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Evaluation of hybridisation on oligonucleotide microarrays for analysis of drug-resistant *Mycobacterium tuberculosis*

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ABSTRACT

A molecular approach was developed to identify drug-resistant strains of *Mycobacterium tuberculosis* by means of biochips with oligonucleotides immobilised in polyacrylamide gel pads. The technique was based on multiplex PCR, followed by hybridisation on an oligonucleotide microarray, and detected >95% of rifampicin-resistant and *c.* 80% of isoniazid-resistant *M. tuberculosis* isolates within 12 h. In total, 220 drug-resistant isolates and 131 clinical samples were tested using biochips. The sensitivity and specificity of the developed method were comparable with those of standard bacteriological testing of *M. tuberculosis* drug resistance.

Keywords Biochips, hybridisation, multidrug resistance, *Mycobacterium tuberculosis*, oligonucleotide microarrays, PCR

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INTRODUCTION

Mycobacterium tuberculosis remains one of the leading causes of human morbidity and mortality worldwide. Early diagnosis, effective treatment and successful prevention of transmission are major strategies in the control of tuberculosis (TB). Current treatment for TB involves a multidrug regimen based on rifampicin and isoniazid, the drugs most effective against *M. tuberculosis* infection. Although use of an appropriate drug with full patient compliance is highly effective in curing pulmonary TB, the emergence of *M. tuberculosis* strains that are resistant to rifampicin and isoniazid reduces the efficacy of standard treatment [1].

Resistance to rifampicin is determined in 96% of cases by polymorphisms in an 81-bp DNA sequence in the *rpoB* gene (RRDR) [2,3]. Isoniazid resistance may be caused by mutations located in several genes (*katG*, *inhA*, *ahpC*) [2,4,5]. Although the frequency of different isoniazid resistance mutations varies between different populations, mutations affect the *katG* gene most frequently; these mutations constitute about 95% of resistance mutations in the Latvian population [6] and about 86% in the Russian population [7]. Several molecular assays have been developed to screen these genes for mutations, including heteroduplex and mismatch analyses, DNA sequencing, real-time PCR, molecular beacons, hybridisation on microarrays, and other assays [8–16]. A commercial test, the Inno-LiPa assay, is available for detection of the four most common mutations in clinical isolates [17,18].

The approach described in the present paper used oligonucleotide microarrays (biochips) to simultaneously detect DNA of the *M. tuberculosis* complex and identify mutations that cause rifampicin and isoniazid resistance in *M. tuberculosis* isolates from clinical specimens. Using microarrays, the analysis of clinical samples requires <12 h. The biochip described in the present work is an improved and more advanced version of a biochip for identification of rifampicin-resistant

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M. tuberculosis isolates described previously [19]. The present study investigated the use of this approach as a tool for large-scale clinical and epidemiological studies, and assessed the specificity, sensitivity and predictive values.

MATERIALS AND METHODS

Mycobacterium isolates and clinical specimens

In total, 220 isoniazid-resistant clinical isolates of *M. tuberculosis*, as well as 113 acid-fast bacillus (AFB)-positive and 20 AFB-negative sputum samples, were analysed. Controls were standard strains of *M. tuberculosis* H37Rv, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium fortuitum*, *Mycobacterium xenopi*, *Mycobacterium gordonae*, *Mycobacterium chelonae* and *Mycobacterium scrofulaceum*.

The 220 clinical isolates were obtained from chronically disabled patients infected with TB from Moscow and the Moscow region (one isolate per patient). All isolates were grown on Lowenstein–Jensen (LJ) medium and were identified by biochemical tests [20]. Isoniazid susceptibility tests were carried out by the proportion method on LJ medium containing isoniazid 0.2 mg/L [20]. Of the 220 isolates, 168 were also resistant phenotypically to rifampicin, confirmed by incubation on LJ medium containing rifampicin 40 mg/L (proportional method) [20]. The isolates were considered resistant if >1% of colonies grew on the antibiotic-containing medium compared with the drug-free medium.

The 113 AFB-positive sputum samples were obtained from primary patients diagnosed with pulmonary tuberculosis by radiological and clinical criteria [21]. Among the 20 AFB-negative sputum samples, six were obtained from chronic TB patients showing poor treatment compliance, while 14 samples were from individuals with suspected TB, but with an unconfirmed diagnosis.

