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Optimization of DNA Hybridization Analysis on Microarrays with Colorimetric Detection

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Abstract—A method of hybridization analysis on a DNA microarray using colorimetric detection on the basis of horseradish peroxidase has been developed. The effectiveness of the incorporation of biotin as a label in the DNA molecule in the PCR process is estimated and the conditions of hybridization of the biotin-labeled DNA with oligonucleotides immobilized on the surface of the array are optimized. The possibility of using the developed method is shown by the example of genotyping of CTX-M β-lactamases.

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Recently, one of the most intensively developing areas of nucleic acid biotechnology has become the use of DNA microarrays for nucleotide sequence analysis. This group of methods has significant advantages over traditional molecular biological methods as it allows one to decrease the size of the studied sample, which considerably decreases the cost and time of analysis. DNA microarray technology is finding wide application in fundamental and applied research such as identification of specific genes and determination of the level of their expression, as well as the study of DNA genetic polymorphism [1–4].

In DNA microarray technology, on a small surface of glass or another carrier, DNA fragments with a known nucleotide sequence are immobilized in the form of regularly arranged micromatrices. For this purpose, two approaches are mainly used: short oligonucleotides are synthesized directly on the surface of the substrate or preliminarily obtained DNA fragments are attached to it by covalent and noncovalent bonds [5, 6]. Then, hybridization of the studied nucleotide sequence, into which a label was preliminarily introduced, with immobilized DNA fragments is performed. If their primary structures coincide, heteroduplexes form on the surface of the microarray, which can be discovered by the appearance on the given segments of analytical signals.

The hybridization results depend on several factors: the length and composition of immobilized oligonucleotides and labeled DNA, the hybridization temperature, the composition of the hybridization mixture, and the type of introduced label [7–9]. The majority of developed DNA microarrays are based on the use of different fluorescent labels [10, 11]. This ensures the necessary sensitivity of the analysis; however, it considerably increases its cost (the high cost of both fluorescent labels and the fluorescent scanners used for recording the signal).

The goal of this study was to develop a method (PCR) of hybridization analysis on a DNA microarray using horseradish peroxidase (HP) colorimetric detection. In this method of detection, a molecule of biotin is introduced into the studied gene during the PCR process as a label, which is then revealed by a streptavidin–HP conjugate with subsequent HP colorimetric detection. In developing the method, we modified the analysis principle proposed in [12] for DNA microarrays with fluorescent detection.

The possibility of using this method is shown by the example of identification of point mutations in genes of CTX-M β-lactamases. These enzymes are the reason for the resistance of microorganisms to third-generation cephalosporins. It has been established that the broadening of the spectrum of activity of β-lactamases is related to the appearance of point mutations in the genes coding them. Studying the data of a mutation, one can draw a conclusion about the subtype of the enzyme and determine its substrate specificity.

EXPERIMENTAL

Forward (5'-ATG GTG ACA AAG AGA GTG C-3') and reverse (5'-CCT TTC GGC GAT GAT TCT CGC-3') primers for the CTX-M-9 β-lactamases and oligonucleotides for identification of point mutations in genes of CTX-M-9 β-lactamases were synthesized by Metabion (Germany). Samples of CTX-M-9 β-lactamases were

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provided by staff of the Institute of Antimicrobial Chemotherapy (Smolensk).

For immobilization, oligonucleotides with amino groups at the 5' end were dissolved in a salt buffer (160 mM Na_2SO_4, 130 mM Na_2HPO_4) to a final concentration of 20 pmol/μL and applied with a MicroGrid II robot (BioRobotics, United Kingdom) to glass plates with epoxy groups (Eppendorf, Germany), after which they were incubated for 30 min at 60°C.

Amplification of the CTX-M-9 β-lactamase gene was performed in a total volume of 25 μL in thin-walled test tubes containing 10 mM TRIS–HCl (pH 8.3 at 25°C); 2.5 mM magnesium acetate; 50 mM KCl; 2.5 units Taq DNA polymerase; 100 μM each dATP, dGTP, and dCTP; 60 μM dTTP (Eppendorf, Germany); 40 μM dUTP–biotin (Roche, Germany); 0.4 μM each forward and reverse primers; and 5 μL of a solution of the DNA matrix. Reactions were carried out in a Tertsik DNA amplifier (DNK-Tekhnologiya, Russia) according to the following procedure: initial denaturation at 94°C (2 min); 30 cycles of denaturation at 94°C (45 s), annealing of primers at 55°C (1 min), and elongation at 72°C (1 min); and a final stage of elongation at 72°C (10 min). Purification of the PCR product was performed on QlAquick columns according to the manufacturer's protocol.

