JOURNAL OF CLINICAL MICROBIOLOGY, July 2001, p. 2531–2540 Vol. 39, No. 7
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.7.2531–2540.2001

Identification of Rifampin-Resistant *Mycobacterium tuberculosis* Strains by Hybridization, PCR, and Ligase Detection Reaction on Oligonucleotide Microchips

VLADIMIR MIKHAILOVICH,[1] SERGEY LAPA,[1] DIMITRY GRYADUNOV,[1] ALEXANDER SOBOLEV,[1] BORIS STRIZHKOV,[1] NIKOLAI CHERNYH,[1] OLGA SKOTNIKOVA,[2] OLGA IRTUGANOVA,[2] ARKADII MOROZ,[2] VITALII LITVINOV,[2] MIKHAIL VLADIMIRSKII,[3] MIKHAIL PERELMAN,[3] LARISA CHERNOUSOVA,[4] VLADISLAV EROKHIN,[4] ALEXANDER ZASEDATELEV,[1] AND ANDREI MIRZABEKOV[1,5]*

Biochip Technology Center, Argonne National Laboratory, Argonne, Illinois,[5] and Moscow Anti-Tuberculosis Center, Moscow Government,[2] Research Institute for Phthisiopulmonology, I. M. Sechenov Moscow Medical Academy,[3] Engelhardt Institute of Molecular Biology,[1] and Central TB Research Institute, Russian Academy of Medical Sciences,[4] Moscow, Russia

Received 22 December 2000/Returned for modification 21 February 2001/Accepted 21 April 2001

Three new molecular approaches were developed to identify drug-resistant strains of *Mycobacterium tuberculosis* using biochips with oligonucleotides immobilized in polyacrylamide gel pads. These approaches are significantly faster than traditional bacteriological methods. All three approaches—hybridization, PCR, and ligase detection reaction—were designed to analyze an 81-bp fragment of the gene *rpoB* encoding the β-subunit of RNA polymerase, where most known mutations of rifampin resistance are located. The call set for hybridization analysis consisted of 42 immobilized oligonucleotides and enabled us to identify 30 mutant variants of the *rpoB* gene within 24 h. These variants are found in 95% of all mutants whose rifampin resistance is caused by mutations in the 81-bp fragment. Using the second approach, allele-specific on-chip PCR, it was possible to directly identify mutations in clinical samples within 1.5 h. The third approach, on-chip ligase detection reaction, was sensitive enough to reveal rifampin-resistant strains in a model mixture containing 1% of resistant and 99% of susceptible bacteria. This level of sensitivity is comparable to that from the determination of *M. tuberculosis* drug resistance by using standard bacteriological tests.

Tuberculosis (TB), one of the most deadly and common infectious diseases, claims 3,000,000 lives a year worldwide (24). Although the disease is found mostly in developing countries, a growing number of cases are diagnosed in the industrialized world as well. The global spread of the disease is further complicated by the ubiquitous appearance of drug-resistant (6) and especially multidrug-resistant strains that, by definition, are resistant to at least rifampin (RIF) and isoniazid. Thus, in 1996 13% of all newly diagnosed cases of TB in the United States were resistant (primary resistance) to at least one first-line drug and 1.6% were multidrug resistant (19).

After radical political changes in the former Soviet Union, the incidence of TB and related mortality in Russia increased to levels that are among the highest in the world. The incidence of TB in Russia in the period between 1991 and 1997 increased from 34 to 82 per 100,000 adults (10), four to seven times higher than that in most European countries. In view of the potential global spread of multidrug-resistant forms of TB, special concerns arise regarding the Russian penitentiary system. Its population presently exceeds 1,000,000, and 10% of the prisoners have active TB (for a review, see reference 10). Among the prisoners, about 25% of all new cases and 92% of nonresponding cases are drug resistant (7); according to another report, 66% of all cases are drug resistant, 50% of which are RIF-resistant (Rif^r) forms (29).

According to the recommendations of the Centers for Disease Control and Prevention, bacteriological laboratories must determine the resistance of all submitted samples of *M. tuberculosis* to all first-line antibiotics. This has to be done using the fastest methods available, and final results must be reported within 30 days after receiving the sample (32). Most laboratories incubate solid medium cultures for 8 weeks to achieve a better sensitivity (9). Several liquid-culture-based strategies, such as MB/BacT (Organon-Teknika), MGIT, BACTEC 460TB, and BACTEC 9000 MB (Becton Dickinson), have been developed that allow the mean time required for detection of *Mycobacterium tuberculosis* to be reduced to 8 to 18 days (1, 3, 22). Subsequent assessment of drug resistance may take another 3 weeks. Even with the most advanced methods the turnaround times are 3 to 15 days for the BACTEC 460TB system (23, 27), 5 to 11 days for the MB/BacT system (5), and 3 to 14 days for MGIT (26). Therefore, there is an urgent need to develop simple, fast, and cost-effective methods to identify drug resistance in mycobacteria that could be used for large-scale population screening, particularly for the screening of prisoners.

We focused our efforts on the resistance of *M. tuberculosis* to RIF, probably the most efficient anti-TB drug and a key component of modern chemotherapeutic cocktails of three to four drugs. RIF resistance could be considered a surrogate marker for multidrug-resistant TB strains (31) and should therefore be subject to the most rapid test performed.

The resistance of *M. tuberculosis* to RIF is caused by a

* Corresponding author. Mailing address: Biochip Technology Center, Engelhardt Institute of Molecular Biology, 32 Vavilova St., Moscow 119991, GSP-1, Russia. Phone: 7 (095) 135 0559. Fax: 7 (095) 135 1405. E-mail: amir@genome.eimb.relarn.ru.

number of mutations. About 95% of these mutations are confined to a short 81-bp-long DNA region in the gene *rpoB* encoding the β-subunit of RNA polymerase B, the so-called RIF-resistance-determining region (RRDR) (20, 31). A number of protocols have been developed to analyze the underlying sequence polymorphisms of RRDR, including direct sequencing (16), dideoxy fingerprinting (11), PCR-heteroduplex analysis (36), single-strand conformation polymorphism (28, 31), DNA probe arrays (13, 34), hybridization of PCR-amplified products to a limited set of probes (8, 15, 25, 35), and RNA mismatch analysis (21).

In the first approach we propose to use hybridization on an oligonucleotide microarray—MAGIChips (38)—to identify Rif^r strains of *M. tuberculosis* in clinical samples within 24 h. This method is based on the difference in stability between perfect and imperfect duplexes formed by the fluorescently labeled target DNA and the probes immobilized in gel pads of the dedicated TB biochip. The relative intensities of fluorescence of the pads indicate the presence and the nature of the mutations.

The second approach involves PCR amplification on MAGIChips. Using this method, the time to identify Rif^r strains is further shortened to 1.5 h. In addition, it does not require any special probe preparation and can be applied to simultaneous analysis of several variable segments of the bacterial genome.

Early detection of low-copy-number mutant DNA against a high background of wild-type DNA is an important practical goal that requires high sensitivity. The third approach, ligase detection reaction (LDR), is applied to identify about 1% of mutant sequences in model samples consisting of mixtures of DNA from wild-type and resistant strains.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides for hybridization analysis and on-chip PCR were designed using the programs Oligo 5 (Molecular Biology Insights, Cascade, Colo.) and Primer Calculator. The procedure included the following steps. First, melting temperatures for perfect matches were determined for prospective oligonucleotides. Second, the length of the oligonucleotides was adjusted to maintain the range of melting temperatures within 3 to 4°C for hybridization probes and 2 to 3°C for PCR primers.

Oligonucleotides were synthesized on an ABI-394 DNA/RNA synthesizer (Applied Biosystems, Foster City, Calif.) using standard phosphoramidite chemistry. Oligonucleotides were purified by reverse-phase high-performance liquid chromatography on C_{18}-Nucleosil columns (Sigma, St. Louis, Mo.) for hybridization and on-chip PCR and in denaturing polyacrylamide gel electrophoresis for on-chip LDR. To immobilize oligonucleotides in gel pads or to attach the fluorescent label Texas Red (Molecular Probes, Inc., Eugene, Oreg.), an amino group was introduced during synthesis using 3′-Amino-Modifier C7 CPG 500 or 5′-Amino-Modifier C6 (Glen Research, Sterling, Va.). The attachment of the fluorescent group to the amino group in oligonucleotides was carried out according to the manufacturer's instructions. Interrogating oligonucleotides were immobilized through their 3′ ends; oligonucleotide primers were immobilized or fluorescently labeled at their 5′ ends; fluorescent oligonucleotides that imitated target DNA with rare mutations were labeled at their 3′ ends.

