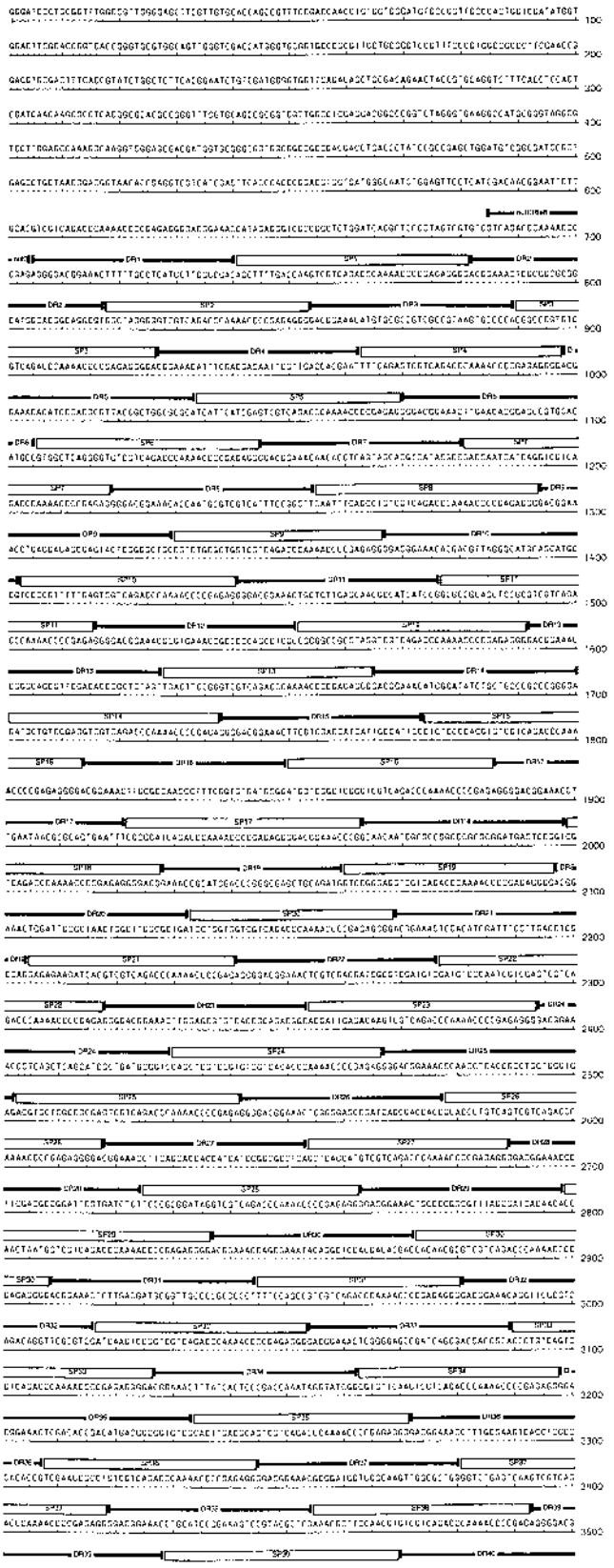
Organism	No. tested	Source[a]
M. africanum	1	ATCC 25420
M. avium	21	VAMR
M. bovis	38	VAMR, ATCC 35720, 19210
M. chelonae	4	ATCC 35751
M. flavescens	1	ATCC 14474
M. fortuitum	3	ATCC 35755, 14467, 6841
M. gastri	1	ATCC 15754
M. genavense	2	U of W
M. gordonae	3	ATCC 35756
M. haemophilum	1	CAP
M. intracellulare	2	VAMR
M. kansasii	4	VAMR, ATCC 12478, 35775, 35778
M. malmoense	1	ATCC 29571
M. marinum	1	ATCC 927
M. phlei	1	ATCC 35784
M. scrofulaceum	1	ATCC 35785
M. smegmatis	1	ATCC 607
M. simiae	1	ATCC 25275
M. szulgai	1	CAP
M. terrae	1	CAP
M. triviale	1	ATCC 23292
M. tuberculosis	171	VAMR, ATCC 27294, 25177, 35801
M. vaccae	1	ATCC 15483
M. xenopi	1	ATCC 19250

