Oligonucleotide Microarray for the Identification of Carbapenemase Genes of Molecular Classes A, B, and D

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Received 23.08.2010

ABSTRACT This work is a report on the development of a method of hybridization analysis on DNA microarrays for the simultaneous identification and typing of carbapenemase-encoding genes. These enzymes are produced by the microorganisms that are responsible for causing infectious diseases. The method involves several steps, including DNA extraction from clinical samples and amplification of carbapenemase genes by multiplex PCR with simultaneous labelling by biotin. Following that, hybridization of the labeled PCR products with oligonucleotide probes immobilized on the surface of a nitrocellulose-based DNA microarray occurs. The biotin molecules attached to the DNA duplexes are detected by using conjugates of streptavidin-horseradish peroxidase, which is then quantified by colorimetric detection of the enzyme. We have designed the required oligonucleotide probes and optimized the conditions of the membrane microarray-based hybridization analysis. Our method allows to identify 7 types of carbapenemase genes belonging to the molecular classes A, B, and D, and it also allows additional typing into genetic subgroups. The microarrays have been tested with the control strains producing the carbapenemase genes which have been characterized by sequencing. The developed method of hybridization analysis was employed to investigate clinical strains of *Pseudomonas* spp. and *Acinetobacter* spp., which produce carbapenemases of different classes based on phenotypic testing. All strains of *Acinetobacter baumanii* resistant to carbapenems were producers of two carbapenemase OXA-type genes (OXA-51, in combination with OXA-23 (1 strain), OXA-40 (5 strains), or OXA-58 (4 strains)). The metallo-β-lactamase VIM-2 type gene was detected in all *Pseudomonas aeruginosa* strains resistant to carbapenems. Testing of carbapenem-sensitive strains did not detect any carbapenemase genes. The microarray method for the identification of carbapenemase genes is very accurate and highly productive. It can be employed in clinical microbiological laboratories for the identification and study of carbapenemase epidemiology.

KEYWORDS DNA microarrays, horseradish peroxidase, colorimetric detection, antibiotic resistance, carbapenemases

INTRODUCTION

During the last 20 years β-lactam antibiotics have been one of the drugs of choice for the treatment of a wide range of severe infectious diseases caused by gram-negative microorganisms. Among these antibiotics, carbapenems are one of the most successful drug groups (chemical structure shown in Fig. 1). This group of antibiotics is characterized by a wide spectrum of sensitive organisms, low toxicity, and good pharmacokinetics [1]. However, their efficiency has recently begun to show limitations with the emergence of drug-resistant strains. Carbapenem-resistant strains are most often found among nosocomial (in-hospital) infections belonging to the *Pseudomonas* spp. and *Acinetobacter* spp. genera. Healthcare facilities in the Russian Federation are, for example, noting a considerable increase in the number of pathogens resistant to carbapenem treatment. 38% of the observed *Pseudomonas aeruginosa* strains are now resistant to these drugs [2].

The emergence of β-lactam resistance in gram-negative bacteria can happen through several mechanisms, which include alteration of membrane permeability due to defective porine channels [3, 4], or activation of efflux systems [5]. However, the most clinically and epidemiologically important mechanism is the production of bacterial enzymes β-lactamases which hydrolyze the β-lactam ring of the antibiotic drug - β-lactamases [6, 7]. The β-lactamases are currently divided into 4 molecular classes – A, B, C, and D, based on their primary structure. The A-, C- and D-class enzymes are serine-type hydrolases, while the B-class enzymes are metallo-hydrolases, which bear one or two zinc atoms in their active site [8]. Several molecular classes of β-lactamase possess carbapenemase activity: however, the most of-

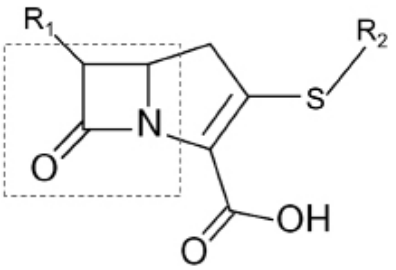

Fig. 1. Chemical structure of carbapenemes. β-Lactam ring in dashed lines.

ten found and clinically important carpbapenemases are KPC-type A-class enzymes [9], 5 groups of metallo-β-lactamases (VIM, IMP, SPM, GIM, SIM) [10], and a number of OXA-type D-class enzymes (subgroups OXA-23, OXA-40, OXA-51, OXA-58) [11].

Of all the numerous β-lactamases, carbapenemases are the most dangerous; they display high catalytic activity and wide substrate specificity, which includes practically every class of β-lactam antibiotics. Since carbapenemase-encoding genes are located on a plasmid, they can spread among pathogenic microorganisms at a rapid pace. Because of the variety of carbapenemases and the danger of their spreading, there is a need for robust methods for the detection of enzyme production, which can then be used for choosing the best suited treatment and for epidemiological control over the spreading of specific drug-resistant types. This is currently accomplished by using microbiological tests [6, 12, 13]. However, these tests take time and are ineffective for the identification of carbapenemase types. Identification of OXA-type enzymes by phenotype-based tests is virtually impossible [14].