***M. tuberculosis* strains.** *M. tuberculosis* strains were isolated from sputum, pleural exudate, bronchial lavage, urine, and cerebrospinal fluid of patients in central Russia (Moscow City and Moscow and Kaluga regions), in the Siberian regions of Russia (Novosibirsk and Tomsk), and in St. Petersburg, Russia. All cultures were grown on Löwenstein-Jensen (LJ) agar slants at 37°C for 8 weeks and were examined for growth rate, gross and microscopic colony morphology, and pigmentation and also were tested for niacin accumulation, nitrate reduction, and catalase and urease activity (18). All cultures underwent standard tests for RIF resistance using the absolute concentration method (14) as follows. The cultures were grown on LJ agar slants, and then the cells were resuspended in sterile saline (0.85% NaCl) to turbidity corresponding to a cell density of 5×10^8 cells per ml. The suspension was further diluted 10-fold with sterile saline, and a 0.2-ml aliquot was plated on LJ solid medium (control) and on the same medium containing 40 μg of RIF/ml. The samples that developed fewer than 20 colonies on RIF-containing medium and showed normal growth on control medium by the end of 4 weeks were considered RIF susceptible.

Preparation of DNA samples from *M. tuberculosis* cultures. One or two colonies of *M. tuberculosis*, 2 to 3 mm in diameter, were resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and centrifuged at 12,000 × *g* for 10 min at 4°C. The pellet was resuspended in 30 μl of TE buffer containing 1% Triton X-100 and incubated for 20 min at 95°C. The extracts were cooled on ice and centrifuged at 12,000 × *g* for 10 min, and 2-μl aliquots of clear supernatant were used for PCR.

Preparation of DNA samples from clinical specimens. Specimens of sputum, pleural exudate, and bronchial lavage (approximately 10 ml each) were decontaminated by treatment with *N*-acetylcysteine and 3% NaOH (17) for 40 min at room temperature. Cell extracts were then obtained by centrifugation and lysis as described above. Other bodily fluids (1.5 ml of urine or 0.5 ml of cerebrospinal fluid) were centrifuged at 10,000 × *g* for 10 min at 4°C. Cell extracts were prepared similarly to the procedure used for pure bacterial culture as described above. Two-microliter aliquots of clear supernatants were used for PCR.

Preparation of target DNA. Target samples of DNA from *M. tuberculosis* were prepared by two-stage PCR. At the first stage, a 193-bp-long fragment of the *rpoB* gene (nucleotides 2288 to 2480; GenBank accession no. L27989) was amplified using primers F105 (5′-CGT GGA GGC GAT CAC ACC GCA GAC GTT G-3′) and R273 (5′-GAC CTC CAG CCC GGC ACG CTC ACG T-3′). The reaction mixture contained 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 U of AmpliTaq DNA polymerase (PE Corporation, Norwalk, Conn.), 0.5 U of uracyl-DNA-glycosylase (Medigen, Moscow, Russia), 100 nM concentrations of each primer, 2 μl of DNA sample, and 0.2 mM (each) dATP, dCTP, dGTP, and dUTP. The reaction was carried out in a MiniCycler (MJ Research, Waltham, Mass.) as follows: 10 min at room temperature; 5 min at 95°C; 30 cycles of 30 s at 95°C and 40 s at 72°C; and 5 min at 72°C. Two microliters of the reaction mixture obtained after the first reaction was used for the second PCR.

In the second reaction, an internal 126-bp-long fragment (nucleotides 2336 to 2461 of the *rpoB* gene) of the first product was amplified using primers F1272 (5′-CGC CGC GAT CAA GGA GTT CT-3′) and R1398 (5′-TCA CGT GAC AGA CCG CCG GG-3′). The reaction mixture contained dTTP instead of the dUTP used in the first reaction mixture, no uracyl-DNA-glycosylase, a 100 nM concentration of fluorescently labeled F1272, and a 10 nM concentration of R1398 in 100 μl. Because of the difference in the concentrations of primers, the reaction yielded predominantly single-stranded DNA. Amplification was carried out as follows: 5 min at 95°C; 35 cycles of 20 s at 95°C, 30 s at 65°C, and 30 s at 72°C; and 5 min at 72°C.

For on-chip LDR, predominantly single-stranded lower-strand (antisense) DNA was amplified in a similar reaction. The F1272 primer was not labeled and was used at 10 nM, while R1398 was used at 100 nM. DNA-polymerase was removed by adding 1 μg of proteinase K (Sigma)/ml. The mixture was incubated for 15 min at 37°C, 10 min at 55°C, and then 10 to 15 min at 95°C to inactivate the proteinase.

PCR products were analyzed by electrophoresis in agarose gels.

Sequencing. The fragment of the *rpoB* gene that determines RIF resistance was amplified with primers F105 and R273 and subjected to automated dideoxy sequencing using one of the terminal primers, a commercial kit (Dye Deoxy Terminator ABI Sequencing Kit with *Taq*-Polymerase FS; PE Corporation), and an ABI-373A automatic sequencer (Applied Biosystems).

TB-MAGIChip with immobilized oligonucleotides. The MAGIChips were prepared as described earlier (38). Each chip consisted of a microscope slide with 169 (13 by 13) polyacrylamide gel pads created on its surface by photopolymerization. Each gel pad is a 100- by 100- by 20-μm block separated from adjacent blocks by a 200-μm-wide strip of hydrophobic surface. Each pad contained 1 pmol of an immobilized oligonucleotide probe. For hybridization, oligonucleotides were immobilized through their 3′ ends; for on-chip PCR and LDR they were immobilized through their 5′ ends.

On-chip hybridization. A hybridization mixture was prepared by adding 12 μl of the second-stage asymmetric PCR mixture to 24 μl of a solution containing 1.5 M guanidine thiocyanate (GuCNS), 0.075 M HEPES (pH 7.5), and 7.5 mM EDTA (unless noted otherwise). Twenty-eight microliters of the hybridization mixture was loaded on the chip and sealed in a hybridization chamber (10 by 10 mm) (in situ frame; Eppendorf Scientific, Westbury, N.Y.). The chamber was incubated for 18 h at 37°C. After hybridization the chip was washed three times at 37°C with 6.67× SSPE buffer, pH 7.4, containing 10% Tween 20, and was air dried.

On-chip PCR. On-chip PCR was described earlier (30). The reaction mixture contained 2.5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mg of bovine serum albumin/ml, 0.2 mM concentrations of each deoxynucleoside triphosphate, 5 U of the Stoffel fragment of *Taq* DNA-polymerase (PE Corporation), a 33 nM concentration of unlabeled forward primer (5′-CGC GAT CAA GGA GTT CTT CGG CAC C-3′), and a 330 nM concentration of fluorescently labeled reverse primer (5′-CCC GGC GGT CTG TAC GTG A-3′). This pair of primers amplified a 133-bp-long fragment of the *rpoB* gene (nucleotides 2339 to 2471). PCR was carried out as follows: 2 min at 95°C and 25 to 35 cycles of 30 s at 95°C, 60 s at 63°C, and 40 s at 72°C. The reaction was monitored in real time in all microchip elements with a fluorescence microscope equipped with a Peltier element (12).

On-chip LDR. For LDR, two interrogating oligonucleotide probes were immobilized through their 5′ ends in separate gel pads. They were designed to discriminate between the wild-type gene with His in position 526 (codon CAC; corresponding probe, 5′-CTA CCC GCT GTC GTG GTT GAC CCA-3′) and a mutant gene with the substitution His-526-Leu (codon CTC; corresponding probe, 5′-CTA CCC GCT GTC GTG GTT GAC CCT-3′). The reaction mixture contained 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, 0.01% Triton X-100, 1 mg of acetylated bovine serum albumin (Sigma)/ml, 40 to 240 nM single-stranded target DNA, 15 U of thermostable *Tth* DNA-ligase (Ampligase; Epicentre Technologies, Madison, Wis.), and 600 to 720 nM detecting oligonucleotide 5′-pCAA GCG CCG ACT GTA GGC ACT GGG-TR-3′. The latter was phosphorylated on the 5′ end and fluorescently labeled with Texas Red on the 3′ end.