[a] ATCC, the American Type Culture Collection; VAMR, John L. McClellan Memorial Veterans Hospital Mycobacteriology Research Laboratory; CAP, College of American Pathologists performance survey strains; U of W, Marie Coyle, University of Washington, Harborview Medical Center, Seattle.

Sequencing of the *M. bovis* 401 DR region. The entire DR region of *M. bovis* 401 was cloned as a *Bam*HI fragment into pUC18 (pDC513). Sequencing was performed on an ABI 373 DNA Sequencer with the Taq DyeDeoxy Terminator Cycle sequencing kit (Perkin-Elmer, Applied Biosystems Inc., Foster City, Calif.). DNA for sequencing was prepared with the Qiagen Mini (Tip 20) or Midi (Tip 100) kit according to the manufacturer's protocol (Qiagen, Chatsworth, Calif.). Sequencing primers were designed with the Oligo 4.0 Primer Analysis software (National Biosciences, Inc., Plymouth, Minn.). Primers were synthesized by the University of Arkansas Medical Sciences Core Facility or the ID Biomedical Corp. Sequence analysis was performed with Seqed (Applied Biosystems Inc.), DNAstar (Madison, Wis.), and the Genetics Computer Group package (Madison, Wis.).

PCR analysis of the DR region. The primers flanking the DR region are indicated on the *M. bovis* 401 DR sequence map (see Fig. 3). Primers for IS*6110* were IS*6110* left (bp 193 to 174, 5′-CGC AGC CAA CAC CAA GTA GA-3′) and IS*6110* right (bp 1302 to 1321, 5′-CAG CCG CCG GCT GAG GTC TC-3′). DNA amplification was performed on a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.) in a 50-μl reaction mixture containing 10 pmol of each primer, 1 to 10 ng of genomic DNA, 200 μM deoxyribonucleoside triphosphates, 1× PCR buffer (pH 8.3, 1.5 mM $MgCl_2$), and 1 U of *Taq* polymerase (Perkin-Elmer). The optimal annealing temperature for each primer pair was determined by PCR. Amplification conditions were as follows: initial denaturation at 95°C for 5 min and 30 cycles of 1 min of denaturation at 95°C, 1 min at an appropriate annealing temperature, and 4 min of extension at 72°C. PCR mixtures were electrophoresed on a 0.8% agarose gel, stained with ethidium bromide, and photographed on the Eagle Eye II gel documentation system (Stratagene, La Jolla, Calif.).

CPT assay. A chimeric ARK2 probe was synthesized based on the ARK2 probe sequence as previously described (1). The four contiguous deoxyriboadenosine residues were replaced by four riboadenosine residues as follows: 5′-d(GTCGTCAGACCC)r(AAAA)d(CCCCGAGAGGG)-3′. The chimeric probe was 5′-labeled with [γ-^{32}P]ATP to a high specific activity (approximately 13 × 10^6 cpm/pmol) with RTG T4 polynucleotide kinase (Pharmacia) (9). CPT reactions (10-μl final volume) were performed with *Thermus thermophilus* RNase H (1). The cycling probe reaction contains buffer (50 mM Tris-HCl [pH 8.1], 8 mM $MgCl_2$, 0.025% Triton X-100), 0.3 fmol of labeled probe, 0.2 μg of RNase H, and the indicated amount of target. Reaction mixtures were incubated at 65°C for 30 min. An equal volume of loading buffer (8 mM urea, 100 mM EDTA, 0.1%