Several PCR-based methods have been suggested for the identification of metallo-β-lactamase genes of the most spread VIM and IMP types [15, 16], and the main OXA-type carbapenemase subgroups [17]. A method for the identification of 5 groups of metallo-β-lactamases, involving multiplex real-time PCR with subsequent analysis of the melting curves of the obtained amplicons, has recently been developed [18]. However, the multiplex capacity of PCR is usually limited, which makes simultaneous detection of a large number of genes impossible.

Hybridization analysis based on a microarray-technology is a promising method of identification that yields quick results of high informative value. This technique has considerable advantages over traditional methods, since it allows a multi-parametric analysis and also uses a miniscule sample, which reduces cost and the time needed to obtain results [19, 20].

The goal of this work was to develop a method for the identification of A-, B-, and D-class carbapenemase genes involving hybridization analysis on membrane-based DNA microarrays which could be visualized by colorimetric detection.

EXPERIMENTAL PROCEDURES

The collection of primers for the amplification of carbapenemase genes and the amino-modified oligonucleotide probes were synthesized by Synthol (Moscow, Russia). Samples of the bacterial DNA extracted from control strains of *A. baumanii*, *Ps. aeruginosa*, *Escherichia coli*, and *Klebsiella pneumonia* were provided by the Institute of Antimicrobial Chemotherapy of the Smolensk State Medical Academy. These samples produced carbopenemases VIM-1, VIM-2, VIM-4, VIM-7, IMP-1, IMP-2, SPM-1, OXA-23, OXA-40, OXA-51, OXA-58, and KPC-3. Cell cultures of microorganisms from the *Enterobacteriaceae family* and *A. baumanii* and *Ps. aeruginosa strains*, either sensitive or resistant to carbapenems, according to phenotypic tests performed on a VITEK automatic analyzer (BioMerieux, France), were provided by the Burdenko Institute of Neurosurgery.

Bacterial DNA extraction

Extraction of bacterial DNA from a cell suspension with no less than 10^5 CFU/ml was performed using temperature lysis in a buffer. 500 μl of suspension was placed in centrifuge tubes, and the cells were pelleted by centrifugation at 10,000 *g* for 1 minute. After removing the supernatant, 100 μl of buffer (10 мМ Tris-HCl, 1 мМ EDTA, pH 7.5) was added to the pellet, which was then re-suspended with a shaker. The tubes were then incubated on a solid-medium thermostat for 20 minutes at a temperature of 99^0 C. After heating, the samples were centrifuged for 1 minute at 10,000 *g*. A PCR sample used 1 μl of the obtained supernatant.

Amplification of carbapenemase genes using multiplex PCR with simultaneous biotin labeling

Amplification of carbapenemase (A-, B-, and D-class) gene-fragments, with simultaneous biotin labeling, was performed in two multiplex PCR reactions (one reaction amplified the genes of all the metallo-β-lactamases and the other amplified the OXA- and KPC-type β-lactamase genes). Each multiplex PCR sample was 25 μl in volume and contained the following: 10 mM Tris-HCl-buffer with 2.5 mM of magnesium acetate, 50 mM KCl pH 8.3, 2.5 units of *Taq*-DNA-polymerase, 100 μM dATP, dGTP, dCTP, 60 μM dTTP, 40 μM dUTP-11-biotin (Fermentas, Germany), 0.4 μM each of the direct and reverse primer for each group of carbapenemase, and 1 μl of the template DNA solution. Amplification was performed in a Mastercycler gradient amplifier (Eppendorf, Germany) according to the following protocol: initial denaturation at 94˚C (2 min), 25 cycles of amplification (20 sec – denaturation at 94˚C, 30 sec – annealing of the primers at 65˚C, 1 minute – elongation at 72˚C), and a final elongation step at 72˚C

(6 min). Horizontal electrophoresis of the PCR products was performed in a 1% agarose gel with TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5) with etidium bromide added to a final concentration of 1.6 μg/ml. Visualization was performed on a UV-transilluminator at a wavelength of 260 nm.

Fragmentation of the PCR-products

The DNA was fragmented at room temperature for 5 minutes. Amplified DNA was diluted to a concentration of 30 ng/microliter with the reaction buffer (40 mM Tris-HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, pH 8.0), and the mixture was then supplemented with DNAase I (Promega, Germany). The reaction was stopped by the addition of 3 mM EDTA and 10-minute incubation at 65°C.

Immobilization of the oligonucleotide probes on a membrane-based DNA-microarray

BioTrace NT nitrocellulose (Pall Corporation, USA) was used as a support for DNA-microarray. Modification of the membranes was performed according to [21], using 1-ethyl-3-(3-dimethylaminopropyl)carboimide (Sigma, USA). The oligonucleotides were diluted in a buffer (160 mM Na_2SO_4, 130 mM Na_2HPO_4) to a final concentration of 20 μM and then applied onto the membranes by an XactII™ Arrayer robot (LabNEXT Inc., USA) using 300 μm pins. After the procedure, the membranes were incubated at 60°C for 30 minutes.