The reaction chamber was incubated for 50 to 60 min at 40 to 42°C and then was placed on a thermal table, and five to eight cycles were carried out as follows: 10 s at 92°C, 16 min at 48 to 50°C. The chamber was rinsed with several changes of 0.15 M NaCl for 5 min at 92°C. The reaction chamber was then disassembled and the chip was rinsed with water and air dried, and its fluorescence was recorded.

Fluorescence measurement. All measurements were taken in real time using a setup of a fluorescence microscope equipped with a CCD camera, a thermal table with step motors and movement controller, and a computerized data acquisition system (12). Data collection and processing were performed using dedicated software, Special Hybridization Experiment Software, based on a LabVIEW interface (National Instruments, Austin, Tex.).

Statistical analysis of the data. The discriminating ability of each pair of oligonucleotides immobilized on the MAGIChip was assessed by the ratio $r = I_m/I_p$, where I_m is the fluorescence of the gel pad corresponding to an imperfect duplex and I_p is the fluorescence of the gel pad corresponding to a perfect duplex. Wild-type oligonucleotide probes were always located in the upper row of the array. Therefore, for wild-type target DNA, fluorescence of the upper gel pad in each column equaled I_p. For mutant target DNA, on the contrary, fluorescence of the gel pad corresponding to the mutation was assumed to equal I_p, while fluorescence of the corresponding wild-type gel pad equaled I_m.

After a series of measurements, a median value (M) of all r values was determined and the scattering of data (L) was assessed as the following quartile deviation (4):

$$L = 0.5 \times |M_2 - M_1|$$

where M_1 is the median for all values below M and M_2 is the median for all values above M.

RESULTS

Hybridization on TB-MAGIChip. The method for identification of Rif^r mutants of *M. tuberculosis* by hybridization on a dedicated MAGIChip includes three successive steps: (i) PCR amplification of clinical sample DNAs, (ii) asymmetric PCR to yield a fluorescently labeled predominantly single-stranded target DNA, and (iii) hybridization of the labeled product to the chip with gel pads carrying immobilized oligonucleotides. These oligonucleotides correspond to either the wild-type or the mutant sequence. As they form, correspondingly, a perfect or an imperfect match, the difference in these structures' stability enables one to discriminate between positive and negative hybridization signals by the intensity of fluorescence.

The TB-MAGIChip for identification of Rif^r strains of *M. tuberculosis* contains a call set of 42 oligonucleotides (Table 1). It can detect 30 of the most common mutations in the *rpoB* gene responsible for the resistance. Gel pads with immobilized oligonucleotides are arrayed in 12 columns (Fig. 1), each column corresponding to a single variable amino acid position. The upper gel pad in each column (a1 through a12) matches the wild-type sequence, i.e., forms a perfect duplex with the wild-type target DNA. Oligonucleotides immobilized in the gel pads below form perfect duplexes with different mutant variants of the same codon.

Figure 1A and C illustrate the hybridization of wild-type target DNA with the TB-MAGIChip. The fluorescence of the gel pads in the upper row is the brightest in each individual column. Only the oligonucleotides in these pads form perfect duplexes with the target DNA.

A mutation in the RRDR segment of the *rpoB* gene results in the formation of a perfect duplex and, therefore, bright fluorescence in a gel pad located in rows b through j. An example of hybridization of a mutant target DNA (His-526-Tyr) is shown in Fig. 1B and D. The fluorescence of the gel pad j10 is much higher than the fluorescence of a10, where the wild-type probe is immobilized, because this is the only pad in the column where a perfect duplex has formed.

Optimization of hybridization on MAGIChip. Melting temperatures of perfect duplexes formed with oligonucleotides immobilized on the TB-MAGIChip in 1 M NaCl are within the range of 58 to 63°C (Fig. 2A). To adapt hybridization to be performed at 37°C, 1 M GuCNS (or 20% formamide) was added to the hybridization buffer, which decreased the melting temperature to about 47°C (Fig. 2A). However, the mechanical strength of the gel pads incubated in the presence of GuCNS is higher than that in formamide.

The difference in the intensity of fluorescence between perfect and imperfect duplexes after hybridization reactions performed at different temperatures (Fig. 2B) was determined. This parameter defines the discrimination ability of the procedure, and it was at its highest level when the hybridization was performed at 37°C. Therefore, the optimal hybridization temperature in 1 M GuCNS was 37°C, 8 to 10°C below the melting temperature, and partial equilibrium was achieved after 14 h of incubation.

Every discriminating pair of oligonucleotides underwent control hybridization with a previously characterized DNA sample carrying the corresponding mutation. Some probes on the microchip corresponded to the mutations that were not available in clinical samples. These probes were successfully tested using synthetic DNA samples.

Analysis of mycobacterial DNA samples on TB-MAGIChip. DNA samples were isolated from 130 Rif^r strains of *M. tuberculosis* and hybridized with the TB-MAGIChip. In 128 of them, mutations in the RRDR were identified. The results of this experiment are summarized in Table 2. The results clearly demonstrate that Ser-531 and His-526 amino acid substitutions are found most frequently, in agreement with the published data (for a review, see reference 20). Nineteen samples representing different mutations as judged from their on-chip hybridization patterns were sequenced; the results of the sequencing fully confirmed the hybridization data. In one of the samples that showed a wild-type pattern when hybridized with

TABLE 1. Immobilized oligonucleotides for TB hybridization technique

Oligonucleotide[a]	Amino acid position	Amino acid substitution[b]	Nucleotide substitution	Sequence 5′→3′[c]	Nucleotide positions
a1	507	WT		GCT GTC TGG TGC CGA AGA	2370–2353
b1	507	DEL (2)	GGCACC	GGC TCA GCT GGC T-- --- -GA AGA A	2376–2352
a2	510	Gln (WT)	CAG	GCT CAG **CTG** GCT GGT GC	2375–2359
b2	510	Gln→His	CAG→CAT	TG GCT CAG **ATG** GCT GGT G	2377–2360
a3	511	Leu (WT)	CTG	AA TTG GCT **CAG** CTG GCT G	2380–2364
b3	511	Leu→Pro	CTG→CCG	AA TTG GCT **CGG** CTG GCT	2380–2364
c3	511	Leu→Arg	CTG→CGG	AA TTG GCT **CCG** CTG GCT	2380–2364
a4	512	Ser (WT)	AGC	AT GAA TTG **GCT** CAG CTG GC	2383–2365
b4	512	Ser→Thr	AGC→ACC	AT GAA TTG **GGT** CAG CTG GC	2383–2365
c4	512	Ser→Arg	AGC→CGC	AT GAA TTG **GCG** CAG CTG	2383–2367
a5	513	Gln (WT)	CAA	TC CAT GAA **TTG** GCT CAG CT	2386–2368
b5	513	Gln→Leu	CAA→CTA	TC CAT GAA **TAG** GCT CAG CT	2386–2368
c5	513	Gln→Lys	CAA→AAA	TC CAT GAA **TTT** GCT CAG CT	2386–2368
d5	513	Gln→Pro	CAA→CCA	TC CAT GAA **TGG** GCT CAG CT	2386–2368
a6	515	Met (WT)	ATG	GTT GTT CTG GTC **CAT** GAA TTG GC	2396–2374
b6	515	Met→Ile	ATG→ATA	GTT GTT CTG GTC **TAT** GAA TTG GC	2396–2374
a7	516	Asp (WT)	GAC	TT GTT CTG **GTC** CAT GAA T	2397–2376
b7	516	Asp→Tyr	GAC→TAC	TT GTT CTG **GTA** CAT GAA T	2397–2376
c7	516	Asp→Gly	GAC→GGC	TT GTT CTG **GCC** CAT GAA T	2397–2376
d7	516	Asp→Val	GAC→GTC	TT GTT CTG **GAC** CAT GAA T	2397–2376
e7	516	Asp→Glu	GAC→GAG	TT GTT CTG **CTC** CAT GAA T	2397–2376
a8	522	Ser (WT)	TCG	T CAA CCC **CGA** CAG CG	2414–2398
b8	522	Ser→Leu	TCG→TTG	T CAA CCC **CAA** CAG CG	2414–2398
a9	524	Leu (WT)	TTG	G CTT GTG GGT **CAA** CCT CGA	2421–2403
b9	524	Leu→Ser	TTG→TCC	G CTT GTG GGT **GGA** CCT CGA	2421–2403
a10	526	His (WT)	CAC	G GCG CTT **GTG** GGT CAA C	2424–2408
b10	526	His→Asp	CAC→GAC	G GCG CTT **GTC** GGT CAA C	2424–2408
c10	526	His→Leu	CAC→CTC	G GCG CTT **GAG** GGT CAA C	2424–2408
d10	526	His→Gln2	CAC→CAA	G GCG CTT **TTG** GGT CAA C	2424–2408
e10	526	His→Gln	CAC→CAG	G GCG CTT **CTG** GGT CAA C	2424–2408
f10	526	His→Cys	CAC→TGC	G GCG CTT **GCA** GGT CAA C	2424–2408
g10	526	His→Asn	CAC→AAC	G GCG CTT **GTT** GGT CAA C	2424–2408
h10	526	His→Arg	CAC→CGC	G GCG CTT **GCG** GGT CAA C	2424–2408
i10	526	His→Pro	CAC→CCC	G GCG CTT **GGG** GGT CAA C	2424–2408
j10	526	His→Tyr	CAC→TAC	G GCG CTT **GTA** GGT CAA C	2424–2408
a11	531	Ser (WT)	TCG	CAG CGC **CGA** CAG TCG	2438–2424
b11	531	Ser→Leu	TCG→TTG	C CAG CGC **CAA** CAG TCG	2439–2424
c11	531	Ser→Trp	TCG→TGG	CAG CGC **CCA** CAG TCG	2438–2424
d11	531	Ser→Gln	TCG→CAG	CAG CGC **CTG** CAG TCG	2438–2424
e11	531	Ser→Cys	TCG→TGT	CAG CGC **ACA** CAG TCG	2438–2424
a12	533	Leu (WT)	CTG	TG CCC **CAG** CGC CGA CAG	2443–2427
b12	533	Leu→Pro	CTG→CCG	TG CCC **CGG** CGC CGA CAG	2443–2427

