METHODS

Detection of Mutations in *Mycobacterium tuberculosis* Genome Determining Resistance to Fluoroquinolones by Hybridization on Biological Microchips

O. V. Antonova, D. A. Gryadunov, S. A. Lapa, A. V. Kuz'min, E. E. Larionova*, T. G. Smirnova*, E. Yu. Nosova, O. I. Skotnikova**, L. N. Chernousova*, A. M. Moroz**, A. S. Zasedatelev, and V. M. Mikhailovich**

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We developed a method of identification of *Mycobacterium tuberculosis* with simultaneous evaluation of the sensitivity to fluoroquinolones on a biological microchip array. The method of multiplex two-staged PCR followed by hybridization of a biochip makes it possible to detect 8 mutant variants of *gyrA* gene occurring in fluoroquinolone-resistant strains (~85% all resistant forms) within 1 day. Using this method we analyzed 107 cultures isolated from patients with tuberculosis and 78 sputum samples. Mutations in *gyrA* gene were detected in 48 (92%) resistant strains. Natural S95T polymorphism in *gyrA* gene was detected in all resistant and in 76% sensitive strains. The sensitivity and specificity of the proposed method calculated on the basis of the analysis of sputum samples (n=78) were 94 and 100%, respectively.

Key Words: *Mycobacterium tuberculosis; biochip; fluoroquinolones; hybridization; polymerase chain reaction*

Patients with pulmonary tuberculosis caused by rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* strains, *i.e.* strains characterized by multiple drug resistance (MDR) and comprising the most epidemiologically dangerous group, need therapy with second-line drugs (reserve drugs), *e.g.* flouroquinolones (FQ), in particular, levofloxacin (ofloxacin L-isomer) and moxifloxacin. These drugs are approved for the treatment of patients with tuberculosis in Russian Federation and exhibit high activity against MDR *Mycobacterium tuberculosis* (MBT) [4,7]

FQ inhibits DNA gyrase (topoisomerase II), which leads to blockade of DNA synthesis. DNA gyrase is a tetramer consisting of two A and two B subunits encoded by *gyrA* and *gyrB* genes, respectively. Point nucleotide substitutions in a small fragment of *gyrA* gene (FQ resistance-determining region) are the main mechanism responsible for *M. tuberculosis* resistance to FQ (75-94% cases) [1,9, 14]. Substitutions in codons 88, 90, 91, and 94 of *gyrA* gene are most prevalent, while mutations in *gyrB* gene determine FQ resistance in rare cases [11,14]. Cross-resistance to FQ was demonstrated, *i.e.* resistance to one drug is usually associated with resistance to other FQ [7].

V. A. Engel'gard Institute of Molecular Biology, Russian Academy of Sciences, Moscow; Central Institute of Tuberculosis, Russian Academy of Medical Sciences, Moscow; Moscow Research-and-Practical Center for Tuberculosis Control. ***Address for correspondence:*** markova@biochip.ru. O. V. Antonova

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Mutations responsible for FQ resistance are usually identified using the methods based on the analysis of the sequence of FQ resistance-determining region: sequencing [14], HPLC [12], hybridization with linear probes [6], and multiplex PCR amplimer conformation analysis [5]. However, these methods are labor-consuming and are not certified for the use in diagnostic laboratories in Russia.

The aim of this work was to evaluate the efficiency of detection of mutations responsible for FQ resistance in MBT genome using a specialized biological microchip. The major advantage of this technique is the possibility of detection of FQ-resistant MBT strains over a short period (within 24 h) using both clinical samples and cultured microorganisms.

MATERIALS AND METHODS

Using this method we analyzed 107 cultures isolated from patients with tuberculosis with MDR and 78 sputum samples. The resistance of MBT strains to ofloxacin was determined by the method of absolute concentrations on Levenstain-Jensen medium. The strain was considered resistant to ofloxacin, if ≥20 CFU grew on a medium with ofloxacin concentration ≥2 μg/ml and abundant grow was observed in the control tube in the absence of the drug. Each strain was inoculated into media containing ofloxacin in concentrations of 2 and 10 μg/ml.