Hybridization on the DNA microarray

Prior to hybridization, the microarrays were washed with PBST buffer (0.01 M K_2HPO_4, 0.15 M NaCl, 0.05% Tween-20, pH 7.0) twice, 10 minutes each time at room temperature, and then blocked in a solution of 1% bovine serum albumine (BSA) and 1% casein (Sigma, USA) in PBS buffer (0.01 M K_2HPO_4, 0.15 M NaCl, pH 7.0) at 37°C for 30 minutes. 500 ng of fragmented and labeled DNA was then diluted in the hybridization buffer - 2x SSPE (0.3 M NaCl, 0.02 M NaH_2PO_4, 2 mM EDTA, pH 7.4), which also included 1.6 pmol/ml of the control biotin-labeled oligonucleotide (positive hybridization control). The microarray was then placed into the hybridization mixture (300 μl per 1 array) and incubated at 45°C for 1 hour in a Thermomixer comfort apparatus (Eppendorf, Germany). After hybridization, the membranes were washed with PBST twice for 15 minutes at room temperature.

Detection and hybridization data analysis

The microarrays were incubated in a solution of streptavidine-peroxidase conjugate (Imtek, Russia) (0.2 μg/ml) in PBST for 30 minutes at 37°C. Then they were washed in PBST for 10 minutes and placed into a substrate solution containing 3,3′,5,5′-tetramethylbenzydene (TMB), H_2O_2 (NVO Immunotech, Russia) and sodium dextransulphate (M_r = 8000, Pharmacia, Sweden) (final concentration - 0.5% (by mass)) for 10 minutes, after which the arrays were washed in distilled water and air-dried. The membrane microarrays were scanned on a Perfection V750 Pro (Epson, Germany) scanner at a resolution of 4,800 dpi. The obtained images (in TIFF format) were analyzed using Scan Array Express (PerkinElmer, version 3.0, Germany) software, and the intensity values of the analytic signals at various spots of the microarray were determined. The absolute values of the signals were then recalculated into relative signals, using the mean intensity of the positive hybridization control used in each array.

RESULTS AND DISCUSSION

Molecular design of the oligonucleotide probes

The Genbank database currently has information on 10 KPC-type enzymes, 52 metallo- β-lactamases (23 from the IMP group, 26 from the VIM group and one member of each of the following groups - SPM, SIM and GIM), and also 70 carbapenemases from the OXA group. Alignment of the amino acid and encoding sequences of these enzymes shows that only enzymes from the KPC group display a high degree of similarity within their group (differ by 1-2 amino acid substitutions), while the numerous members of the IMP, VIM, and OXA groups differ considerably from their group members. Because of this, each group was split into separate subgroups, which included enzymes whose genes were highly similar. Thus, the VIM group was divided into 3 subgroups (VIM-1, VIM-2, and VIM-7); the IMP group, into 6 subgroups (IMP-1, IMP-2, IMP-5, IMP-11, IMP-12, and IMP-14); while carbapenemases from the OXA groups were divided into 4 subgroups (OXA-23, OXA-40, OXA-51 and OXA-58). Group and subgroup classification of carbapenemases based on their amino acid sequence alignments and their β-lactamase molecular classification are shown in Fig. 2.

One of the main stages of DNA microarray development was the design of the oligonucleotide probe sequences required to detect various groups of carbapenemase genes. The selection of an oligonucleotide probe for the identification of a group of genes is based on the alignment of the coding sequences of all the carbapenemase genes in this group. What is needed is a sufficiently long fragment of the gene that is conserved in all of the members of this group and is no less than 18 nucleotides long. These regions were then analyzed in terms of melting temperature, G/C content and secondary structure formation. In order to perform microarray-based hybridization analysis, we selected the oligonucleotides that were unlikely to form secondary structures and

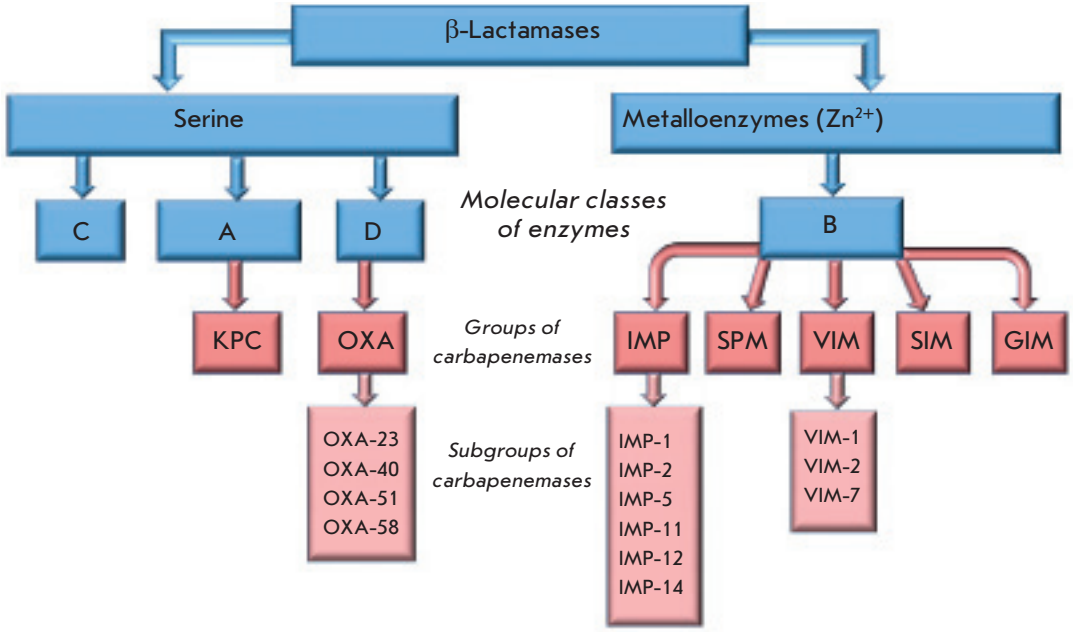

Fig. 2. Classification of carbapenemases into groups and subgroups based on the alignment of amino acid sequences and their correspondence to molecular classes of β-lactamases.