Horizontal electrophoresis of PCR products was performed in a 1% agarose gel using TAE buffer (40 mM TRIS, 20 mM acetic acid, 1 mM EDTA, pH 8.5). A solution of ethidium bromide was added to the gel to a concentration of 1.6 μg/mL. Visualization was performed on a UV transilluminator at a wavelength of 260 nm.

DNA fragmentation was performed at room temperature for 5 min. For this purpose, amplified DNA was dissolved to a final concentration of 30 ng/μL in reaction buffer (40 mM TRIS–HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, pH 8.0) and DNase I was added (Promega, Germany). The reaction was stopped by adding 3 mM EDTA and incubating for 10 min at 65°C.

Before DNA hybridization was carried out, DNA microarrays with nucleotides applied to them were washed at room temperature (5 min in 0.1% Triton X-100 in ddH_2O, 4 min in an HCl solution, 10 min in 100 mM KCl) and then free epoxy groups were blocked (incubation of glasses in a 25% solution of ethylene glycol at 50°C for 15 min). The hybridization mixture, consisting of fragmented DNA, buffer (20 × SSPE: 3.0 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA, pH 7.7), and water taken in a specific ratio, was applied (in an amount of 70 μL) to a DNA microarray, covered with a frame, and incubated for 4 h at a specific temperature. Then the glasses were washed: 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) containing 0.1% SDS and then 2 × SSC and 0.2 × SSC (each stage lasted 10 min at room temperature). After blocking of the free centers of protein binding (incubation in a 1% BSA solution in phosphate buffered saline (PBS) for 30 min at 37°C), the microarrays were incubated for 1 h at room temperature in a solution of a streptavidin–HP conjugate in PBS Tween-20 (PBST) (dilution 1/1000). After this, the glasses were washed (PBST, two times for 5 min each) and placed in a substrate solution containing 4 mM *o*-dianisidine, 4 mM 4-chloro-1-napthol, and 1 mM hydrogen peroxide in 0.1 M potassium citrate buffer (pH 4.7). The microarrays were scanned on a Nikon Coolscan 4000 film scanner with a resolution of 4000 dpi. For determining the intensity of staining of spots, the obtained image was processed using the ScanArray Express program (Perkin-Elmer, v. 3.0).

RESULTS AND DISCUSSION

The principle of the method of hybridization analysis on a DNA microarray with colorimetric detection for identification of CTX-M β-lactamases was as follows. Single-stranded oligonucleotides were covalently immobilized on glass in a specific order. For identification of each mutation, a set of four oligonucleotides with a unique sequence of bases corresponding to the structure of the gene of β-lactamases in the given segment and differing from each other only by the nucleotide in the central position (A, G, C, or T) was used. The determination of the presence of mutations in the β-lactamase gene involved the amplification of the gene isolated from the clinical sample with the simultaneous introduction of a molecule of biotin (in the composition of dUTP–biotin) as a label, the fragmentation of the obtained PCR product, and its subsequent hybridization with specific oligonucleotides on the surface of a diagnostic DNA microarray (Fig. 1).The introduced biotin was revealed using a streptavidin–HP conjugate and HP colorimetric detection. For this purpose, *o*-dianisidine and 4-chloro-1-napthol were chosen as substrates, which upon combined enzymatic oxidation with hydrogen peroxide form an insoluble dark violet product well absorbed on the surface of the glass near the location of enzyme molecules. After scanning, an image of the micromatrix was obtained with points whose staining intensity was proportional to the enzymatic activity of the peroxidase and, consequently, the number of heteroduplexes forming on hybridization. The quantitative processing of the results consisted of the construction of profiles of the absolute intensity of staining estimated using the ScanArray Express program. It was supposed that, in each group, the oligonucleotide fully complementary to the fragment of the labeled DNA target gives a much stronger signal than the remaining three, differing from it in the central position. To determine the specificity of identification of a mutation (the ratio of the signal of complementary hybridization to the signal of nonspecific hybridization), the absolute values of intensities were converted