Clinical samples were processed and DNA isolation from MBT was performed as described previously.

The choice of sequences of discriminating oligonucleotides for immobilization of the biochip and primers for amplification of *gyrA* gene fragment, synthesis and purification of probes, and incorporation of a fluorescent label IMD-515 (IMB Biochip) into primers were carried out by the method of identification of rifampicin- and isoniazid-resistant *M. tuberculosis* strains on biological microchips [8]. The biochip contained 20 immobilized oligonucleotides for detection of mutations in *gyrA* gene (Table 1), 3 marker points for image positioning (acquisition) by the computer software, and 2 control wells with empty gel (Fig. 1, *a*).

Hybridization on biochips was preceded by two-stage multiplex PCR for obtaining of single-stranded fluorolabeled fragments using primers spe-

TABLE 1. Sequence of Oligonucleotides Immobilized on Biochip

Oligonucleotide	Position of amino acid	Amino acid substitution	Nucleotide substitution	Sequence 5'→3'	Position of oligonucleotide
A1	88	Gly(WT)	GGC	CCC GCA CGG CGA CGC G	7557-7572*
A2	88	Gly>Cys	GGC>TGC	CCC GCA CTG CGA CGC G	7557-7572*
B1	90	Ala (WT)	GCG	CGA CGC GTC GAT CTA CG	7566-7582*
B2	90	Ala>Val	GCG>GTG	CGA CGT GTC GAT CTA CG	7566-7582*
C1	91	$Ser(WT)_1$	TCG	CGA CGC GTC GAT CTA CGA	7566-7583*
C2	91	$Ser>Pro_1$	TCG>CCG	CGA CGC GCC GAT CTA CG	7566-7582*
D1	91	$Ser(WT)_2$	TCG	CGA CGT GTC GAT CTA CGA	7566-7583*
D2	91	$Ser>Pro_2$	TCG>CCG	CGA CGT GCC GAT CTA CG	7566-7582*
E1	95	Ser(WT)	AGC	TAC GAC AGC CTG GTG CG	7579-7595*
E2	95	Ser>Thr	AGC>ACC	TAC GAC ACC CTG GTG CG	7579-7595*
A3	94	$Asp(WT)_1$	GAC	CGA TCT ACG ACA GCC TG	7574-7590*
B3	94	$Asp>His_1$	GAC>CAC	CGA TCT ACC ACA GCC TG	7574-7590*
C3	94	$Asp>Asn_1$	GAC>AAC	TCG ATC TAC AAC AGC CTG	7573-7590*
D3	94	Asp>Gly	GAC>GGC	GAT CTA CGG CAG CCT G	7575-7590*
E3	94	Asp>Ala	GAC>GCC	GAT CTA CGC CAG CCT G	7575-7590*
F3	94	Asp>Tyr	GAC>TAC	TCG ATC TAC TAC AGC CTG	7573-7590*
A4	94	$Asp(WT)_2$	GAC	TCT ACG ACA CCC TGG T	7577-7592*
B4	94	$Asp>His_2$	GAC>CAC	CGA TCT ACC ACA CCC TG	7574-7590*
C4	94	$Asp>Ala_2$	GAC>GCC	TCT ACG CCA CCC TGG T	7577-7592*
F4	—	—	WT	CCG GAG CTG CGT GAG CG	1359-1375**

Note. Code of oligoducleotides corresponded to their position on the biochip. *Relative to sequence of *gyrA* gene (Genbank Acc.No NC_000962), **relative to the sequence of IS6110 element (Genbank Acc.No AF189827).

