METHODS

Detection of Rifampicin-Resistant *Mycobacterium tuberculosis* Strains by Hybridization and Polymerase Chain Reaction on a Specialized TB-Microchip

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Two alternative methods for identification of rifampicin-resistant strains of *Mycobacterium tuberculosis* on biological microchips are developed. The methods are based on detection of point mutations and other rearrangements in the *rpoB* gene region determining rifampicin resistance. Hybridization on TB-microchip detects 30 mutant variants of DNA in rifampicin-resistant strains (about 95% of all resistant forms). Allele-specific microchip PCR shortens the duration of analysis to 1.5 h. These methods can be used in clinical diagnostic laboratories for evaluating drug resistance/sensitivity of tuberculosis agent and for monitoring of the efficiency of antibiotic therapy.

Key Words: *Mycobacterium tuberculosis; microchip; rifampicin; hybridization; polymerase chain reaction*

According to WHO data, tuberculosis is responsible for 3 million deaths in the world annually [8]. The disease is highly prevalent in countries with low economic level and in developed counties. The situation is aggravated by overall prevalence of drug-resistant and polyresistant forms of *Mycobacterium tuberculosis* [1].

Laboratory diagnosis of tuberculosis and subsequent evaluation of drug resistance of the isolated agent by routine methods takes 6-9 weeks [7,9]. At the same time, rapid evaluation of drug resistance is significant for choosing effective drug therapy and preventing the propagation of resistant strains. Therefore, development of new rapid methods for evaluation of *M. tuberculosis* drug resistance is an important problem.

Resistance to rifampicin, one of the most effective and widely used antimycobacterial drugs, in 96% cases develops due to point mutations or short insertions or deletions in a 81 b.p. fragment of *rpoB* gene (rifampicin resistance determining region, RRDR) [6]. Apart from routine methods of evaluation of rifampi-

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cin resistance, molecular genetic methods based on the analysis of RRDR sequence are used [2,3,5,11,13,14].

The method of microchip hybridization proposed here differs from the previously described method [12] by low costs and the possibility of commercial manufacture and wide use in clinical diagnostic laboratories.

The other method described in this paper is based on allele-specific polymerase chain reaction (PCR) performed directly on the microchip. Its obvious advantage is that the data on rifampicin resistance of mycobacterial strains in the clinical sample can be obtained within 1.5 h.

MATERIALS AND METHODS

Oligonucleotides were synthesized on an automated 394 DNA/RNA synthesizer (Applied Biosystems) and contained a spacer with a free amino acid group 3'Amino-Modifier C7 CPG 500 (oligonucleotides for hybridization) or 5'-Amino-Modifier C6 (primers for microchip PCR) (both Glen Research). Oligonucleotides were labeled with fluorescent label Texas Red (Molecular Probes Inc.) as recommended by the manufacturer.

A total of 131 cultures of *M. tuberculosis* with known rifampicin sensitivity were collected from patients from Moscow, Moscow region, Tomck, Novosibirsk, Kaluga, Bryansk, and St.-Petersburg.

Mycobacterial cells were washed with a buffer containing 10 mM Tris-HCl, 1 mM EDTA (pH 8.0), precipitated by centrifugation at 10,000*g* for 10 min at 4°C, and heated in the initial buffer with 1% Triton X-100 at 95°C for 10 min. The lysates were centrifuged at 10,000*g* for 10 min, and the supernatant (1-2 μl) was used in PCR.