[a] The designations of oligonucleotides correspond to their locations in Fig. 1.
[b] WT, wild type. DEL, deletion.
[c] The sequences belong to the antisense strand. Dashes indicate deleted nucleotides.

the chip, no mutations in the RRDR were found by sequencing (see Discussion).

Comparison between detection of RIF resistance in sputum by hybridization with TB-MAGIChip and by conventional drug susceptibility testing. Thirty-one samples of sputum from patients with clinically confirmed TB were each divided in two parts. One part was used to prepare target DNA; the amplified DNA was then hybridized with the TB-MAGIChip. The other part was used to isolate and identify *M. tuberculosis* by using standard clinical methods and then to determine its RIF resistance by the absolute concentration method.

The results of this experiment are summarized in Table 3. According to the hybridization results, 20 samples were categorized as RIF susceptible, while 11 contained mutations (Ser-531-Leu, 8 samples; Asp-516-Val, 2 samples; His-526-Arg, 1 sample).

Statistical evaluation of the hybridization results. The discrimination ability of the TB-MAGIChip was assessed by the *r* value (see Materials and Methods) for 18 pairs of wild-type and mutant oligonucleotide probes. In six independent series of experiments, hybridization was carried out with wild-type DNA and DNA from five different mutants: His-526-Tyr (CAC→TAC); Ser-522-Leu (TCG→TTG); Leu-533-Pro (CTG→CCC); His-526-Asn (CAC→AAC); and double mutant Leu-511-Arg (CTG→CGG) and Asp-516-Tyr (GAC→TAC). Each hybridization was performed eight times. Data for each individual pair were then grouped together, and median values and quartile deviations were determined (Table 4).

Identification of Rif^r mutants by on-chip PCR. The on-chip PCR approach is a modification of allele-specific PCR that had been considered as a tool for the detection of point mutations (30). The sequences of allele-specific immobilized primers and

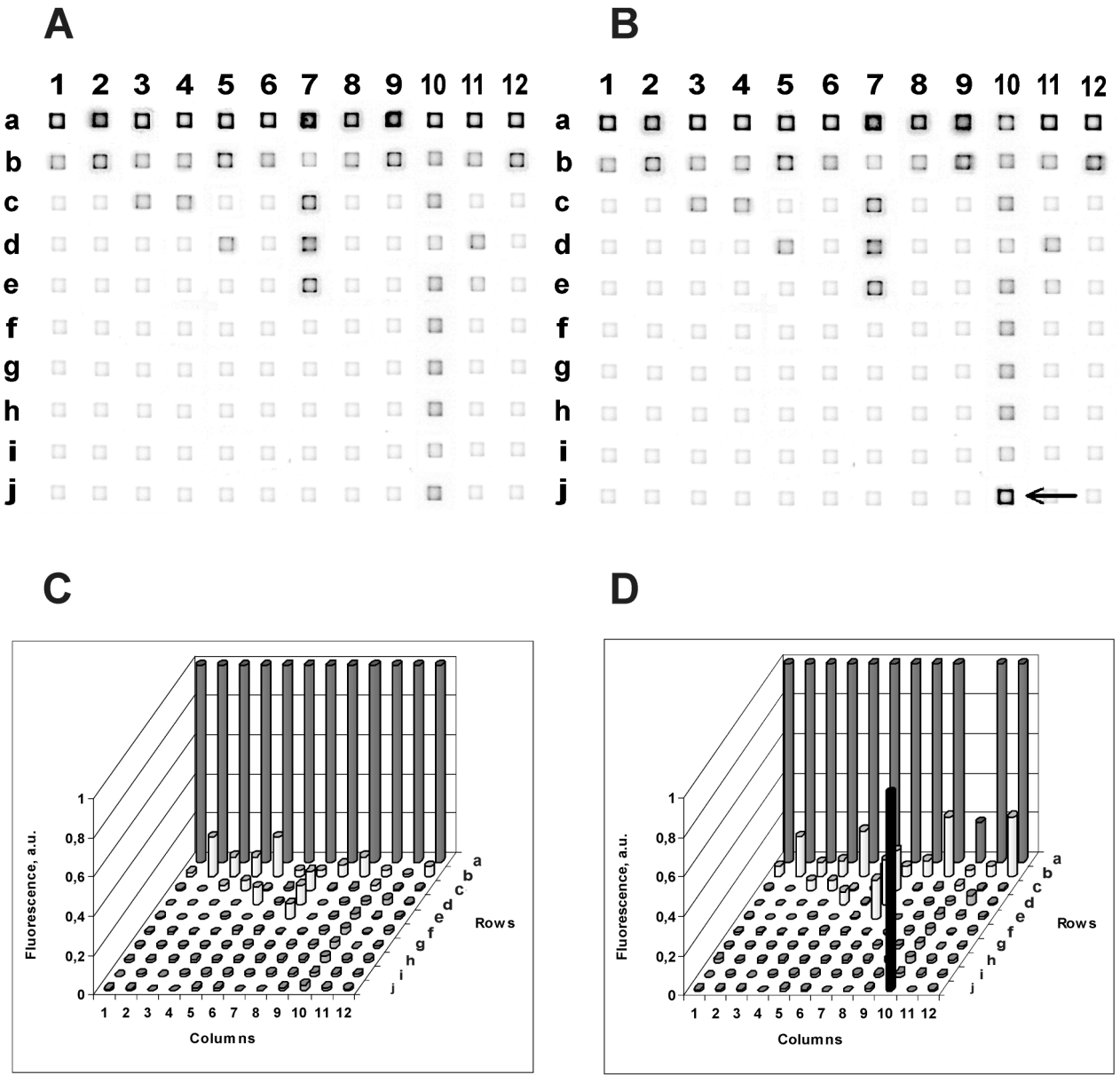

FIG. 1. The images (A and B) and intensities (C and D) of hybridization. The immobilized probes are listed in Table 1. (A and C) Wild-type target DNA; (B and D) His-526-Tyr mutant target DNA. The fluorescence intensities within each column were normalized to a maximal fluorescence signal corresponding to a perfect hybridization duplex. a. u., arbitrary units. The arrow in panel B points to the only gel pad where a perfect duplex formed, resulting in a higher level of fluorescence.

corresponding nucleotide and amino acid substitutions are listed in Table 5. During the first cycles, asymmetric PCR occurs in the liquid covering the chip and results in a single-stranded product with a fluorescently labeled primer at its 5′ end. Accumulation of this product leads to its hybridization to specific primers immobilized inside the gel pads and their extension. Long perfect duplexes formed during this process have significantly higher melting temperatures than do the short duplexes between the single-stranded PCR product and immobilized primers. Therefore, when the temperature exceeds that of primer annealing, fluorescence can be observed only in those gel pads in which the immobilized primer has been extended. Similar to the setup described for hybridization experiments, the interrogating sets for individual codons of RRDR were placed in columns with wild-type primers on top. For wild-type target DNA, the upper gel pads must be the brightest; for mutant samples, the most intense fluorescence was seen in one of the pads described below. An example of on-chip PCR with DNA from wild-type *M. tuberculosis* DNA and from the His-526-Asp (CAC→GAC) mutant is shown in Fig. 3. In further experiments, we tested DNA from 30 different mutant strains of *M. tuberculosis*, and the results were fully concordant with the hybridization analysis of the same samples.