cific to IS6110 and *gyrA* gene fragments. At the first stage, PCR was performed according to the following protocol: 5 min at 95°C (36 cycles): 30 sec at 95°C, 20 sec at 67°C, and 20 sec at 72°C, and then 5 min at 72°C. The reaction mixture (30 μl) for the first stage contained 10× buffer for PCR (Sileks), 0.2 mM each deoxynucleoside triphosphate (Silekc), 100 nM primers gyrA_f1 (GCC ACG CCA AGT CGG CCC GGT CGG TT) and gyrA_r1 (TAC CTC ATC GCC GCC GGT GGG TCA), 50 nM IS_f and IS_r1 [8], 5 U Taq polymerase (Sileks), and 1 μl DNA sample. At the second stage (50 μl reaction mixture) we used primers gyrA_f2 (GGC CCG GTC GGT TGC CGA GAC C) and gyrA_r2* (GCA GCG ACC AGG GCT GGG CCA TG) in concentrations of 10 and 100 nM, respectively, and 5 nM IS_f and 50 pM IS_r2* [8]. PCR product obtained at stage 1 (1 μl) was used as the template. Amplification was performed according to the following protocol: 30 sec at 95°C, 30 sec at 65°C, and 20 sec at 72°C (40 cycles). The time of denaturation in the first cycle and the time of elongation in the last cycle were prolonged to 5 min.

Hybridization on biochips was performed as described previously [8]. The results of hybridization were analyzed using a Universal hardware and software complex for biochip analysis (Institute of Molecular Biology, Russian Academy of Sciences). For interpretation of the results, the gel cells containing nucleotides were combined into 6 groups (Fig. 1, *a*) in such a way that comparison of the intensity of fluorescent signals from cells in each group makes it possible to detect formed hybridization complex in one cell and confirm the presence/absence of mutation (minor polymorphism) leading to substitution of the corresponding amino acid residue.

During hybridization on the biochip of a DNA sample not containing mutations, the cells with oligonucleotides complimentary to wild-type DNA had higher intensity of the fluorescent signal in comparison with other cells in each group (Fig. 1, *b*). Fluorescent signal in cell F4 confirmed the fact that the analyzed strain belonged to MBT complex.

During hybridization of DNA fragments isolated from FQ-resistant MBT strain, the intensity of the fluorescent signal in the cell with oligonucleotide complimentary to wild-type DNA in each group of elements of the biochip was higher than in other cells. The exception was groups of elements E1-E2 and A3-F3, A4-C4 (Fig. 1, *c*). The maximum fluorescence intensity in the group E1-E2 was recorded in element E2. Hence, DNA of the studied strain had a point nucleotide substitution G>C in position 283 of *gyrA* gene, which, in turn, leads to substitution of Ser to Thr in codon 95 (S95T). However, this substitution was found in both sensitive and resistant MBT strains. This substitution does not determine FQ resistance and is considered as a

TABLE 2. Identification of Mutations in *gyrA* gene Isolated from Clinical Samples with Different Ofloxacin Sensitivity

Type of mutation determined by hybridization on biochips	Number of strains determined by the method of absolute concentrations as		
	sensitive	resistant to ofloxacin in a concentration of	
		2 μg/ml	10 μg/ml
Wild type[1]	10	—	—
S95T[2]	42	3	1
G88C[3]	—	—	1
A90V[3]	3	6	2
S91P[3]	—	3	—
D94G[3]	—	13	5
D94Y[3]	—	3	—
D94A[3]	—	4	2
D94N[3]	—	2	3
D94H[3]	—	—	1
A90V and S91P[3]	—	1	1
S91P and D94G[3]	—	1	—
Total	55	36	16

Note. [1]No mutations and polymorphism were detected; [2]nucleotide polymorphism leading to substitution of serine (S) to threonine (T) in codon 95; [3]mutations linked with S95T polymorphism (see text).

natural polymorphism [1,5,6,14]. Element C4 is characterized by the maximum intensity of fluorescence in the group (A3-F3, A4-C4). Hence, DNA of the studied strain has a nucleotide substitution A>C in position 280 of *gyrA* gene corresponding to amino acid substitution Asp to Ala in codon 94 (D94A), which leads to the development of FQ resistance of the studied strain. Thus, a conclusion is made that the analyzed clinical sample contains MBT resistant to FQ. The resistance is determined by amino acid substitution Asp to Ala in codon 94 of *gyrA* gene.