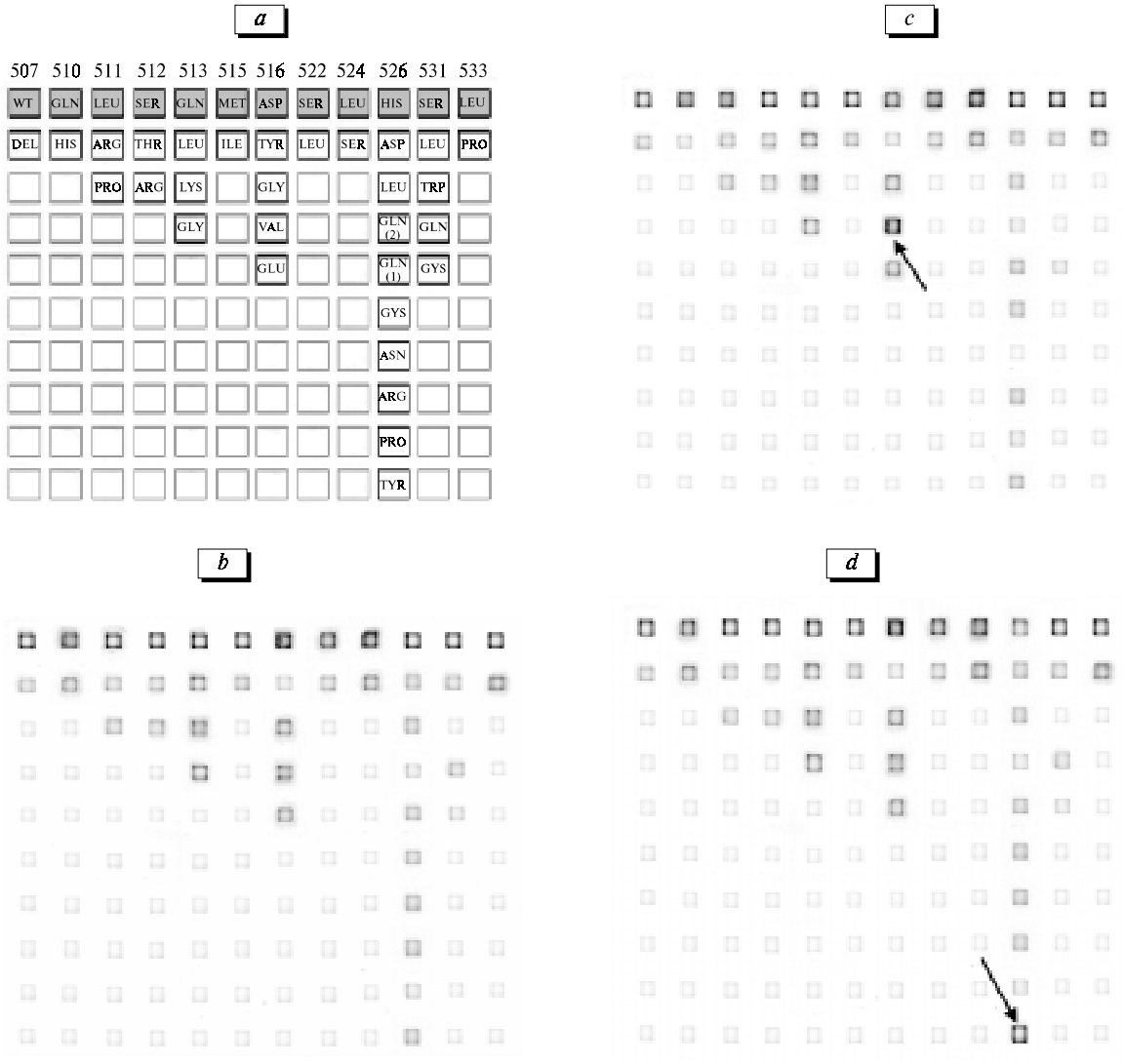

Fig. 1. Position of discriminating oligonucleotides on microchip (*a*) and hybridization patterns obtained in analysis of wild type DNA samples (*b*), Asp516>Val (*c*), and His526>Tyr (*d*). Arrows show mutation positions.