Identification of Rif^r mutants by on-chip LDR. To test the LDR-based approach to identify Rif^r mutations, a chip was designed with two interrogating immobilized oligonucleotides. They discriminated between a wild-type sequence encoding

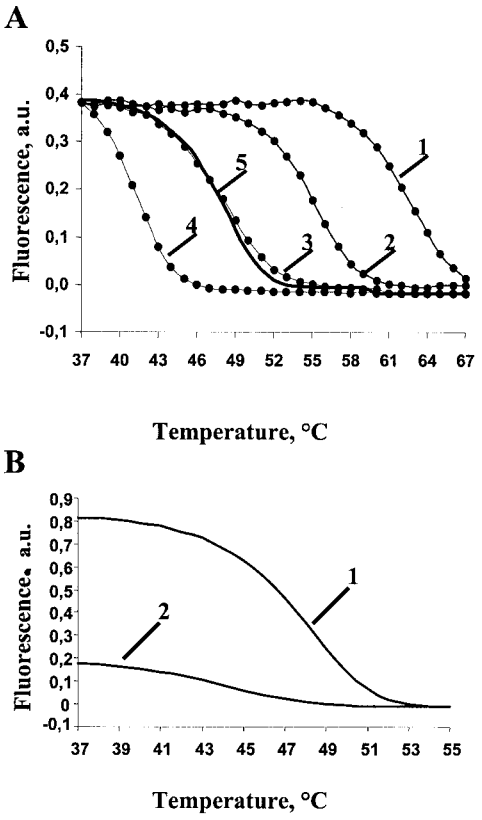

FIG. 2. Melting curves of hybridization duplexes. (A) Effect of buffer composition on the melting temperature of a perfect duplex formed between oligonucleotide probe a3 (Table 1) and a PCR-amplified fragment of the wild-type *rpoB* gene (labeled target DNA). The samples were melted in 1 M NaCl (curve 1), 1 M NaCl with 10% formamide (curve 2), 20% formamide (curve 3), 30% formamide (curve 4), or 1 M GuCNS (curve 5). (B) Melting curves of perfect (curve 1) and imperfect (curve 2) duplexes in 1 M GuCNS. The duplexes were formed by a PCR-amplified fragment of the wild-type *rpoB* gene and oligonucleotide probe a3 (perfect duplex) or b3 (imperfect duplex). a. u., arbitrary units.

His-526 (codon CAC) and a mutant sequence encoding Leu (codon CTC). The interrogating oligonucleotides had T or A in the 3′ position, resepectively. The reaction mixture contained the detecting oligonucleotide which hybridized with the target DNA immediately adjacent to the immobilized oligonucleotides and carried 5′ phosphate to make the ligation reaction possible and 3′-fluorescent label for detection. When the target DNA formed a perfect hybridization duplex with the immobilized interrogating nucleotide, the ligase covalently linked its 3′-terminal base to the 5′-terminal base of the detecting oligonucleotide which became an integral part of the immobilized oligonucleotide. To enhance the positive fluorescence signal, the reaction was carried out at elevated temperature and all participating compounds underwent multiple cycles of annealing, ligation, and melting. Therefore, thermostable ligase has been used.

The results of on-chip LDR are shown in Fig. 4. The gel pads on the right contained the oligonucleotide corresponding to the wild-type sequence, and those on the left contained the oligonucleotide corresponding to the mutant sequence. Using either wild-type or mutant DNA, the signal accumulated almost exclusively in the corresponding pads (rows a and e). When the samples were mixed in different proportions, various levels of fluorescence were detected in both gel pads. In particular, there was a definite signal in the left (mutant) gel pad when mutant DNA made up just 1% of the total target DNA (row b). In control experiments, neither variations in the amount of oligonucleotides immobilized in the gel pads nor changes in the concentration of detecting oligonucleotide and target DNA resulted in false-positive or -negative signals (not shown).

TABLE 2. Distribution of *rpoB* mutations in Rif^r *M. tuberculosis* strains

Mutation type(s)	No. of strains
Leu511→Pro	1
Gln513→Leu	1
Asp516→Tyr	2
Asp516→Val	5
Ser522→Leu	2
His526→Arg	1
His526→Asp	7
His526→Asn	5
His526→Cys	1
His526→Leu	1
His526→Tyr	5
Ser531→Leu	85
Ser531→Trp	1
Leu533→Pro	10
Leu511→Pro, His526→Asn	1
Wild type	2
Total	130

DISCUSSION

Molecular analysis of *M. tuberculosis* is a promising alternative to bacteriological assays. Using the state-of-the-art technology of oligonucleotide microchips, we developed three approaches to the detection of *M. tuberculosis* resistance to RIF in both cultures and clinical samples: hybridization, on-chip PCR, and on-chip LDR on dedicated TB-MAGIChips.

The use of the MAGIChip offers several advantages for RIF resistance testing over alternative microchip technologies (34), e.g., GeneChips developed by Affymetrix, Inc. (Santa Clara, Calif.), and manufactured by solid-phase chemical synthesis of oligonucleotides with photolithographic fabrication techniques. The MAGIChips are lower in cost, simpler to use, and more efficient in discrimination of the perfect and mismatched duplexes (12). Because of the better discrimination capacity, the analysis of specific sequences can be performed using a

TABLE 3. Comparison of RIF susceptibilities of *M. tuberculosis* in sputum specimens as assessed by on-chip hybridization and conventional drug susceptibility testing

On-chip hybridization result	Standard drug susceptibility testing result[a]		
	No. resistant	No. susceptible	Total
Resistant	11	0	11
Susceptible	2	18	20
Total	13	18	31

[a] Absolute concentration method was used. For details, see Materials and Methods.

TABLE 4. Discriminatory ability of the TB-MAGIChip for different mutations in the *rpoB* gene

Amino acid substitution	Nucleotide sequences of the probes[a]	Statistical analysis of *r* values[b]	
		M	*L*
Gln510→His	GCT CAG (**C**/A)TG GCT GGT GC	0.41	0.09
Leu511→Arg	AAT TGG CTC (**A**/C)GC TGG CT	0.03	<0.01
Leu511→Pro	AAT TGG CTC (**A**/G)GC TGG CT	0.33	0.02
Asp516→Val	GGT TGT TCT GG(**T**/A) CCA TGA ATT G	0.23	0.02
Asp516→Glu	GGT TGT TCT G(**G**/C)T CCA TGA ATT G	0.01	<0.01
Asp516→Tyr	GGT TGT TCT GGT (**C**/A)CA TGA ATT G	0.03	0.06
Asp516→Gly	GGT TGT TCT GG(**T**/C) CCA TGA ATT G	0.02	0.04
Ser522→Leu	GGT CAA CCC C(**G**/A)A CAG CG	0.06	0.02
His526→Asp	GGC GCT TGT (**G**/C)GG TCA AC	0.01	<0.01
His526→Leu	GGC GCT TG(**T**/A) GGG TCA AC	0.16	<0.01
His526→Gln2	GGC GCT T(**G**/T)T GGG TCA AC	0.15	0.02
His526→Gln1	GGC GCT T(**G**/C)T GGG TCA AC	0.01	<0.01
His526→Cys	GGC GCT TG(**T**/C) (**G**/A)GG TCA AC	0.01	<0.01
His526→Asn	GGC GCT TGT (**G**/T)GG TCA AC	0.01	0.01
His526→Arg	GGC GCT TG(**T**/C) GGG TCA AC	0.02	<0.01
His526→Pro	GGC GCT TG(**T**/G) GGG TCA AC	0.08	0.01
His526→Tyr	GGC GCT TGT (**G**/A)GG TCA AC	0.01	<0.01
Leu533→Pro	TGC CCC (**A**/G)GC GCC GAC AG	0.01	0.01

[a] Nucleotides corresponding to the wild-type sequence are shown in bold.
[b] $r = I_m/I_p$, where I_m and I_p are the fluorescence intensity of imperfect and perfect duplexes, respectively. *M*, median; *L*, scattering of data (for details, see Materials and Methods).

very limited call set of oligonucleotides necessary and sufficient for identification of known mutations. The latter consideration dramatically decreases the cost of chip manufacturing and eliminates the need for cumbersome mathematical interpretation of the results.