Sequencing of *gyrA* gene fragment obtained using primers gyrA_f1 and gyrA_r1 and ABI PRISM®

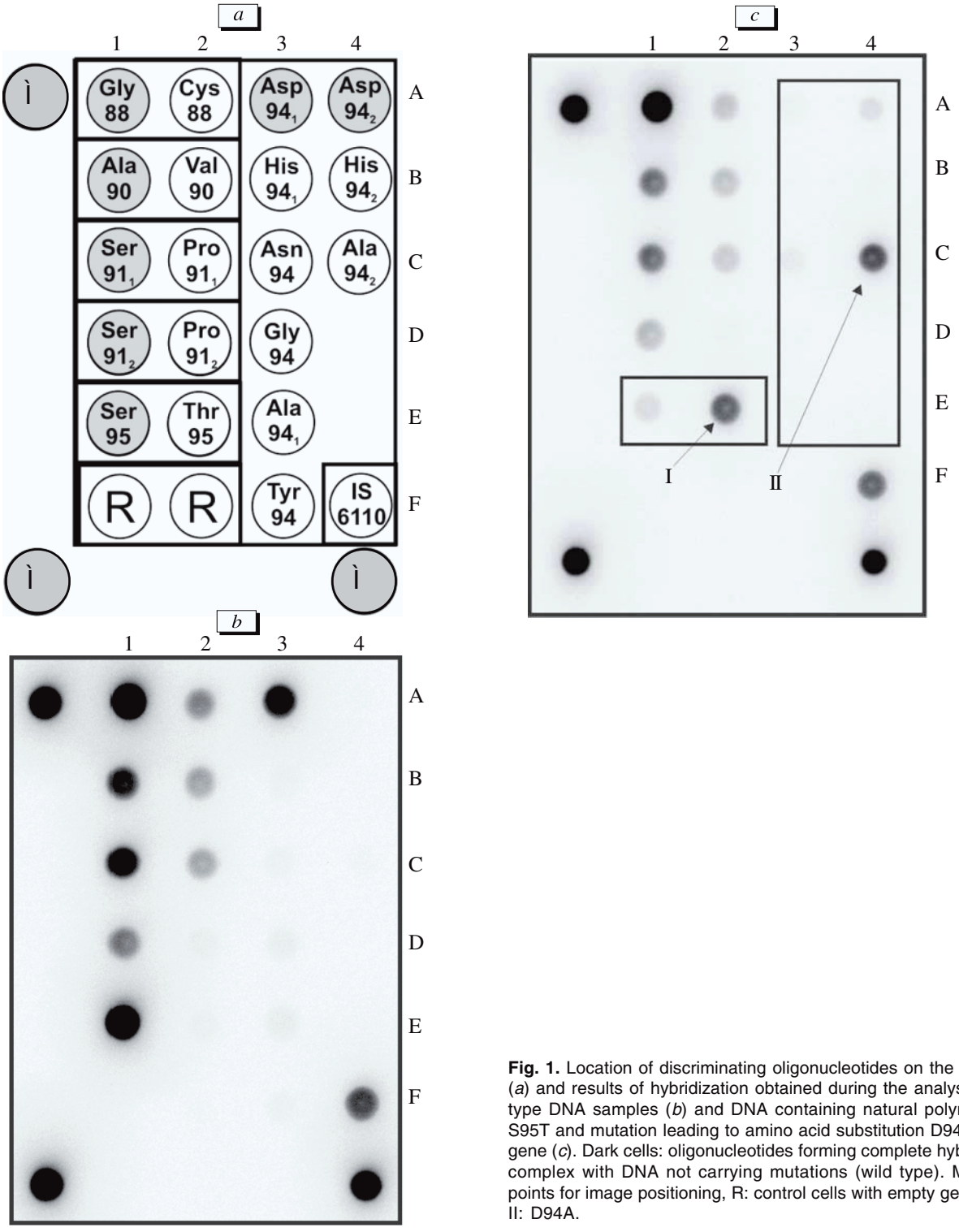

Fig. 1. Location of discriminating oligonucleotides on the microchip (*a*) and results of hybridization obtained during the analysis of wild type DNA samples (*b*) and DNA containing natural polymorphism S95T and mutation leading to amino acid substitution D94A in *gyrA* gene (*c*). Dark cells: oligonucleotides forming complete hybridization complex with DNA not carrying mutations (wild type). M: marker points for image positioning, R: control cells with empty gel, I: S95T; II: D94A.