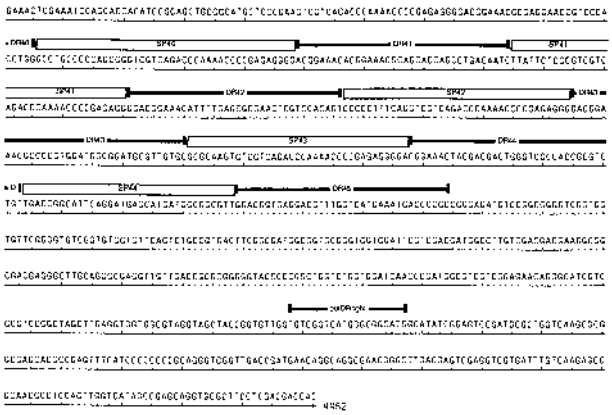


FIG. 3. Sequence of the 36-bp DR region of *M. bovis* 401. The DRs are numbered from left to right and are indicated by black lines under the sequence. The spacer sequences are numbered from left to right and are indicated by open boxes under the sequence. Primers out DR left and out DR right are indicated by black lines under the sequence.

bromophenol blue, 0.1% xylene cyanol) was added to each sample and incubated at 90°C for 5 min to stop the reaction. Reaction products were resolved by polyacrylamide gel electrophoresis on a denaturing gel (20% polyacrylamide–7 M urea), and the gel was exposed to a phosphor screen and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The percentage of probe cut in each reaction was estimated by integration of the band corresponding to intact probe and the bands corresponding to the cut probe fragments. The net percent cut product was determined by subtracting the percent cut in reactions containing DNA isolated from control samples from the percent cut in reactions with *M. tuberculosis* DNA.

Nucleotide sequence accession number. The nucleotide sequence reported here will appear in the GenBank nucleotide sequence database under accession number U47864.

RESULTS AND DISCUSSION

Specificity of the DR. Ninety *M. tuberculosis* complex strains and 65 nontuberculous mycobacteria (Table 1) were examined by Southern blot hybridization with the DR probes (ARK1, ARK2, and ARK3). Genomic DNA was restricted with *Bam*HI, for which there is a single restriction site in IS*6110* (10). If IS*6110* is in the DR region, it is cleaved by *Bam*HI and two hybridizing fragments are visualized. None of the strains of nontuberculous mycobacteria hybridized with the three DR probes, whereas all the *M. tuberculosis* complex strains hybridized with these probes (Fig. 2). The majority of *M. tuberculosis* complex strains showed two hybridizing fragments with considerable variation in their sizes. This indicates that IS*6110* is located within the DR region or that a *Bam*HI site is present in the DR region. A few strains (lanes 7, 9, and 15) showed one hybridizing fragment, indicating that IS*6110* is not inserted in the DR region. In some cases (lane 2), the probes hybridized with three fragments, suggesting that these strains contain two copies of IS*6110* in the DR region or one copy of IS*6110* and a *Bam*HI site in the DR region. Interestingly, five strains of *M. tuberculosis* which do not contain IS*6110* showed the one hybridizing fragment. The 36-bp DR is specific for the *M. tuberculosis* complex and is found in strains that lack IS*6110.*

Sequence of the DR region of *M. bovis* 401. *M. bovis* 401 contains three copies of IS*6110* with one residing in the DR region (2). The sequence of the DR region of *M. bovis* 401 is presented in Fig. 3. This includes 604 bp to the left and 578 bp to the right of the DR region. There are 45 DRs and 44 spacer sequences. The sequence of IS*6110*, which is not included, is located within the 27th DR. The sequence of DR19 lacks GTCG at the beginning of the sequence; however, the sequences of the remaining DRs are identical.