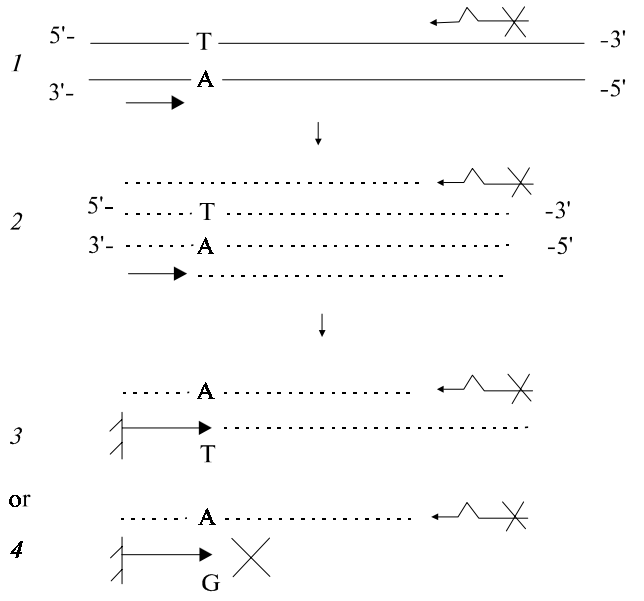

Fig. 2. Principal scheme of microchip PCR. Explanations in the text.

Samples of mycobacterial DNA for hybridization were prepared by two-step PCR. First, a 193 b.p. fragment of *M. tuberculosis rpoB* gene was amplified with 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') on a MiniCycler amplifier (MJ Research). For preventing cross contamination, 0.5 U uracil-DNA glycosylase (Medigen) was added to the buffer for PCR and dUTP was used instead of dTTP. The temperature regimen was as follows: 10 min at 18-20°C, denaturation 5 min at 95°C, 30 amplification cycles (30 sec at 95°C and 40 sec at 72°C), and final 5-min incubation at 72°C. The reaction mixture (2 μl) after PCR stage 1 was used as the template for stage 2.

Stage 2 of PCR was carried out with primers f1272 (5'- cgc cgc gat caa gga gtt ct -3') labeled with Texas Red fluorescent label and r1398 (5'- tca cgt gac aga ccg ccg gg -3'). The reaction mixture (final volume 100 μl) contained 10 and 1 pmol each primer, respectively. Uracil-DNA glycosylase was not added. Amplification was carried out in the following regimen: denaturation 5 min at 95°C, 35 cycles (20 sec at 95°C, 30 sec at 65°C, and 30 sec at 72°C), and final 5-min incubation at 72°C.

Gel preparation and oligonucleotide immobilization in wells were previously described [15].

A set of discriminating oligonucleotides with close melting points was selected for each amino acid position with known mutations responsible for the RR-phenotype. Each nucleotide in the set can form a perfect hybridization duplex with only one mutant DNA and imperfect duplexes with other variants. In hybridization the variable bases are situated in the middle part of discriminating oligonucleotide, while in PCR on the chip they are located in the 3'-terminal position. The length of oligonucleotides was so chosen that melting points differed no more than by 3-4°C. Oligonucleotides forming highly stable secondary pin-type structures were not used.

After stage 2 of PCR, concentrated hybridization buffer was added to 12 μl of reaction mixture containing primarily single-strand DNA with a fluorescent label (about 150 fmol) so that the final concentration of guanidine thiocyanate were 1 M, HEPES 50 mM, and EDTA 5 mM, pH 7.5.

Hybridization mixture (28 μl) was transferred to the microchip and covered with hybridization Cover-Well Incubation Chamber (Grace Bio-Labs). Hybridization was carried out at 37°C for 14-18 h. After hybridization the microchip was washed three times with a buffer containing 1 M NaCl, 67 mM Na-phosphate-buffer (pH 7.4), 6.7 EDTA, and 10% Twin-20 (37°C) and dried.