whose melting temperature differed by no more than 10°C. Based on the selected sequences, we synthesized two oligonucleotide probes which were complementary to the direct and reverse strands of the gene. For additional typing of IMP, VIM, and OXA carbapenemase gene-subgroups, we chose regions with high similarity which bore no mutations inside a given subgroup and had low similarity with genes from other subgroups. In order to increase the specificity of the analytical procedure, we chose two oligonucleotide probe variants which corresponded to different regions of the gene. In this case, we synthesized the probes which were complementary to the reverse strand of the gene.

The sequence of the chosen oligonucleotide probes and their characteristics are presented in Table 1. The length of the probes varied from 18 to 27 nucleotides, the G/C-content was 30- 60%, and the melting temperature was 63–72°C. Each probe was supplemented by additional spacers at the 5'-terminus, which helped to distance the probe from the array surface, thus removing steric barriers for hybridization and increasing the availability of the probe for the DNA-target. Optimization procedures showed that the best spacer contained 13 thymidine residues. The additional thymidines did not have any significant effects on the intensity of the hybridization signals, or on the specificity of hybridization (data not shown).

Amplification of carbapenemase genes of various molecular classes with simultaneous labeling

Amplification of A-, B-, and D-class carbapenemase genes utilized multiplex PCR with simultaneous biotin labeling. Biotin-labeled deoxyribouridine triphosphate (dUTP) was used as a labeling reagent and was incorporated into the DNA, along with unlabeled deoxyribothymidine triphosphate (dTTP). The templates for the PCR reaction were bacterial DNA samples extracted from the control strains of microorganisms producing β-lactamases VIM-1, VIM-2, VIM-7, IMP-1, IMP-2, SPM-1, OXA-23, OXA-40, OXA-51, OXA-58, and KPC-3.

Design of primers for the amplification of various carbapenemase genes was based on an alignment of the coding regions of these genes. Primers for amplification of the full-size carbapenemase genes from the KPC group and metallo-β-lactamase groups SPM, SIM, and GIM were chosen from regions which were conserved in this group, namely the gene termini. We could not find conserved regions longer than 20 bp for metallo- β-lactamases from the IMP and VIM groups, nor could we find any for OXA-type carbapenemases, since these groups showed a low degree of in-group similarity, which is why separate primers had to be selected for each subgroup.

The length of the primers (20 – 28 nucleotides) was chosen so as to push their melting temperature to 62 – 68°C, which would allow simultaneous amplification of all the types of genes simultaneously and with equal efficiency. Primer selection also factored in G/C-content, and we chose structures that had a G/C content of 30 – 60%. We also estimated the possibility of primer-dimer and secondary structure formation and favored the sequences which were least likely to do so. As a result, each group was fitted with several direct and reverse primers with various parameters. Various combinations of these primers were tested in PCR reactions in order

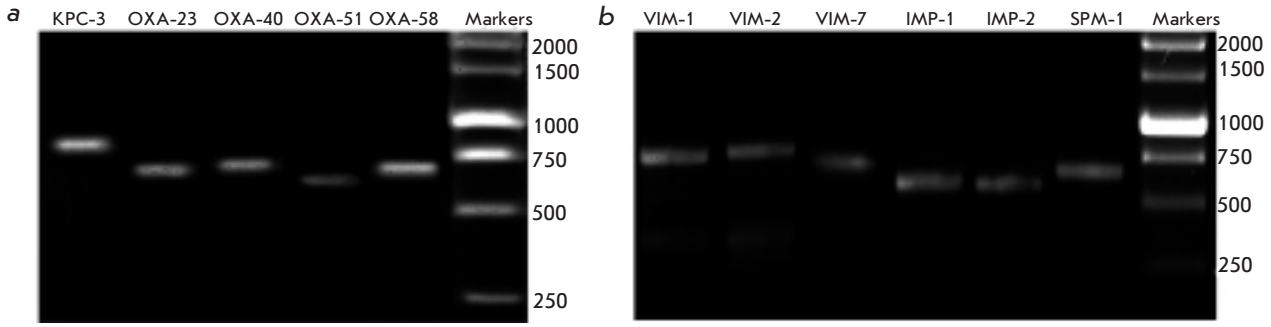

Fig. 3. Electrophoretic separation of multiplex PCR products after amplification of OXA-, KPC-type carbapenemases (a) and metallo-β-lactamases (b).

to test the specificity of gene amplification. The primers which showed the highest specificity with a good output were used for the multiplex procedure. The primer sequences are presented in Table 2.