Furthermore, the readout of the results may be carried out not only by using the fluorescence microscope setup described earlier (12) but also by a simplified version of the chip analyzer developed in our laboratory (2). The latter device can be equipped with either a CCD camera or a Polaroid camera and costs under $2,000. In particular, the use of a Polaroid camera enables one to analyze the results visually without any computations.

Finally, three-dimensional polyacrylamide gel pads offer much higher capacity and therefore a stronger fluorescence signal than the two-dimensional glass surface. Although there are certain steric hindrances that limit even distribution of the reacting molecules throughout the gel pads and result in higher intensity of the fluorescence at their periphery, this does not affect signal discrimination. Presently we are investigating ways to increase the porosity of the gel in order to make it more accessible for various components of chemical reactions, particularly larger DNA molecules.

Optimization of hybridization on TB-MAGIChip for clinical laboratories. The conditions were optimized to perform hybridization at 37°C (Fig. 2), which is convenient for clinical laboratories.

Initially, we found that the absolute fluorescence intensities in individual gel pads of the TB-MAGIChip varied widely, and this made visual interpretation of the results rather difficult. We attempted to equalize the intensity of positive signals by adjusting the length of the probes. However, for some probes this still did not help. Apparently, under nonequilibrium conditions the formation of secondary structures by both target DNA and immobilized oligonucleotides affects the yield of annealed duplexes and therefore the intensity of the corresponding fluorescence signals.

Sensitivity and specificity of hybridization and on-chip PCR. In our experiments with Rif^r strains, 128 out of 130 samples were mutant as judged by hybridization with the TB-MAGIChip; therefore, in a real clinical situation they would be correctly classified as resistant. Hence, the accuracy of the hybridization protocol was better than 98%. Sequencing of the RRDR from one of the Rif^r samples that gave a wild-type hybridization pattern on the TB-MAGIChip confirmed the absence of any mutations. This is not surprising, since in about 4% of all Rif^r *M. tuberculosis* strains the resistance is not determined by mutations in RRDR (20).

In primary specimens, the detection of RIF susceptibility by hybridization with the MAGIChip showed good correlation with bacteriological testing obtained by the absolute concentration method (accuracy was better than 93%). The limited number of tested specimens (31 altogether) calls for cautious interpretation of the results. Nevertheless, the specificity of the procedure (i.e., the ability to detect true susceptibility) was 100% and the sensitivity of the procedure (i.e., the ability to detect true resistance) was reasonably high (85%). The positive predictive value of a resistant result was 100%, while the negative predictive value was 90%.

These data indicate that hybridization with the TB-MAGIChip could be a promising approach to fast detection of RIF sensitivity of TB pathogens directly in clinical samples. The analysis takes less than 24 h and is much faster than the most advanced bacteriological methods. False-negative results obtained by hybridization with the TB-MAGIChip may be caused by several factors: low content of Rif^r mycobacteria in the primary population, resistance independent of mutations in RRDR, and rare mutations that do not have complementary probes in the standard call set on the chip. Despite all these drawbacks, the high positive predictive value of the hybridiza-

TABLE 5. Immobilized primers for on-chip PCR amplification

Oligonucleotide[a]	Amino acid position	Amino acid substitution[b]	Nucleotide substitution	Sequence (5′→3)[c]	Sequence position
a1pcr	513	Gln (WT)	CAA	TTCGGCACCAGCCAGCCAGCTGAGCCA	2355–2378
b1pcr	513	Gln→Leu	CAA→CTA	TTCGGCACCAGCCAGCCAGCTGAGCCt	2355–2377
c1pcr	513	Gln→Lys	CAA→AAA	TTCGGCACCAGCCAGCCAGCTGAGCa	2355–2376
d1pcr	513	Gln→Pro	CAA→CCA	TTCGGCACCAGCCAGCCAGCTGAGCCc	2355–2377
a2pcr	515	Met (WT)	ATG	TCGGCACCAGCCAGCTGAGACAATTCATG	2356–2384
b2pcr	515	Met→Ile	ATG→ATA	TCGGCACCAGCCAGCTGAGACAATTCATa	2356–2384
a3pcr	516	Asp (WT)	GAC	GCACCAGCCAGCTGAGACAATTCATGGAC	2359–2387
b3pcr	516	Asp→Tyr	GAC→TAC	GCACCAGCCAGCTGAGCCAATTCATGt	2359–2385
c3pcr	516	Asp→Gly	GAC→GGC	GCACCAGCCAGCTGAGACAATTCATGGg	2359–2386
d3pcr	516	Asp→Val	GAC→GTC	GCACCAGCCAGCTGAGACAATTCATGGt	2359–2386
e3pcr	516	Asp→Glu	GAC→GAG	GCACCAGCCAGCTGAGACAATTCATGGAg	2359–2387
a4pcr	522	Ser (WT)	TCG	GCCAATTCATGGACCAGAACAACCCGCTGTC	2374–2404
b4pcr	522	Ser→Leu	TCG→TTG	GCCAATTCATGGACCAGAACAACCCGCTGTt	2374–2404
a5pcr	524	Leu (WT)	TTG→TCC	CATGGACCAGAACAACCCGCTGTCGAGTTG	2381–2410
b5pcr	524	Leu→Ser	TTG→TCC	CATGGACCAGAACAACCCGCTGTCGAGTTc	2381–2420
a6pcr	526	His (WT)	CAC	CCAGAACTACCCGCTGTCGTGGTTGACCC	2387–2415
b6pcr	526	His→Leu	CAC→CTC	CCAGAACTACCCGCTGTCGTGGTTGACCCt	2387–2416
c6pcr	526	His→Asp	CAC→GAC	CCAGAACTACCCGCTGTCGTGGTTGACCg	2387–2415
d6pcr	526	His→Gln2	CAC→CAA	CCAGAACTACCCGCTGTCGTGGTTGACCCAa	2387–2417
e6pcr	526	His→Gln	CAC→CAG	CCAGAACTACCCGCTGTCGTGGTTGACCCAg	2387–2417
f6pcr	526	His→Cys	CAC→TGC	CCAGAACTACCCGCTGTCGTGGTTGACCTg	2387–2416
g6pcr	526	His→Asn	CAC→AAC	CCAGAACTACCCGCTGTCGTGGTTGACCa	2387–2415
h6pcr	526	His→Arg	CAC→CGC	CCAGAACTACCCGCTGTCGTGGTTGACCCg	2387–2416
i6pcr	526	His→Pro	CAC→CCC	CCAGAACTACCCGCTGTCGTGGTTGACCCc	2387–2416
j6pcr	526	His→Tyr	CAC→TAC	CCAGAACTACCCGCTGTCGTGGTTGACCt	2387–2415
a7pcr	531	Ser (WT)	TCG	GGTTGACCAACAAGCGCCGACTGTC	2407–2431
b7pcr	531	Ser→Leu	TCG→TTG	GGTTGACCAACAAGCGCCGACTGTt	2407–2431
c7pcr	531	Ser→Trp	TCG→TGG	GGTTGACCAACAAGCGCCGACTGTGg	2407–2431
d7pcr	531	Ser→Gln	TCG→CAG	GGTTGACCAACAAGCGCCGACTGca	2407–2431
e7pcr	531	Ser→Cys	TCG→TGT	GGTTGACCAACAAGCGCCGACTGTGt	2407–2432
a8pcr	533	Leu (WT)	CTG	TGACCCACAAGCGCCGACTGTCGGAGCT	2413–2437
b8pcr	533	Leu→Pro	CTG→CCG	TGACCCACAAGCGCCGACTGTCGGAGc	2413–2437

[a] The designations of oligonucleotides correspond to their locations in Fig. 3.
[b] WT, wild type.
[c] The sequences belong to the antisense strand. The variable 3′ nucleotides are shown in lowercase.

tion results enables physicians to exclude RIF from the treatment regimen and seek an alternative drug.