The spacer sequences that have been previously described by other investigators are those designated SP20 to SP40 (5–7). The spacer sequences that have not previously been identified are SP1 to SP19 and SP41 to SP44. SP38 is also a unique sequence between two previously identified sequences. The first two spacer sequences to the left and right of IS*6110* were

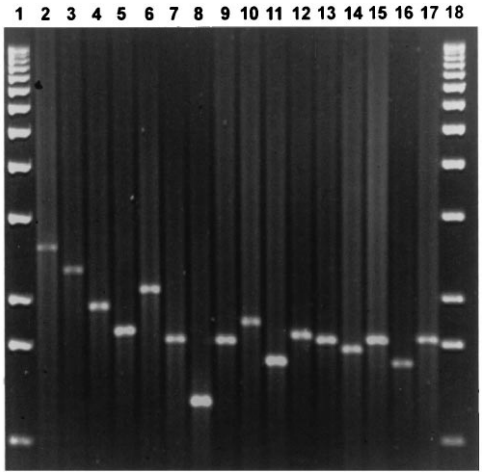


FIG. 4. Left- and right-side PCR of seven *M. tuberculosis* isolates and *M. bovis* 401. Lanes 1 and 18, 1-kb ladder. The first lane of each set is the left-side product followed by the right-side product. The source of each isolate is indicated and followed by the copy number of IS*6110*. Lanes 2 and 3, Colorado, 1; lanes 4 and 5, California, 2; lanes 6 and 7, *M. bovis* 401, 3; lanes 8 and 9, California, 4; lanes 10 and 11, Texas, 9; lanes 12 and 13, Texas, 12; lanes 14 and 15, Spain, 15; lanes 16 and 17, Brazil, 17.

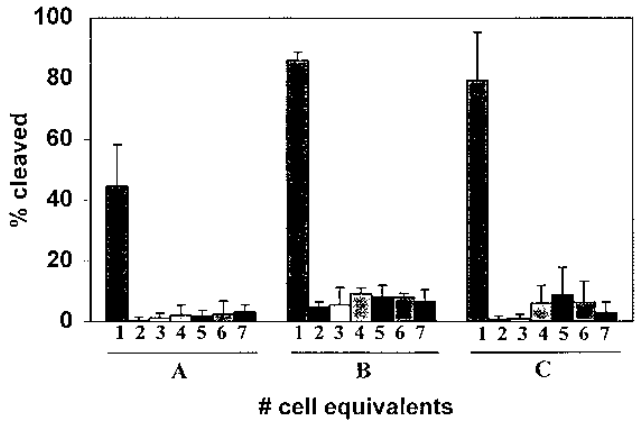


FIG. 5. Specificity of the cycling reaction. Percent probe cleaved in cycling reactions with concentrations of *M. tuberculosis* genomic DNA corresponding to 10^4 cells (A), 10^5 cells (B), and 10^6 cells (C) was compared with percent probe cleaved in reactions with the same concentrations of DNA from each of six nontuberculous mycobacteria (1, *M. terrae*; 2, *M. avium*; 3, *M. simiae*; 4, *M. kansasii*; 5, *M. intracellulare*; 6, *M. gordonae*). The average of four independent experiments is reported, and the standard deviation between experiments is indicated by the error bars.

identical to those published for *M. tuberculosis* and *M. bovis* BCG in the same position (3–5). In addition, during the process of isolating the flanking regions of IS*6110*, the right side of the DR region was sequenced in three strains of *M. tuberculosis*. In all cases, the first spacer sequence was identical to that found in *M. bovis* 401 (4). This indicates that IS*6110* is located at an identical site in the DR region in these strains.

Further characterization of the DR region. Experiments were performed to determine if the DR region occurs in the same site on the chromosome, the frequency with which IS*6110* resides in the DR region, and the number of copies of the DR in a variety of strains. The left-side PCR primer set consists of IS*6110* left and out DR left and the right-side primer PCR set consists of IS*6110* right and out DR right. *M. bovis* 401 produces a left-side PCR product of approximately 2.1 kb and a right-side PCR product of approximately 1.7 kb.