The chamber and equipment for microchip PCR were described previously [10]. A buffer for PCR (30 μl) containing 2.5 mM $MgCl_2$, 10 mM KCl, 10 mM tris-HCl (pH 8.3), 0.1% BSA (Sigma), 200 μM of each dNTP (Sigma), 5 U Stoffel AmpliTaq DNA polyme-

TABLE 1. Distribution of *rpoB* Alleles in DNA Samples Isolated from Clinical RR Strains (Results of Microchip Hybridization)

Type of mutation	Nomber of samples
Leu511>Pro	1
Gln513>Leu	2
Asp516>Tyr	2
Asp516>Val	9
Ser522>Leu	2
His526>Arg	1
His526>Asp	9
His526>Cys	1
His526>Leu	1
His526>Tyr	5
Ser531>Gln	1
Ser531>Leu	78
Ser531>Trp	1
Leu533>Pro	9
Asp516>Gly, His526>Asn	1
Leu511>Arg, Asp516>Tyr	1
Leu511>Pro, Asp516>Gly	1
Leu511>Pro, His526>Asn	1
Leu511>Pro, His526>Gln	1
Wild type	4
Total	131

rase fragment (Perkin Elmer), 1 pmol direct unlabeled primer (5'- cgc gat caa gga gtt ctt cgg cac c -3'), and 10 pmol reverse labeled primer (5'- ccc ggc ggt ctg tac gtg a -3') was added to reaction chamber. After preheating at 95°C for 120 sec, 25-30 amplification cycles were carried out (30 sec at 95°C, 60 sec at 63°C, and 40 sec at 72°C).

Wells of the upper horizontal row of the microchip contain oligonucleotides complementary to the wild type sequences determining rifampicin resistance (Fig. 1). Each column contains immobilized oligonucleotides necessary for identification of mutations affecting the specified amino acid residue. The highest fluorescence intensity in the column indicates the well containing perfect hybridization duplex.

The microchip contains 42 immobilized oligonucleotides and can be used for identification of 30 allele variants of RRDR sequence, which covers by incidence more than 95% of all known mutations responsible for rifampicin resistance [6]. Oligonucleotides and conditions of hybridization were optimized so as to ensure reliable interpretation of the hybridization picture by means of computer software and visually. The chip can identify mutations involving more than one amino acid residue, which was demonstrated by detection of 5 samples carrying double amino acid substitutions, and on synthetic DNA imitating a triple substitution (Leu522, Leu526, Leu531).

Microchip PCR is a modification of allele-specific PCR (Fig. 2). At the initial stage, PCR runs in the reaction volume with the formation of a labeled product carrying fluorescent label due to incorporation of a 5'-labeled primer (Fig. 2, *1*). Accumulated amplification product is hybridized with specific primers immobilized on the microchip (Fig. 2, *2*). If the 3'-terminal fragment of the immobilized primer is complementary to the hybridized labeled amplification product, polymerase reaction takes place. In the resultant hybridization duplex with high melting point one chain is represented by hybridized fluorescent-labeled amplification product and the other by completed immobilized primer (Fig. 2, *3*). By contrast, in case when 3'-terminal sequence of the immobilized primer is not complementary to the sequence of hybridized product, polymerase reacton is blocked, and the hybridization duplex is stable only at lower temperatures close to the