There was full concordance between the analysis by hybridization on TB-MAGIChip and on-chip PCR; in addition, the latter protocol can be carried out in just 1.5 to 2 h.

Reproducibility of hybridization results. For statistical purposes, the *r* values were averaged using the median, which is less sensitive to random deviations than other averages (4). One of the reasons for observed experimental deviations could be minor differences in the process of chip manufacturing.

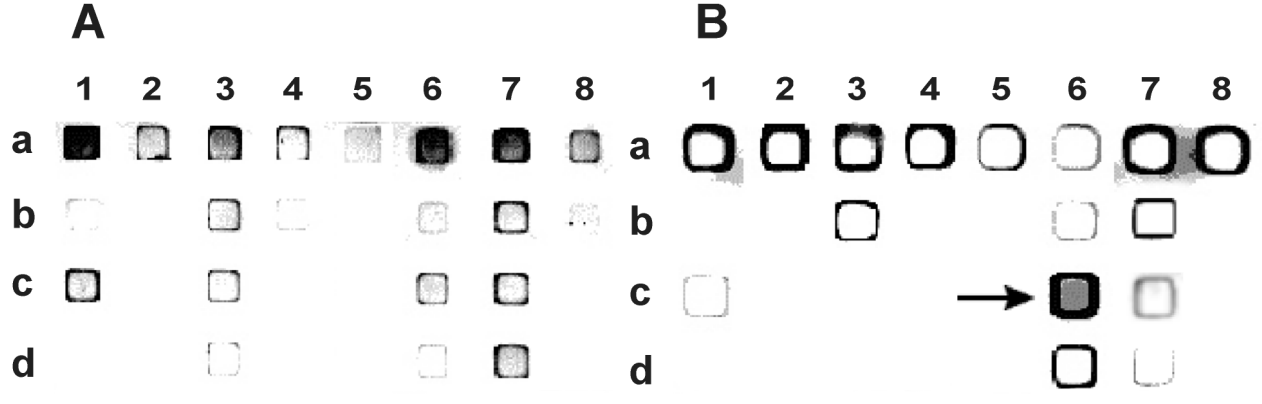

FIG. 3. Allele-specific on-chip PCR. A strong fluorescence signal is observed when the 3′ nucleotide of the immobilized primer is complementary to target DNA, extended by *Taq* polymerase, and forms a stable duplex with the fluorescently labeled target DNA. Immobilized primers are listed in Table 5. (A) Wild-type target DNA; (B) His-526-Asp mutant target DNA (CAC→GAC); the corresponding gel pad is marked with an arrow.

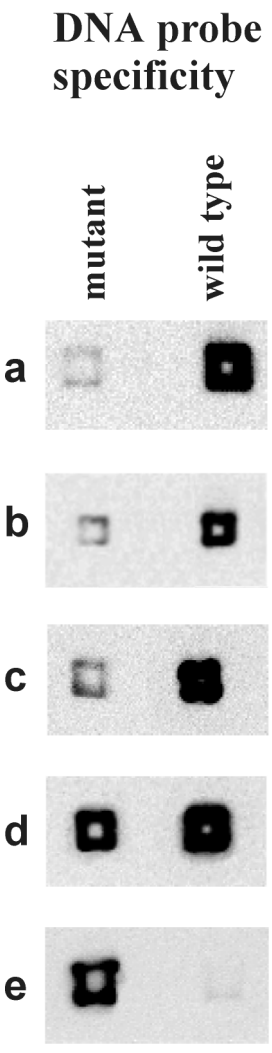

FIG. 4. Detection of mutant DNA by on-chip LDR. Each reaction mixture contained a total of 3 pmol of single-stranded DNA. Reaction a was performed with wild-type DNA; reaction e was performed with His-526-Leu mutant DNA; other reactions contained 3 pmol of wild-type DNA with a mixture of 1% (b), 2% (c), or 10% (d) of the mutant DNA.

Even for mutations with the most significant scattering of data (substitutions Gln-510-His and Leu-511-Pro), the quartile deviation was well within the limit of confidence (Table 4), i.e., it allowed for reliable discrimination between positive and negative signals. Indeed, positive signals in these cases were still 2.5 and 3 times stronger than negative signals, respectively. This is sufficient to identify mutations visually without any special equipment. In other words, in no case did chip-to-chip or probe-to-probe variations result in the inversion of positive and negative readings, regardless of the observed scattering of data and fluctuations of fluorescence intensity for individual oligonucleotide probes.

On-chip LDR. The method of on-chip LDR is especially attractive for simultaneously identifying several variants of the *rpoB* gene against a high background of the wild-type gene. We succeeded in detecting a variant gene that made up only 1% of the target sample. It has to be noted that this level of sensitivity has been chosen as an arbitrary threshold for the bacteriological definition of RIF sensitivity (37).

Several advantages offered by TB-MAGIChips and their potentially low cost make them attractive enough for large-scale commercial production. The on-chip PCR technique can also be extended to analysis of genes responsible for resistance to other drugs for which genetic determinants of resistance are known. One such method was described recently (33). It enables one to simultaneously analyze several mutations in three different genes responsible for resistance to RIF, isoniazid, and streptomycin.

ACKNOWLEDGMENTS

This work was supported by the Moscow Government (a262), by grant no. 5/2000 from the Russian Human Genome Program, and by the U.S. Department of Energy at the initial stage of research.

We are grateful to V. Chupeeva and E. Kreindlin for the manufacturing of microchips, to I. Taran, S. Surzhikov, and B. Chernov for the synthesis of oligonucleotides, to E. Vishnevskaja and S. Tatkov for bacterial DNA samples, and to V. Barsky for kindly providing the portable chip analyzer prior to publication of the manuscript. The assistance of Health Front Line, Ltd. (Champaign, Ill.), in the preparation of the manuscript is appreciated.

REFERENCES

1. **Badak, F. Z., D. L. Kiska, S. Setterquist, C. Hartley, M. A. O'Connell, and R. L. Hopfer.** 1996. Comparison of mycobacteria growth indicator tube with BACTEC 460 for detection and recovery of mycobacteria from clinical specimens. J. Clin. Microbiol. **34:**2236–2239.
2. **Bavykin, S. G., J. P. Akowski, V. M. Zakhariev, V. E. Barsky, A. N. Perov, and A. D. Mirzabekov.** 2001. Portable system for microbial sample preparation and oligonucleotide microarray analysis. Appl. Environ. Microbiol. **67:** 922–928.
3. **Benjamin, W. H., Jr., K. B. Waites, A. Beverly, L. Gibbs, M. Waller, S. Nix, S. A. Moser, and M. Willert.** 1998. Comparison of MB/BacT system with a revised antibiotic supplement kit to the BACTEC 460 system for detection of mycobacteria in clinical specimens. J. Clin. Microbiol. **36:**3234–3238.
4. **Bocharov, P. P.** 1994. Series criterion based on median, p. 164. *In* A. B. Pechinkin (ed.), Mathematical statistics. RUDN, Moscow, Russia.
5. **Brunello, F., and R. Fontana.** 2000. Reliability of the MB/BacT system for testing susceptibility of *Mycobacterium tuberculosis* complex isolates to antituberculosis drugs. J. Clin. Microbiol. **38:**872–873.
6. **Cohn, D. L., F. Bustreo, and M. C. Raviglione.** 1997. Drug-resistant tuberculosis: review of the worldwide situation and the W. H. O./IUATLD global surveillance project. Clin. Infect. Dis. **24:**S121–S130.
7. **Coninx, R., G. E. Pfyffer, C. Mathieu, D. Savina, M. Debacker, F. Jafarov, I. Jabrailov, A. Ismailov, F. Mirzoev, R. de Haller, and F. Portaels.** 1998. Drug resistant tuberculosis in prisons in Azerbaijan: case study. Br. Med. J. **316:** 1423–1425.
8. **De Beenhouwer, H., Z. Lhiang, G. Jannes, W. Mijs, L. Machtelinckx, R. Rossau, H. Traore, and F. Portaels.** 1995. Rapid detection of rifampin resistance in sputum and biopsy specimens from tuberculosis patients by P. C. R. and line probe assay. Tuber. Lung Dis. **76:**425–430.
9. **Drobniewski, F. A., B. Watt, E. G. Smith, J. G. Magee, R. Williams, J. Holder, and J. Ostrowski.** 1999. A national audit of the laboratory diagnosis of tuberculosis and other mycobacterial diseases within the United Kingdom. J. Clin. Pathol. **52:**334–337.
10. **Farmer, P. E., A. S. Kononets, S. E. Borisov, A. Goldfarb, T. Healing, and M. McKee.** 1999. Recrudescent tuberculosis in the Russian Federation, p. 41–83. *In* The global impact of drug resistant tuberculosis. Program in Infectious Disease and Social Change. Harvard Medical School, Boston, Mass.
11. **Felmlee, T. A., Q. Lin, and A. C. Whelen.** 1995. Genomic detection of *Mycobacterium tuberculosis* rifampin resistance: comparison of single-strand conformation polymorphism and dideoxy fingerprinting. J. Clin. Microbiol. **33:**1617–1623.
12. **Fotin, A., A. Drobyshev, D. Proudnikov, A. Perov, and A. Mirzabekov.** 1998. Parallel thermodynamic analysis of duplexes on oligodeoxyribonucleotide microchips. Nucleic Acids Res. **26:**1515–1521.
13. **Gingeras, T. R., G. Ghandour, E. Wang, A. Berno, P. M. Small, F. Drobniewski, D. Alland, E. Desmond, M. Holodniy, and J. Drenkow.** 1998. Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic *Mycobacterium* DNA arrays. Genome Res. **8:**435–448.
14. **Heifets, L. B.** 1991. Drug susceptibility tests in the management of chemotherapy of tuberculosis, p.90–121. *In* L. B. Heifets (ed.), Drug susceptibility