In order to determine the optimal primer-annealing temperature for the multiplex amplification of all the carbapenemase gene types, we first calculated the optimal annealing temperature for each pair of primers in a specific reaction and then selected the lowest temperature value for the multiplex PCR reaction. The range of the studied primer T_a 's was 52 – 68°C. The optimal T_a turned out to be 60°C. Reactions at this temperature resulted in the efficient synthesis of specific PCR-products.

After determining the optimal carbapenemase gene amplification conditions, we evaluated the possibility of performing multiplex PCR with 16 pairs of primers in order to amplify the genes of all of the studied groups simultaneously. However, under these conditions the specific product for most of the carbapenemase groups was either absent or the yield was very low, which made further microarray hybridization analysis impossible. Because of this, we decided to amplify the carbapenemase genes using a two step multiplex PCR procedure: the first used a mixture of primers specific to metallo-β-lactamases (11 pairs of primers), and the second used a mixture of primers specific to OXA and KPC carbapenemases (5 pairs of primers). The results of electrophoretic analysis of the PCR-products obtained during the amplification of various carbapenemase genes from control microorganism strains are presented in Fig. 3.

The yield of labeled specific products from the multiplex PCR procedure was approximately 40-50 ng/μl for each type of carbapenemases gene, which was sufficient for further microarray-based hybridization analysis. Amplification of nonspecific products was detected only for genes from the VIM-1 and VIM-2 subgroups: however, these products had low yields and, as it follows from later experiments, their presence did not affect the specificity of the hybridization analysis.

Oligonucleotide microarray for detecting A-, B-, and D-class carbapenemase genes

The DNA microarray for the identification of the major types of carbapenemases is made on a support of nitrocellulose and has a size of 6.0 x 9.5 mm. On its surface there are 40 immobilized oligonucleotide probes (14 probes for the identification of 7 distinct groups of carbapenemases and 26 probes for additional typing of these genes into subgroups). Each microarray also includes 3 types of control oligonucleotides: an immobilization control (a biotin labeled oligonucleotide), a positive hybridization control (an oligonucleotide whose sequence is complementary to a biotin-labeled oligonucleotide which is added to the hybridization mixture), a negative hybridization control (an oligonucleotide with a random base sequence). In order to increase the reproducibility of this procedure, each oligonucleotide probe is present on the microarray in three copies. A schematic of the layout of the specific and control oligonucleotide probes on the surface of the DNA microarray is presented in Fig. 4.

The carbapenemase gene identification procedure involved hybridization analysis on a DNA microarray and included the following stages: 1) amplification of the β-lactamase gene from DNA isolated from the clinical strain (by two multiplex PCR reactions); 2) hybridization of the biotin-labeled DNA with oligonucleotide probes on the surface of the microarray; 3) visualization of the hybridization results using a streptavidin-peroxidase conjugate followed by colorimetric detection of the enzyme.

We also optimized hybridization conditions. We analyzed hybridization efficiency at temperatures ranging from 40 to 50°C; the temperature could not be higher than the melting temperature (T_m) of any oligonucleotide probe and was limited by the high level of nonspecific hybridization at lower temperatures. Hybridization at 40°C displayed strong signals: however, most of the probes showed cross-hybridization with the genes of various carbapenemases. Hybridiza-