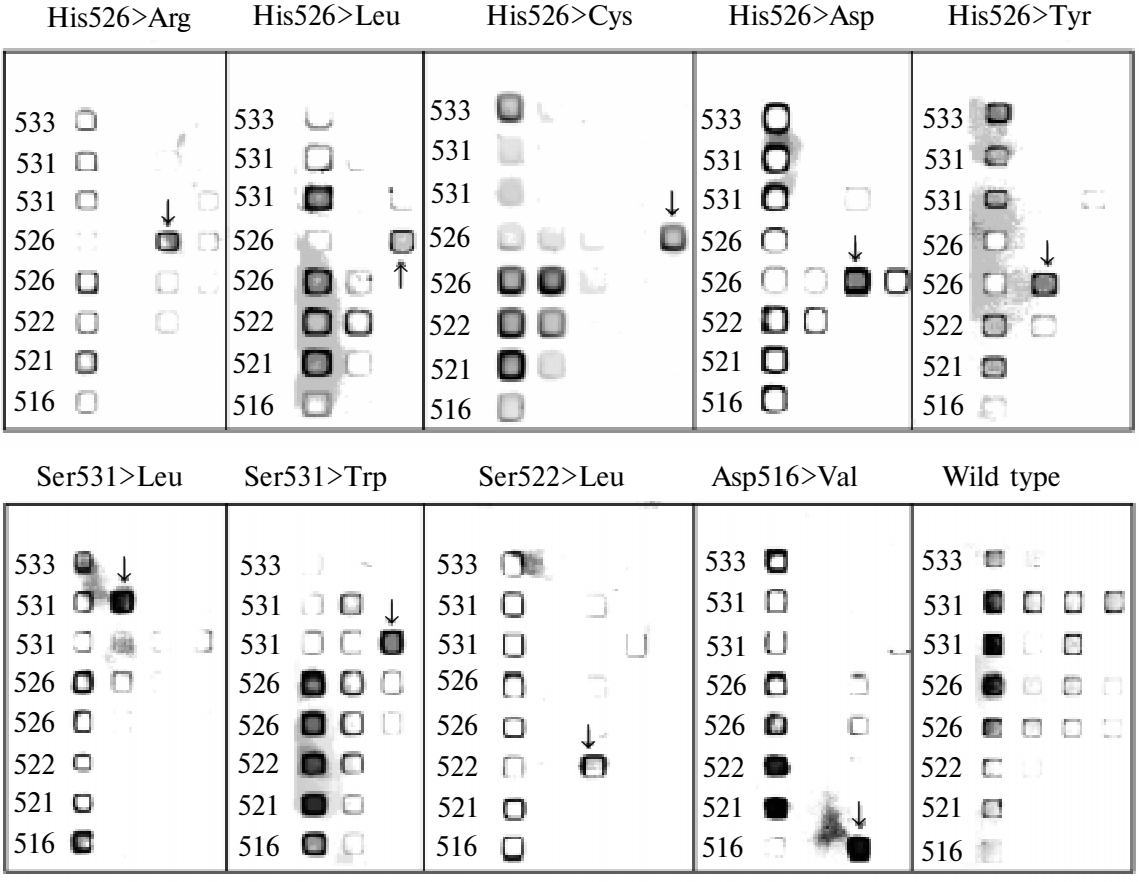

Fig. 3. Microchip PCR assay of 10 DNA samples (wild type and mutant variants). Sets of specific primers needed for identification of mutations involving specified amino acid residues were placed in the horizontal row of the microchip wells. Primers specific for wild type sequence are situated in the left column. Arrows show fluorescing wells in the microchip, which indicate the presence of mutations.

annealing temperature of the primer (Fig. 2, *4*). At high temperatures the fluorescent signal is observed only in wells with completed primer (Fig. 2, *3*). Comparison of the intensity of fluorescence of the wells, as was described for microchip hybridization, indicates the presence/absence of mutations in the *rpoB* gene and their type, and hence, it allows to determine rifampicin resistance/sensitivity of the analyzed *M. tuberculosis* sample.

All experiments were carried out in the real-time mode on an experimental setup including fluorescent microscope, digital chamber, thermocontrolled table based on the Peltie element, temperature controller, and computer for data recording [4]. Software based on the LabVIEW interface (National Instruments) was used during experiments and for data processing.

RESULTS

DNA of a rifampicin-sensitive *M. tuberculosis* strain, presented by wild type sequence, forms perfect hybridization duplexes with oligonucleotides immobilized in the upper row of the microchip (Fig. 1, *b*). Hybridization of samples containing mutations in RRDR of the *rpoB* gene yields perfect hybridization duplexes in a well below the upper row of the microchip wells (Fig. 1, *c, d*).