BigDye™ Terminator v 3.1 kits (Applied Biosystems) followed by analysis of the reaction products on an automated DNA sequenator ABI PRISM® 3100-Avant (Applied Biosystems) served as the control method.

RESULTS

Cultural analysis showed that 55 strains were sensitive to ofloxacin and 52 were resistant to this drug. Hybridization on biochips showed that 10 DNA samples had no substitutions in the analyzed fragments of *gyrA* gene and 97 samples carried natural polymorphism in codon 95. The majority of resistant strains (63%) had mutations in codon 94 (Table 2).

Three sensitive strains (according to bacteriological tests) carrying mutation in codon 90 (A90V) of *gyrA* gene and polymorphism S95T comprised a special group. We considered these results as false positive despite the fact that some FQ-sensitive strains have mutations in codon 90 linked with mutations in other regions of *gyrA* gene (*e.g.* in codon 80) or other genes [2]. The effect of linked mutations on the development of drug resistance requires special investigation.

Among MBT strains that were resistant by the results of cultural analysis (*i.e.* grew in a medium with 2 μg/ml ofloxacin) 4 samples had only natural S95T polymorphism (Table 2). For these strains additional sequencing of *gyrA* gene fragment was performed. Sequencing confirmed the presence of this polymorphism and the absence of other substitutions in the analyzed fragment. The resistance of strains not carrying mutations in the analyzed fragment of *gyrA* gene can be caused by substitutions in other genes associated with FQ resistance or other defense mechanisms, *e.g.* active pumping out of the drug from MBT or decreased permeability of the cell wall for FQ [1,12].

Analysis of sputum by the method of absolute concentrations revealed 63 ofloxacin-sensitive and 15 ofloxacin-resistant strains. Hybridization on biochips of MBT DNA obtained from clinical samples showed that 8 DNA samples had no substitutions in the analyzed region, 56 samples contained only S95T substitution, and 14 samples had mutations leading to the development of FQ resistance: A90V-S95T (3 samples), D94G-S95T (5 samples), D94Y-S95T (2 samples), D94N-S95T (3 samples), D94A-S95T (1 sample).

The results of the hybridization results for DNA isolated directly from clinical samples and from cultures grown from these samples coincided in 98.7% cases (n=78). One sample containing natural polymorphism S95T (by the analysis of DNA from the sputum) and assigned to sensitive MBT strain was identified as resistant by the analysis of DNA isolated from the corresponding culture. Apart from S95T polymorphism, A90V substitution was shown. This discrepancy can be explained by the presence of 2 MBT subpopulations (or strains) in the sputum, the amount of the subpopulation carrying additional mutation being insufficient for the detection by the proposed method (against the background of predominance of the other subpopulation) [3]. Thus, the sensitivity and specificity of the method of hybridization were 94 and 100%, respectively.

For evaluation of the analytic sensitivity of the method we used DNA isolated from clinical samples with known concentrations of MBT (determined by real-time PCR). It was shown that the results of hybridization on biochips were reproducible if the sample added to the reaction mixture contained 500 or more copies of MBT genome. The specificity of the method for detection of MBT strains was tested on samples obtained from patients with non-tuberculosis inflammatory process (12 clinical samples) and on non-tuberculosis MBT strains: *M. avium*, *M. kansasii*, *M. intracelulare*, *M. scrofulaceum,* and *M. marinum.* No false-positive results of hybridization were obtained.

Thus, the developed method of hybridization on biochips allows effective detection of MBT directly in clinical material and simultaneous evaluation of their resistance to FQ. The results can be obtained during a short period of time. The procedure is adapted for the use in diagnostic laboratories of specialized phthisiological departments. The developed method was tested in medical settings and was registered by the Federal Service for Supervision in the Sphere of Health Care and Social Development.

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