The specimens were decontaminated with an equal volume of *N*-acetyl-L-cysteine and NaOH 2% w/v, homogenised by centrifugal swirling, and then incubated for 15 min [22]. The reaction was neutralised by adding 0.067 M phosphate-buffered saline (pH 6.8) to a final volume of 50 mL. The specimens were concentrated by centrifugation at 3000 *g* for 15 min. The supernatant was discarded, and the sediment was resuspended in 5 mL of sterile water. Part of the sediment from each specimen was used to inoculate an LJ slant, and the remaining portion was stored at −20°C until used for DNA extraction. LJ slants were incubated at 37°C for 6 weeks and inspected weekly for growth. When growth was detected, a smear was prepared to confirm the presence of AFB in suspect colonies by Ziehl–Neelsen staining. Rifampicin and isoniazid susceptibility tests were carried out by the proportion method on LJ medium containing rifampicin 40 mg/L and isoniazid 0.2 mg/L, respectively.

Preparation of DNA samples

Frozen decontaminated samples of sputum, or one or two colonies of *M. tuberculosis* (2–3 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 Triton X-100 1% v/v and incubated for 30 min at 95°C. The extracts were cooled on ice and centrifuged at 12 000 *g* for 10 min, after which 2-μL aliquots of clear supernatant were used in PCRs.

Oligonucleotides

Oligonucleotides for immobilisation on a biochip (Table 1) and primers for amplification (Table 2) were designed and synthesised as described previously [19]. To immobilise oligonucleotides in gel pads or to attach the indodicarbocyanine dye (Biochip-IMB Ltd, Moscow, Russia), an amino group was introduced during synthesis using 3′-Amino-Modifier C7 CPG 500 or 5′-Amino-Modifier C6 (Glen Research, Sterling, VA, USA). The attachment of the fluorescent group to the oligonucleotide amino group was carried out according to the manufacturer's instructions. Oligonucleotides and primers were purified by reverse phase high-performance liquid chromatography on C_{18}-Nucleosil columns (Sigma, St Louis, MO, USA). The molecular mass of each oligonucleotide was measured with a MALDI-TOF mass spectrometer (COMPACT MALDI 4; Kratos Analytical, Chestnut Ridge, NY, USA) using sinapinic acid or 2-amino-5-nitropyridine as a matrix.

Preparation of target DNA for hybridisation on biochips

Target samples of DNA from *M. tuberculosis* were prepared by two-stage multiplex PCR. In the first stage, five genome segments of *M. tuberculosis* were amplified simultaneously. The lengths of the fragments were 300 bp (IS*6110* loci), 212 bp (*rpoB*), 166 bp (*katG*), 143 bp (*inhA*) and 126 bp (*ahpC*). Primers used for amplification of these segments are listed in Table 2. Each reaction mix (25 μL) contained 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.2 mM each dNTP, 5 U of *Taq* DNA polymerase (Sileks, Moscow, Russia), 0.5 U of uracyl-DNA-glycosylase to prevent contamination (Sileks), 50 nM primers p105f, p293r, katG_f, katG_r1, IS_f and IS_r1, 100 nM primers inhA_f, inhA_r1, ahpC_f and ahpC_r1, and 2 μL of DNA sample (10–100 pg). The reactions were carried out in a MiniCycler (MJ Research, Waltham, MA, USA), with 5 min at 95°C, followed by 36 cycles of 30 s at 95°C and 40 s at 72°C, and a final extension at 72°C for 5 min. Following amplification, 1 μL of the reaction mixture was used as a template for the second PCR round.

In the second reaction, internal fragments of about 100 bases were amplified for each target. The primers used in the reaction are listed in Table 2. Each PCR mix (50 μL) contained the following concentrations of primers: p1272f*/p1398r, 100 nM/10 nM; katG_f/katG_r2*, 5 nM/50 nM; inhA_f/inhA_r2*, 10 nM/100 nM; ahpC_f/ahpC_r2*, 5 nM/50 nM; and IS_f/IS_r2*, 5 nM/50 nM. The difference in the concentrations of forward and reverse primers within each pair meant that each reaction yielded predominantly single-stranded fluorescently-labelled product. PCRs comprised 5 min at 95°C, followed by 42 cycles of 30 s at 95°C, 30 s at 65°C and 20 s at 72°C, with a final extension at 72°C for 5 min.

Sequencing

Fragments of genes that determine resistance were amplified with the corresponding primers (Table 2) and subjected to dideoxy-sequencing using one of the terminal primers and a Big Dye Deoxy Terminator Sequencing Kit with *Taq* Polymerase