We examined 85 clinical strains of *M. tuberculosis* and 35 strains of *M. bovis* isolated from California, Kansas, Texas, Colorado, Uganda, Spain, Brazil, Switzerland, Canada, and Thailand. Results with seven of these strains are shown in Fig. 4.

The PCR products varied in size compared with the control, *M. bovis* 401, which indicates that there is variation in the number of DRs in this region. The results also indicate that the DR region is located in the same place in the chromosome with IS*6110* inserted in the same orientation. In 23 samples, no products were observed, indicating that either IS*6110* is not present in the DR region or the sequence of the flanking DNA is variable. These strains were examined by PCR with the left (out DR left) and right (out DR right) flanking region primers combined with primers based in the 36-bp repeat. Both of these reactions result in PCR products extending from each DR to the primer based in the flanking DNA (data not shown). In all cases, such products were produced, indicating that the sequence flanking the DR region is the same, and IS*6110* either is not present in the DR region or is inverted. Additional experiments demonstrated that IS*6110* was not located in the DR region (data not shown).

The size of the products allowed estimation of the number of DRs present in each strain. Since the products are large, it is not possible to resolve small differences in the number of DRs. The approximate sizes of the fragments produced by the right- and left-side PCR were summed, and the base pairs representing IS*6110* and regions outside the DR were deducted. This value was divided by 74 (36-bp repeat plus 38-bp average spacer sequence) to obtain the approximate number of DRs. In the strains examined, the number of DRs varied from 14 to 63. The average number of DRs for all strains was 38.

The 36-bp DR provides an attractive target for detecting *M. tuberculosis* because the sequence is present in high copy numbers in the genome, is specific for the *M. tuberculosis* complex, and is found in strains that lack IS*6110*.

The observation that strains vary in the number of repeats in the DR region is interesting for the potential application to strain differentiation. Presently, we have been further examining the DR region in a number of *M. tuberculosis* complex strains by PCR. If the DR number is determined to be a suitable strain-specific marker, then it would be worthwhile modifying the CPT assay to quantify the number of DRs.

Evaluation of CPT for detection of *M. tuberculosis*. To confirm that the ARK2 chimeric probe shows specificity in the cycling reaction, the signal generated in reactions containing genomic DNA from *M. tuberculosis* was compared with that observed with DNA from each of six species of nontuberculous mycobacteria (Fig. 5). A good signal-to-noise ratio (at least 9:1) was observed between *M. tuberculosis* and the six nontuberculous species at each of the three high concentrations of DNA tested. This indicates that this sequence maintains specificity in the cycling reaction.

The sensitivity of the cycling with the ARK2 chimeric probe sequence was assessed by testing dilutions of DNA isolated from *M. tuberculosis* and the same amounts of DNA isolated from a negative control, *Mycobacterium gordonae* (Fig. 6A). Signal was observed in reactions containing *M. tuberculosis* DNA with a lower limit of detection of approximately 100 cell equivalents and a signal-to-noise ratio of 2:1 at 250 cell equivalents. Furthermore, when the net percentage of cut probe is plotted versus the number of cell equivalents of DNA, the linearity of the cycling reaction is confirmed (Fig. 6B).

