METHODS

Application of Nested-PCR Technique for the Diagnosis of Tuberculosis

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A test-system based on amplification of IS 986 fragment (nested-PCR) was developed for the detection of *Mycobacterium tuberculosis* and *M. bovis* in different biological samples. We constructed external primers and selected appropriate amplification parameters (annealing temperatures for stages I and II, the number of cycles for each amplification stage, components of the amplification mixture, and pretreatment conditions for different biological samples). The developed parameters make the detection of mycobacteria more efficient and less expensive compared to commercial Cobas Amplicor system.

Key Words: *tuberculosis; pathogen; nested-PCR; specificity; efficiency*

Polymerase chain reaction (PCR) is widely applied in the diagnosis of infectious diseases [1,2]. Several PCR systems were described for the detection of *Mycobacterium tuberculosis* (MBT), among them the most widely used are the test-systems based on amplification of IS 6110 element [1,5,11]. PCR radically accelerates detection of the pathogen and considerably improves sensitivity of the analysis. When using PCR in practical diagnosis some peculiarities of specimens affecting the detection of pathogen should be taken into account: heterogeneity of clinical samples (different content of pathogens), the presence of factors inhibiting amplification, and loss of the pathogen during processing of clinical samples. In this connection the commonly used one-stage PCR not always provides necessary sensitivity and reliability.

Nested PCR (N-PCR), a variant of PCR characterized by high sensitivity and specificity, is less frequently used for the diagnosis of infections. This technique is based on application of two pairs of primers (internal and external) and includes two amplification stages. Each pair of primers participates in a distinct stage characterized by specific conditions of denaturation, annealing, and synthesis [1,3].

In this study, N-PCR was applied to reveal *M. tuberculosis* and *M. bovis* in different clinical samples. Our test-system based on amplification of IS 6110 fragment is a modification of standard PCR system [7]. The efficiency of the proposed system for the detection of MBT in clinical samples was compared with that of commercial Cobas Amplicor system (Hoffman La Roche).

MATERIALS AND METHODS

M. tuberculosis, M. bovis, M. bovis BCG, M. avium, M. fortuitum, M. kansasii, M. terrae, and M. gordonae strains were obtained from the collection of Moscow Research Center for Tuberculosis. More than 20 bacterial strains obtained from the L. A. Tarasevich Institute of Biomedical Problems were analyzed to determine the specificity of selected primers.

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Biological samples from patients with different forms of tuberculosis (Clinical Department of the Moscow Research Center for Tuberculosis) were analyzed. The samples (sputum, exudate, bronchoalveolar lavage) were mixed 1:1 with fresh 0.5% acetylcysteine in 20% NaOH, carefully vortexed, incubated for 40 min at room temperature, diluted 1:5 (v/v) with phosphate buffer (pH=6.8), and centrifuged for 30 min at 3000 rpm. Cerebrospinal fluid (CSF) was centrifuged for 10 min at 10,000 rpm. Blood lymphocytes were isolated as described elsewhere [4]. After that all samples were processed similarly: the sediment was washed with TE-buffer (10 mM Tris-HCl and 1 mM EDTA, pH=8.0), resuspended in the same buffer with 1% Triton X-100, heated in a dry thermostat for 15 min at 95°C, and centrifuged for 10 min at 10,000 rpm. The supernatant was used for PCR.

Samples for PCR in the Cobas Amplicor system were treated according to manufacturer's instructions.

Amplification was performed in a Hybaid thermocycler in 30 μl medium containing 10 mM Tris-HCl (pH=8.8), 50 mM KCl, 0.5% Tween-20, 2.5 mM $MgCl_2$, 5% formamide, 200 μM nucleoside risphosphates (each), 1-2 units Taq polymerase, 0.1-0.15 μM oligonucleotide primers (each) and 3 μl sample; 30 μl mineral oil was added to each tube to prevent evaporation. INS 5 and INS 6 primers were used in amplification stage I consisting of 20 cycles: 40 sec at 95°C, 1 min at 60°C, and 1 min at 72°C. INS 1 and INS 2 primers were used for stage II amplification consisting of 25 cycles at a higher annealing temperature (72°C).