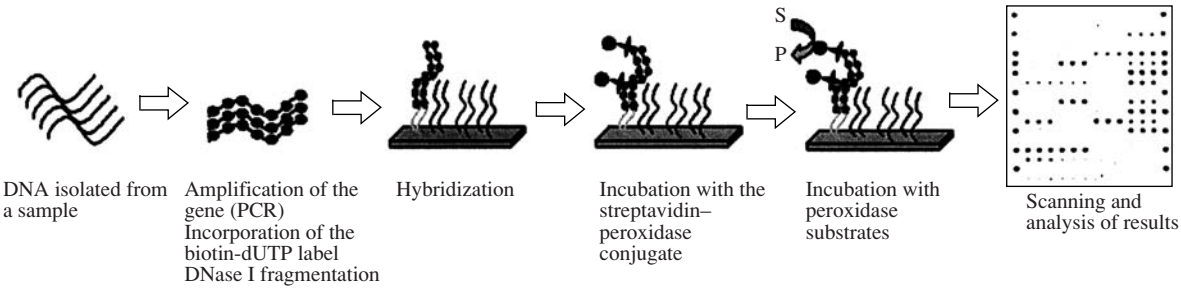

Fig. 1. Scheme of the method of hybridization analysis on a DNA microarray with colorimetric detection on the basis of horseradish peroxidase.

into relative values with the signal of complementary hybridization being taken as a unity.

Obtaining a Biotin-Labeled CTX-M-9 β-Lactamase Gene by the PCR Method

To ensure a high sensitivity of the hybridization analysis, the probe for hybridization should have a high specific incorporation of the label. The PCR method is efficient and highly reproducible as it allows considerable quantities of a specific probe to be obtained within a short period of time with minimal amounts of the DNA sample being used as the initial matrix. However, the molecules of the label bound to dNTP can prevent the incorporation by DNA polymerase of modified bases into the synthesized DNA chain. In this study, an estimate of the efficiency of incorporation of biotin molecules into the CTX-M-9 β-lactamase gene during PCR was performed using the results of determination of the molecular mass of PCR products by the gel electrophoresis method (Fig. 2). It is known that the CTX-M-type β-lactamase gene has a length of 876 bp. Since the average molecular mass of one base pair equals 614, the molecular mass of the CTX-M-9 β-lactamase gene is approximately 540000. On introduction of biotin molecules, the mass of the amplified fragment increases by approximately 45000, which corresponds to 80 molecules of biotin. Thus, the number of nucleotides in a single-stranded DNA molecule per label molecule was 20.

Earlier it was shown that, on introduction of the fluorescent label Cy3 into the TEM β-lactamase gene at the same ratio of concentrations of labeled/unlabeled dNTP, this value usually amounted to 50–150 depending on the quality of the DNA sample [12]. Thus, biotin is incorporated into the DNA molecule during PCR 2.5–7 times more efficiently in comparison with the fluorescent dye Cy3, frequently used as a label in DNA microarray technology.

Optimization of the Conditions of Hybridization on a DNA Microarray

The problem of analysis on a DNA microarray is the simultaneous determination of all mutation positions described for the given enzyme. Therefore, subsequently, we carried out the optimization of hybridization conditions on a DNA microarray with the goal of selecting the conditions under which it is possible to identify the maximum number of mutations. For this purpose, we used a microarray containing oligonucleotides for determining the 13 mutations described for β-lactamases of the CTX-M-9 subgroup, located in the following positions of the amino acid sequence: 7, 21, 29, 52, 121, 167, 183, 220.1, 220.2, 231, 240, 274, and 288.

It is known that the results of hybridization depend on several parameters, the main of which, at a fixed length of immobilized oligonucleotides, are the hybridization temperature and the molarity of the hybridization buffer.

The main criterion in choice of the hybridization temperature is that it should be significantly lower than the melting temperature (T_m) of the heteroduplexes forming. However, it cannot be greatly decreased

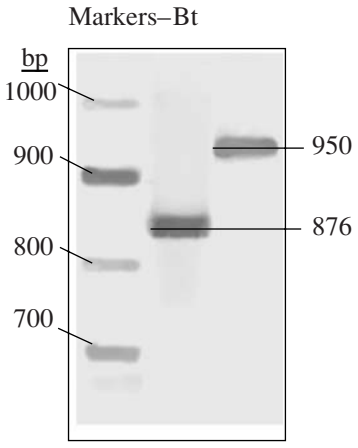

Fig. 2. Results of gel electrophoresis of the unlabeled and the biotin-labeled CTX-M-9 β-lactamase gene.