CPT has potentially multiple applications in infectious dis-

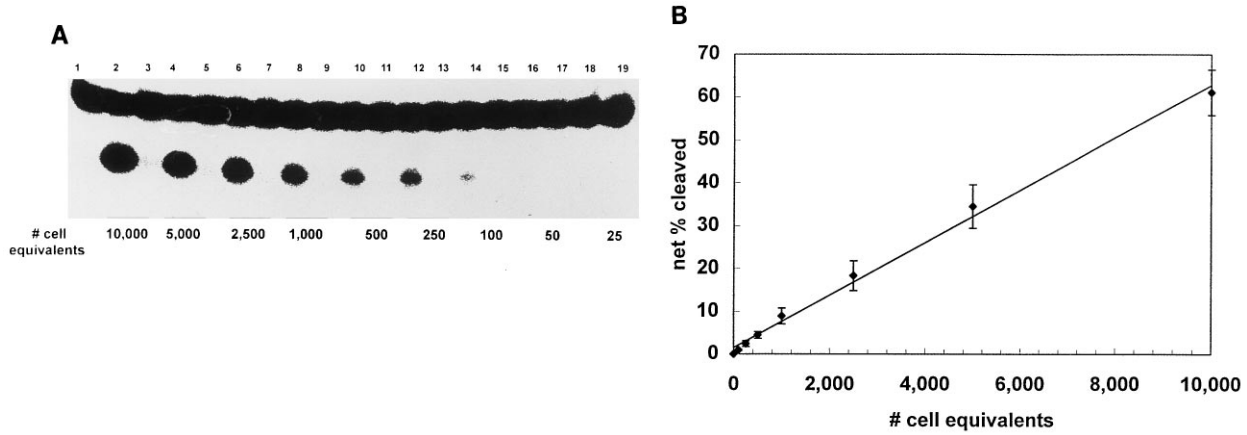


FIG. 6. (A) Sensitivity of the cycling probe reaction. Lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18 show cycling reactions performed with DNA isolated from *M. tuberculosis* cells and diluted to the number of cell equivalents indicated. Lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19 similarly show reactions with *M. gordonae* DNA. Lane 1 shows a control reaction with probe and RNase H, but no target DNA. The upper band represents intact probe, and the lower band represents cleaved probe fragments. (B) Linearity of the cycling probe reaction. The percent probe cleaved at each of the number of cell equivalents for *M. tuberculosis* in panel A was corrected for the background cleavage in reactions containing the same amounts of *M. gordonae* DNA to give the net percentage cleaved of cut probe. The average of seven independent experiments is plotted, and the standard deviation between experiments is indicated by the error bars.

ease diagnostics. Clearly, the assay format must be modified to adapt CPT to the clinical laboratory setting. Currently, work is ongoing to simplify the test procedure and convert the assay to a nonisotopic format. When testing clinical samples, a major consideration is the level of sensitivity and specificity. The sensitivity of the CPT reaction is limited by the turnover number, which is the number of probe molecules cleaved per target molecule. (In a 30-min reaction, the turnover number is approximately 1,000). To increase sensitivity, one must use high concentrations of RNase H, which is required for detection of low amounts of target DNA. Specificity is enhanced by using probes which are designed with minimal potential for the formation of inter- and intraprobe reactions. Further assay development is focused on improving assay sensitivity and eliminating nonspecific cleavage of probe. Another consideration is that the clinical samples may contain nonspecific RNases and/or high amounts of human genomic DNA which would effect cleavage of the probe. Assays have been performed with a limited number of clinical samples, and there has been no evidence of cleavage of the probe in the absence of RNase H. Also, experiments with RNase A indicate that the probe is not a substrate for this enzyme. Preliminary experiments demonstrated that high amounts of human DNA which are present in sputum sediments resulted in high background. However, recent assay modifications have overcome this problem.

In conclusion, the DR sequence is highly conserved and found in multiple copies in all strains of the *M. tuberculosis* complex tested. It has been demonstrated to be specific and is found even in strains that are shown to lack IS*6110*. ARK2 was tested for specificity and sensitivity in cycling reactions containing dilutions of crude DNA from *M. tuberculosis* isolates and a number of nontuberculous mycobacterial strains. Results have demonstrated that ARK2 can be used to discriminate between these strains in reactions containing DNA corresponding to as many as one million cells. The lower limit of detection with a signal-to-noise ratio of at least 2:1 was approximately 250 cell equivalents. CPT is certainly a viable option in the collection of amplification technologies because of its attractive features (rapidity, simplicity, and not being subject to amplicon contamination).

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