Of 131 DNA samples isolated from RR strains of *M. tuberculosis*, 127 (97%) carried mutations in the *rpoB* gene as determined by the hybridization method. The types of detected mutations are presented in Table 1. Results of direct sequencing (19 samples) coincided in 100% cases with the results of microchip hybridization. As for the rest 4 DNA samples isolated from RR strains (by the results of standard culturing test) referred to wild rifampicin-sensitive type by hybridization, this result is not unexpected, since in about 4% rifampicin-resistant strains this sign is not associated with mutations in the *rpoB* gene RRDR [6]. Sequencing of one sample confirmed the absence of mutations in RRDR.

Distribution of mutations by types detected in this study is in line with the data obtained by other authors [6], in particular, predominance of mutations involving amino acid residues Ser531 and His526.

Figure 3 presents the results of microchip PCR identification of 10 allele variants of RRDR sequences. Results of analysis of 30 mutant strains of *M. tuberculosis* by microchip PCR and hybridization coincided completely.

The results of identification of RR *M. tuberculosis* strains obtained by the methods of hybridization and PCR on microchip developed by us strictly correlate with the results of evaluation of antibiotic sensitivity by traditional methods and can be obtained much sooner. Hybridization microchips can be commercially manufactured, which will reduce their cost and allow the use of the method at clinical diagnostic laboratories, while microchips can be used for primary identification of rifampicin-sensitive and RR strains of *M. tuberculosis* and for monitoring of the treatment efficiency. Our modification of allele-specific PCR on microchip can be used for simultaneous analysis of many variable genome sites not connected with each other, which is particularly important for identification of polyresistant forms of *M. tuberculosis*.

REFERENCES

1. D. L. Cohn, F. Bustreo, and M. C. Raviglione, *Clin. Infect. Dis.,* **24,** S121-S130 (1997).
2. H. De Beenhouwer, Z. Lhiang, G. Jannes, *et al., Tubercle Lung. Dis.,* **76,** 425-230 (1995).
3. T. A. Felmlee, Q. Lin, and A. C. Whelen, *J. Clin. Microbiol.,* **33,** 1617-1623 (1995).
4. A. Fotin, A. Drobyshev, D. Proudnikov, *et al., Nucleic Acids Res.,* **26,** 1515-1521 (1998).
5. I. Nachamkin, C. Kang, and M. P. Weinstein, *Clin. Infect. Dis.,* **24,** 894-900 (1997).
6. S. Ramaswamy and J. M. Musser, *Tubercle Lung Dis.,* **79,** 3-29 (1998).
7. N. Rastogi, K. S. Goh, and H. L. David, *Res. Microbiol.,* **140,** 405-417 (1989).
8. M. C. Raviglione, D. E. Snider, and A. Kochi, *J. Am. Med. Ass.,* **273,** 220-226 (1999).
9. S. H. Siddiqi, J. E. Hawkins, and A. Laszlo, *J. Clin. Microbiol.,* **22,** 919-923 (1985).
10. B. N. Strizhkov, A. L. Drobyshev, V. M. Mikhailovich, and A. D. Mirzabekov, *BioTechniques,* **29,** 844-857 (2000).
11. A. Telenti, P. Imboden, F. Marchesi, *et al., Lancet,* **341,** 647-650 (1993).
12. A. Troesch, H. Nguyen, C. G. Miyada, *et al., J. Clin. Microbiol.,* **37,** 49-55 (1999).
13. T. C. Victor, A. M. Jordaan, A. van Rie, *et al., Tubercle Lung Dis.,* **79,** 343-348 (1999).
14. D. L. Williams, C. Waguespack, K. Eisenach, *et al., Antimicrob. Agents Chemother.,* **38,** 2380-2386 (1994).
15. G. Yershov, V. Barsky, A. Belgovskiy, *et al., Proc. Natl. Acad. Sci. USA,* **93,** 4913-4918 (1996).