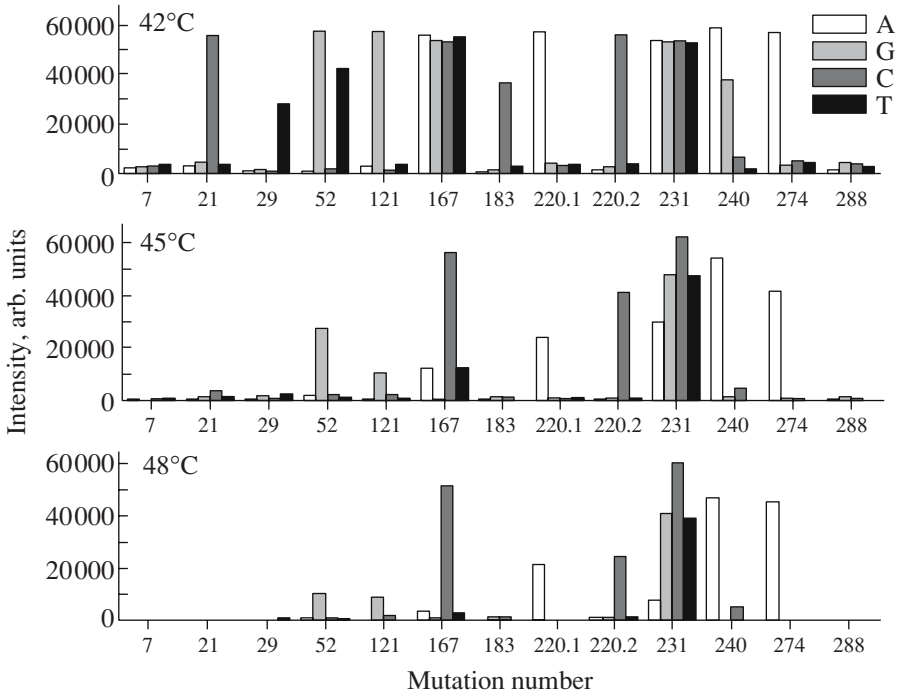

Fig. 3. Results of testing of CTX-M-9 I-lactamase on a DNA microarray with colorimetric detection obtained at different hybridization temperatures. Hybridization conditions: 0.3 M NaCl; fragment size 100–200 np.

because in this case the specificity of the analysis is lost. Since T_m of oligonucleotides for detection of mutations in CTX-M-type β-lactamase genes is 52.4–84.1°C, it was decided to study the range of hybridization temperatures 42–48°C. Data on testing of the CTX-M-9 β-lactamase gene on DNA microarrays with colorimetric detection obtained at different hybridization temperatures are shown in Fig. 3.

In comparison of the data of the diagrams, it is seen that, with a decrease in temperature from 48 to 42°C, the absolute intensity of signals grows, which indicates an increase in the hybridization efficiency and, thus, the sensitivity of the analysis. Thus, at 42°C, the results of hybridization can be seen for a number of positions of mutations that are impossible to identify at higher temperatures (21, 29, 183) due to very weak signals comparable in intensity with the background. Therefore, for the subsequent performance of a multianalysis on a DNA microarray, the temperature 42°C was chosen. However, with a decrease in the temperature, a decrease in the specificity of determination of a mutation was observed, and at 42°C we could not reliably determine the type of nucleotide in positions 52, 167, 231, and 240.

One of the means of eliminating nonspecific interactions in performing hybridization analysis is increasing the molarity of the hybridization buffer. In Fig. 4, the results of hybridization of the CTX-M-9 β-lactamase gene obtained at a different content of NaCl in the hybridization buffer are presented. For positions 52 and 240, the specificity of determination improves considerably with an increase in the concentration of NaCl to 0.6 M, while for positions 167 and 231 a further increase in the concentration of the salt to 1.0 M was required. But, since at a concentration of 1.0 M for positions 29 and 183 the intensity of hybridization signals was very low, for the subsequent performance of hybridization, an NaCl concentration of 0.6 M was chosen.

Yet another parameter influencing the efficiency and specificity of hybridization is the size of molecules of the studied DNA. An increase in the size of fragments enables the amount of label introduced per unit area of the carrier to be increased. However, in molecules of DNA having a great length, secondary structures unavoidably form, causing steric hindrances during hybridization and lowering its specificity. Therefore, subsequently we increased the degree of fragmentation of labeled DNA, for which we increased the concentration of DNase from 0.2 mU/ng DNA (fragment size 100–200 np) to 0.5 mU/ng DNA (fragment size 40–150 np). The intensity of signals decreased inconsiderably, which had little effect on the sensitivity of the analysis, but the specificity of hybridization in positions 52, 167, and 240 improved (Fig. 5).