Table 1. Sequences of the specific and control oligonucleotide probes

Name	Nucleotide sequence, 5'→3'	Length, nucleotides	G/C, %	T_m °C
Control oligonucleotides				
Immobilization control	TCTAGACAGCCACTCATA-Biotin	18	44.4	60.4
Positive hybridization control	GATTGGACGAGTCAGGAGC	19	57.9	66.1
Negative hybridization control	TCTAGACAGCCACTCATA	18	44.4	60.4
Oligonucleotide probes for determining the carbapenemase group				
KPC_direct	GCTTCCCACTGTGCAGCTCATTC	23	56.5	72.0
KPC_reverse	GAATGAGCTGCACAGTGGGAAGC	23	56.5	72.0
VIM_direct	GGAGATTGAAAAGCAAATTGGACT	24	37.5	66.6
VIM_reverse	AGTCCAATTTGCTTTTCAATCTCC	24	37.5	66.6
IMP_direct	GGAATAGAGTGGCTTAATTCTCG/A	23	41.3	64.7
IMP_reverse	C/TGAGAATTAAGCCACTCTATTCC	23	41.3	64.7
SPM_direct	GATGGGACCGTTGTCATTG	19	52.6	64.9
SPM_reverse	CAATGACAACGGTCCCATC	19	52.6	64.9
SIM_direct	CCTTGGCAATCTAAGTGACGCAA	23	47.8	69.7
SIM_reverse	TTGCGTCACTTAGATTGCCAAGG	23	47.8	69.7
GIM_direct	CACACTGGGAAATGGGCTTATA	22	45.5	66.7
GIM_reverse	TATAAGCCCATTTCCCAGTGTG	22	45.5	66.7
OXA_direct	CCACAA/GGTG/AGGC/TTGGTTG/AAC	20	55.0	67.0
OXA_reverse	GTC/AAACCAG/ACCC/TACT/CTGTGG	20	55.0	67.0
Oligonucleotide probes for determining the carbapenemase sub-group				
VIM-1_568	TCAGCGAACGTGCTATACGG	20	55.0	68.3
VIM-1_590	GTTGTGCCGTTCATGAGTTGT	21	47.6	67.9
VIM-2_568	TCTGCGAGTGTGCTCTATGG	20	55.0	67.9
VIM-2_590	GTTGTGCGATTTATGAGTTGT	21	38.1	63.7
VIM-7_127	GTTCGGCTGTACAAGATTGGCG	22	54.5	70.0
VIM-7_181	CTCGGTGACACGGTGTAC	18	61.1	65.8
IMP-1_135	GTGGGGCGTTGTTCCTAAACATG	23	52.2	70.2
IMP-1_387	GGTTCAAGCCACAAATTCATTTAGC	25	40.0	67.8
IMP-2_264	TCAAAGGCACTATTTCCTCACATTTC	26	38.5	68.2
IMP-2_497	TACCTGAAAAGAAAATTTTATTCGGTG	27	29.6	65.7
IMP-5_506	AATAGAGTTTTGTTCGGTGGTT	22	36.4	65.0
IMP-5_459	TGGTCCAGGGCACACTCC	18	66.7	70.4
IMP-11_570	TGTTGAAGCATGGCCACATT	20	45.0	67.6
IMP-11_621	TGCAAAACTGGTTGTTCCAAGCC	23	47.8	70.9
IMP-12_226	AAATTAGTTGCTTGGTTTGTAGGG	24	37.5	66.4
IMP-12_495	GCTACCTGAAAACAAAATTTTATTCG	26	30.8	64.8
IMP-14_292	GGTGACAGTACGGCTGGAATAG	22	54.5	68.4
IMP-14_374	AAAAAGACAATAAGGTACAAGCTA	24	29.2	63.4
OXA-23_225	AAATACAGAATATGTGCCAGCCTCT	25	40.0	68.8
OXA-23_309	GAAGGGCGAGAAAAGGTCATTTAC	24	45.8	68.0
OXA-40_225	AAATAAAGAATATGTCCCTGCATCA	25	32.0	65.6
OXA-40_329	GAACTTATCCTATGTGGGAGAAAG	24	41.7	64.8
OXA-51_225	TTCGACCGAGTATGTACCTGCTTCG	25	52.0	71.7
OXA-51_578	GCCCAAAAGTCCAAGATGAAG	21	47.6	65.8
OXA-58_225	AAAAACAGCTTATATTCCTGCATCT	25	32.0	66.0
OXA-58_206	GCACGCATTTAGACCGAGC	19	57.9	67.7

Table 2. Primer sequences for the multiplex PCR-amplification of carbapenemase genes

Type		Sequence 5'→3'	Length, nucleotide	G/C, %	T_m, °C	Length of PCR-product, bp
KPC	direct	TTCTGCTGTCTTGTCTCTCATGG	23	47.8	64.7	801
	reverse	CCTCGCTGTGCTTGTCATCC	20	60.0	65.7	
IMP-1	direct	GGCGTTTATGTTCATACTTCGTTTG	25	40.0	64.4	584
	reverse	GTAAGTTTCAAGAGTGATGCGTCTCC	26	46.2	65.6	
IMP-2	direct	GGTGTTTATGTTCATACATCGTTCG	25	40.0	63.8	584
	reverse	GTACGTTTCAAGAGTGATGCGTCCCC	26	53.8	67.8	
IMP-5	direct	GGTGTTTATGTTCATACTTCGTTTG	25	36.0	62.5	584
	reverse	GTACGTTTCAAGAGTGATACATCTCC	26	42.3	63.4	
IMP-11	direct	GGTGTTTATGTTCATACATCGTTTG	25	36.0	62.6	584
	reverse	GTAAGCTTCAAGAGCGACGCATCTCC	26	53.8	67.8	
IMP-12	direct	GGTGTTTATCTTCATACATCTTTTG	25	32.0	60.5	584
	reverse	GTAAGTTTCAAGAGTGATGCGTTCCC	26	46.2	66.0	
VIM-1	direct	GTAGTTTATTGGTCTACATGACCGCGTC	28	46.4	66.9	743
	reverse	CGCTGTGTGCTGGAGCAAGTC	21	61.9	68.1	
VIM-2	direct	GTAAGTTATTGGTCTATTTGACCGCGTC	28	42.9	65.9	743
	reverse	CGTTGTGTGCTTGAGCAAGTC	21	52.4	64.7	
VIM-7	direct	AGCATATTCCGCACAGCCTGG	21	57.1	67.5	685
	reverse	CCGGGCGGTCTGGAATTGCTC	21	66.7	67.7	
SPM	direct	CGTTTTGTTTGTTGCTCGTTGCGGG	25	52.0	67.4	648
	reverse	CCTTCACATTGGCATCTCCCAGATAAC	27	48.1	67.2	
SIM	direct	GTTTGCGGAAGAAGCCCAGCC	21	61.9	68.6	613
	reverse	CTCCGATTTCACTGTGGCTTGGG	23	56.5	67.6	
GIM	direct	CTTGTAGCGTTGCCAGCTTTAGCTC	25	52.0	67.8	638
	reverse	CTGAACTTCCAACTTTGCCATGCC	24	50.0	66.9	
OXA-23	direct	GAAACCCCGAGTCAGATTGTTCAAG	25	48.0	65.8	686
	reverse	GGCATTTCTGACCGCATTTCC	21	52.4	64.8	
OXA-40	direct	GTTTCTCTCAGTGCATGTTCATC	23	43.5	62.3	714
	reverse	CATTTCTAAGTTGAGCGAAAAGGGG	25	44.0	64.6	
OXA-51	direct	CGAAGCACACACTACGGGTG	20	60.0	65.4	649
	reverse	CTCTTTTCGAACAGAGCTAGGTATTC	26	42.3	63.4	
OXA-58	direct	CTTGTGCTGAGCATAGTATGAGTC	24	45.8	63.3	684
	reverse	CCACTTGCCCATCTGCCTTTTC	22	54.5	66.5	