of mycobacterial infections. CRC Press, Boca Raton, Fla.
15. **Hirano, K., C. Abe, and M. Takahashi.** 1999. Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. J. Clin. Microbiol. **37:**2663–2666.
16. **Kapur, V., L. Li, S. Iordanescu, M. R. Hamrick, A. Wanger, B. N. Kreiswirth, and J. M. Musser.** 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase B subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. J. Clin. Microbiol. **32:**1095–1098.
17. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology. A guide for the level III laboratory. U. S. Department of Health and Human Services. Centers for Disease Control and Prevention, Atlanta, Ga.
18. **Metchok, B., F. S. Nolte, and R. J. Wallace, Jr.** 1999. Mycobacterium, p. 399–437. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C.
19. **Moore, M., I. M. Onorato, E. McCray, and K. G. Castro.** 1997. Trends in drug-resistant tuberculosis in the United States, 1993–1996. JAMA **278:**833–837.
20. **Musser, J. M.** 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin. Microbiol. Rev. **8:**496–514.
21. **Nash, K. A., A. Gaytan, and C. B. Inderlied.** 1997. Detection of rifampin resistance in *Mycobacterium tuberculosis* by means of a rapid, simple, and specific RNA/RNA mismatch assay. J. Infect. Dis. **176:**533–536.
22. **Pfyffer, G. E., C. Cieslak, H.-M. Welscher, P. Kissling., and S. Ruesch-Gerdes.** 1997. Rapid detection of *Mycobacteria* in clinical specimens by using the automated BACTEC 9000 MB system and comparison with radiometric and solid-culture systems. J. Clin. Microbiol. **35:**2229–2234.
23. **Rastogi, N., K. S. Goh, and H. L. David.** 1989. Drug susceptibility testing in tuberculosis: a comparison of the proportion method using Löwenstein-Jensen, Middlebrook 7H10 and 7H11 agar media and a radiometric method. Res. Microbiol. **140:**405–417.
24. **Raviglione, M. C., D. E. Snider, and A. Kochi.** 1995. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. JAMA **273:**220–226.
25. **Rossau, R., H. Traore, H. de Beenhouwer, W. Mijs, G. Jannes, P. de Rijk, and F. Portaels.** 1997. Evaluation of the INNO-LIPA Rif TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob. Agents Chemother. **41:**2093–2098.
26. **Rüsch-Gerdes, S., C. Domehl, G. Nardi, M. R. Gismondo, H.-M. Welscher, and G. E. Pfyffer.** 1999. Multicenter evaluation of the mycobacteria growth indicator tube for testing susceptibility of *Mycobacterium tuberculosis* to first-line drugs. J. Clin. Microbiol. **37:**45–48.
27. **Siddiqi, S. H., J. E. Hawkins, and A. Laszlo.** 1985. Interlaboratory drug susceptibility testing of *Mycobacterium tuberculosis* by radiometric procedure and two conventional methods. J. Clin. Microbiol. **22:**919–923.
28. **Sreevatsan, S., J. B. Bookout, F. M. Ringpis, S. L. Mogazeh, B. N. Kreiswirth, R. R. Pottathil, and R. R. Barathur.** 1998. Comparative evaluation of cleavase fragment length polymorphism with PCR-SSCP and PCR-RFLP to detect antimicrobial agent resistance in *Mycobacterium tuberculosis.* Mol. Diagn. **3:**81–91.
29. **Stepanshina, V. N., E. A. Panfertsev, O. V. Korobova, I. G. Shemyakin, Y. G. Stepanshin, I. M. Medvedeva, and I. R. Dorozhkova.** 1999. Drug-resistant strains of Mycobacterium tuberculosis isolated in Russia. Int. J. Tuberc. Lung Dis. **3:**149–152.
30. **Strizhkov, B. N., A. L. Drobyshev, V. M. Mikhailovich, and A. D. Mirzabekov.** 2000. PCR amplification on a microarray of gel-immobilized oligonucleotides: detection of bacterial toxin- and drug-resistant genes and their mutations. BioTechniques **29:**844–854.
31. **Telenti, A., P. Imboden, F. Marchesi, D. Lowrie, S. Cole, M. J. Colston, L. Matter, K. Schopfer, and T. Bodmer.** 1993. Detection of rifampin-resistance mutations in *Mycobacterium tuberculosis.* Lancet **341:**647–650.
32. **Tenover, F. C., J. T. Crawford, R. E. Huebner, L. J. Geiter, C. R. Horsburgh, Jr., and R. C. Good.** 1993. The resurgence of tuberculosis: is your laboratory ready? J. Clin. Microbiol. **31:**767–770.
33. **Tillib, S., B. Strizhkov, and A. Mirzabekov.** Integration of multiple PCR amplifications and DNA mutation analysis by using oligonucleotide microchip. Anal. Biochem., in press.
34. **Troesch, A., H. Nguyen, C. G. Miyada, S. Desvarenne, T. R. Gingeras, P. M. Kaplan, P. Cross, and C. Mabilat.** 1999. *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays. J. Clin. Microbiol. **37:**49–55.
35. **Victor, T. C., A. M. Jordaan, A. van G. Rie, D. van der Spuy, M. Richardson, P. D. van Helden, and R. Warren.** 1999. Detection of mutations in drug resistance genes of *Mycobacterium tuberculosis* by a dot-blot hybridization strategy. Tuber. Lung Dis. **79:**343–348.
36. **Williams, D. L., C. Waguespack, K. Eisenach, J. T. Crawford, F. Portaels, M. Salfinger, C. M. Nolan, C. Abe, V. Sticht-Groh, and T. P. Gillis.** 1994. Characterization of rifampin resistance in pathogenic mycobacteria. Antimicrob. Agents Chemother. **38:**2380–2386.
37. **World Health Organization/IUATLD Global Working Group on Tuberculosis Drug Resistance Surveillance.** 1997. Guidelines for surveillance of drug resistance in tuberculosis. World Health Organization publication WHO/TB/96.216. World Health Organization, Geneva, Switzerland.
38. **Yershov, G., V. Barsky, A. Belgovskiy, E. Kirillov, E. Kreindlin, I. Ivanov, S. Parinov, D. Guschin, A. Drobyshev, S. Dubiley, and A. Mirzabekov.** 1996. DNA analysis and diagnostics on oligonucleotide microchips. Proc. Natl. Acad. Sci. USA **93:**4913–4918.