Thus, the use of DNA microarrays with colorimetric detection enables identification of 11 out of 13 oligonucleotides for determination of the mutations described for the given subtype of β-lactamases. One position (231) is characterized by a low specificity of determination (Fig. 5); the signals in positions 7 and 288 remain close to the background values under any hybridization conditions. It is possible that this explains the low effi-

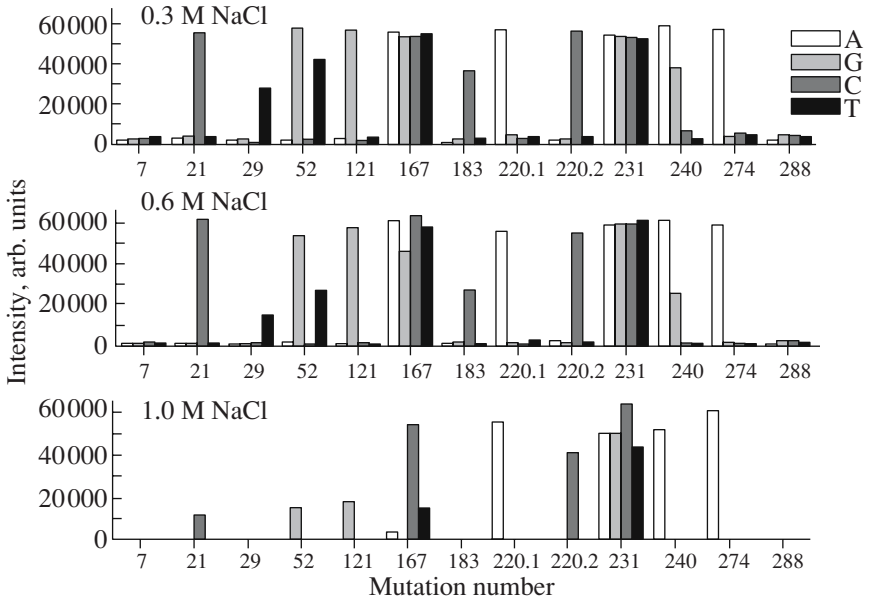

Fig. 4. Results of testing of CTX-M-9 I-lactamase on a DNA microarray with colorimetric detection obtained for different NaCl concentrations in the hybridization buffer. Hybridization conditions: 42°C; fragment size 100–200 np.

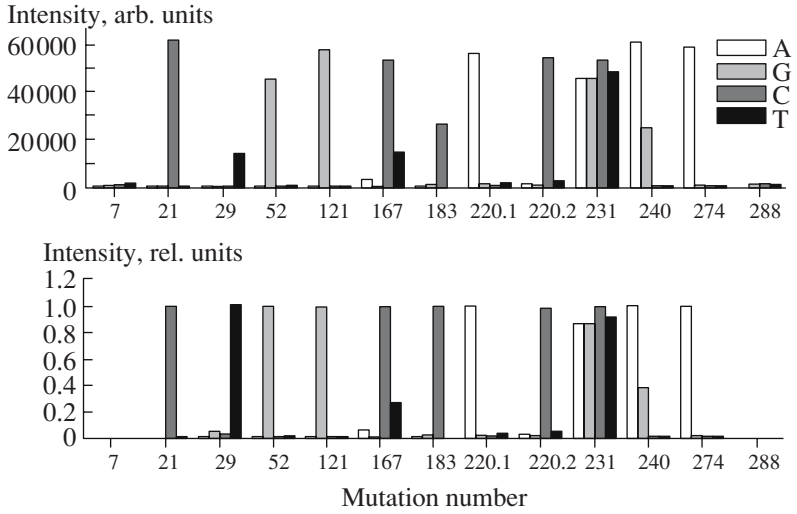

Fig. 5. Results of testing of CTX-M-9 I-lactamase on a DNA microarray with colorimetric detection. Hybridization conditions: 42°C, 0.6 M NaCl; fragment size 40–150 np.

ciency of incorporation of the label in these segments since they are the end segments in the gene and are close to the region of annealing of primers.

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