Table 3. Results on clinical sample testing on DNA microarrays

Type of microorganism	Carbapenemase sensitivity as determined by phenotypical tests	Number of samples	Detected carbapenemase types				
			OXA-23	OXA-40	OXA-51	OXA-58	VIM-2
A. baumanii	Resistant	10	1	5	10	4	-
Ps.aeruginosa		11	-	-	-	-	11
A. baumanii	Sensitive	2	-	-	-	-	-
K. pneumonia		3	-	-	-	-	-
E. coli		2	-	-	-	-	-

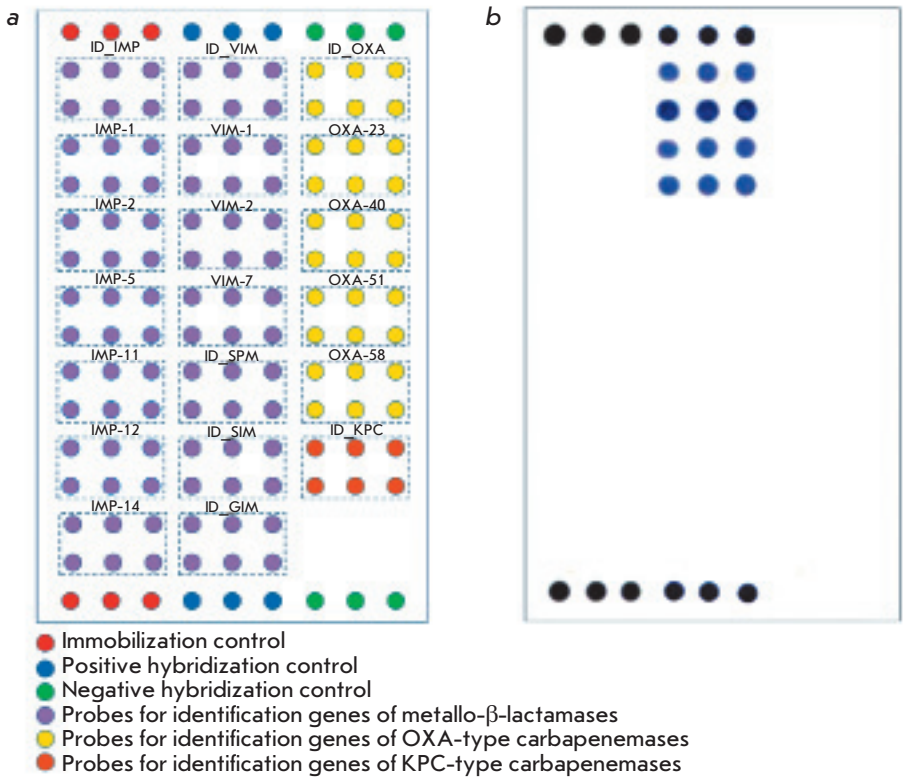

Fig. 4. Layout of specific and control oligonucleotide probes on the surface of the DNA microarray for the identification of the A-, B-, D-class carbapenemase genes.

tion at 50°C displayed weak signal intensities for some of the probes. For these reasons, we chose 45°C as the optimal temperature. The hybridization buffer consisted of 2x SSPE supplemented by 0.2 % sodi dodecylsulphate (SDS) in order to improve the membrane's wetting properties.

The size of the labeled DNA-target proved to be a critical parameter for hybridization. Hybridization of labeled PCR-products whose size was 580–800 nucleotides with the appropriate oligonucleotide probes proved to yield weak signals. Additional fragmentation by DNAase (yielding fragments of 50–150 nucleotides) proved to increase hybridization signal intensity for most of the probes.

Hybridization duration was assayed within a range of 0.5 to 4.0 hours. It was observed that the hybridization of biotin-labeled DNA onto immobilized probes reaches equilibrium after two hours of incubation with active mixing. We also noticed that conducting the reaction in kinetic conditions (1 hour) does not dramatically weaken the signals as compared to the equilibrium state (about 10 – 20% depending on the probe). It also did not lower the specificity of the analytic procedure, which allowed the positive identification of all types of carbapenemase genes in the hybridization mixture.

Figure 5 shows the results of an experiment in which control microorganism strains producing VIM-1 and IMP-1 metallo- β-lactamases and carbapenemases OXA-51 and OXA-40 were tested on our DNA microarray. Identification of the β-lactamase group was assayed by the hybridization intensity with a group-specific probe, while additional typing was assayed by the hybridization intensity with subgroup-specific probes. The advantage of the microarray-based hybridization analysis is the possibility of simultaneously detecting several genes, which is demonstrated by testing the control *A. baumanii strain* for OXA-type carbapenemases.

Testing clinical strains of microorganisms resistant to carbapenems

The DNA microarray developed was tested on clinical strains of gram-negative microorganisms, either resistant or sensitive to carbapenems, as assayed by a phenotypical test. Table 3 shows the results for 28 clinical strains of *Ps. aeruginosa*, *A. baumanii* and *Enterobacteriaceae* spp., which display various levels of carbapenem sensitivity (strains were provided by the N.N. Burdenko Institute of Neurosurgery and Institute of Antimicrobial Chemotherapy of Smolensk State Medical Academy). All the *A. baumanii* strains which proved resistant to carbapenems (as assayed by phenotyping) expressed two carbapenemase genes (OXA-51 and OXA-23 (1 strain), OXA-40 (5 strains), OXA-58 (4 strains)). All the carbapenem-resistant *Ps. aeruginosa* strains happened to possess a VIM-2-type metallo-β-lactamase gene. Testing of carbapenem-sensitive

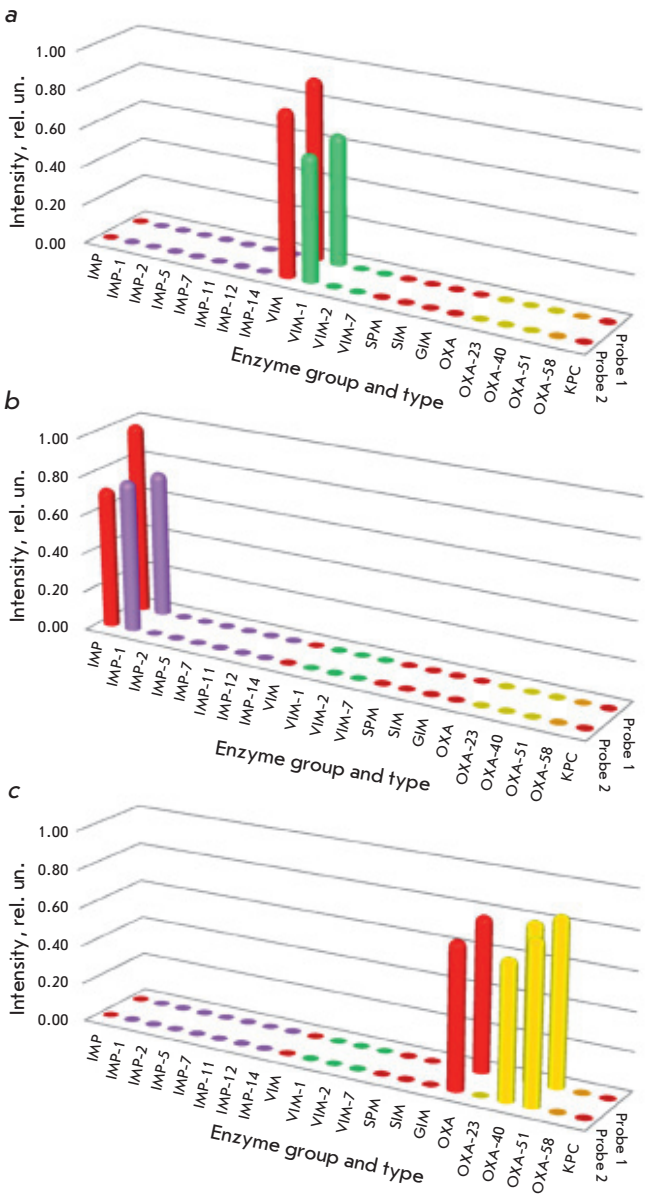

Fig. 5. Results of a DNA microarray-based hybridization analysis for control *Ps. aeruginosa (a, b) and A. baumanii (c)* strains, producing carbapenemases VIM-1 (a), IMP-1(b), OXA-51 and OXA-40 (c).

strains revealed no carbapenemase genes. Thus, the results of the microarray-based hybridization analysis are in accordance with the phenotyping tests. Moreover, the structure of the observed carbapenemases was confirmed by gene sequencing for two carbapenem-resistant samples - one strain of *A. baumanii* and one strain of *Ps. aeruginosa*, which expressed OXA-40, OXA-51 and VIM-2 genes.

Thus, our method of hybridization analysis based on DNA microarray for the identification and typing of carbapenemase genes is highly accurate, productive, and can be used in clinical microbiological laboratories for the identification of carbapenemases and for studying their epidemiology. The phenotyping tests currently being used take time (from 24 to 48 hours) and are not always effective for determining carbapenemase types, such as OXA-type carbapenemases. Identification of carbapenemase genes on DNA microarrays allows rapid diagnostics, with the whole procedure taking only 4.5 hours, including 0.5 hours for bacterial DNA extraction, 1.5 hours for amplifying the carbapenemase genes and fragmenting the PCR-products, 1.5 hours for the hybridization and washing steps, and 1 hour for colorimetric detection of the hybridization results. An important feature of this method is the possibility of simultaneously identifying several genes in one sample. ●

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