External primers were selected using OLIGO.4 software. External primers INS 5 (5'-*ggtttgcggtggggtgtcg*-3') and INS 6 (5'-*gttggatgcctgcctcgg*-3') yielded a 450-bp amplicon; internal primers INS 1 (5'-*cgtgagggcatcgaggtggc*-3') and INS 2 (5'-*gcgtaggcgtcggtgacaaa*-3') yielded a 245-bp amplicon. These internal primers were previously used for PCR-identification of MBT [7].

TABLE 1. Detection of MBT (in %) by Different Laboratory Techniques

Sample	Growth on a Levenstein—Jenssen medium	PCR	
		Cobas	N-PCR
Sputum (*n*=42)	63	87	98
Exudate (*n*=30)	No	84	97
CSF (*n*=20)	No	17	90
Blood (*n*=25)	No	85	99
Bronchial secretion (*n*=27)	No	77	96

After amplification the samples were separated in a 1.5% agarose gel and analyzed as described previously [8].

Electrophoregrams were photographed, DNA fragments were transferred to Zeta-probe membrane filters (Bio-Rad) using an alkaline technique [8]. The amplicon obtained from control MBT strain was used as a hybridization probe. Amplicons were purified with a standard Jet-sorb kit (Genomed) according to manufacturer's instructions. Terminal sequences corresponding to primers were removed with Taq-1 restrictase (Fermentas), the products were purified using a Jet-sorb system.

The probe was labeled with ^{32}P using a Prime-a-Gene Labeling System kit (Promega).

Hybridization was performed in a RI-2D hybridizer at 65°C in a 5-fold standard EDTA saline containing 0.25% sodium dodecyl sulfate, 0.1% sodium pyrophosphate, and 100 μg/ml heparin. The samples were washed 2 times with 2-fold standard salt solution (SSC) at 68°C and then with 0.2 SSC at the same temperature.

The restriction analysis was performed with Pvu-1 restrictase (Fermentas) according to manufacturer's protocol.

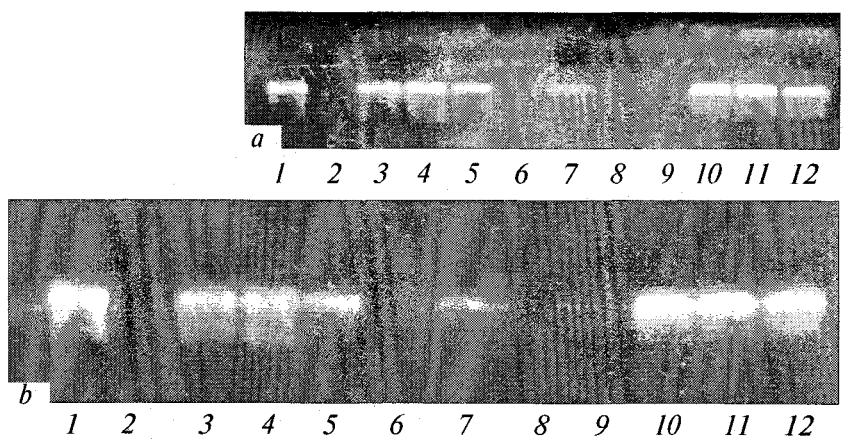

Fig. 1. DNA/DNA hybridization of amplification products. Ethidium bromide staining, without magnification. *a)* electrophoregram of amplification products; *b)* hybridization. *1) M tuberculosis*; *2)* empty; *3-5, 7, 10-12* CSF samples; *6) Streptococcus pneumoniae*; *8) Neisseria meningitidis*; *9) Staphylococcus aureus*.

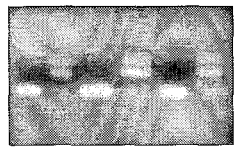

Fig. 2. Restriction analysis of N-PCR products. Ethidium bromide staining, without magnification. *1, 2*) *M. tuberculosis*; *3, 4*) blood; *5, 6*) CSF; *1, 3, 5*) untreated amplification products; *2, 4, 6*) amplification products treated with restrictase.

RESULTS

In previous studies we developed a test-system based on PCR-amplification of IS 6110 element and designed to detect tuberculosis mycobacteria in patients with different forms of tuberculosis [7,13]. Clinical tests showed that its sensitivity depended on sample quality. A great variety of biological samples used for the detection of pathogen required either more complex pretreatment the sample or introduction of additional stage (hybridization or restriction), which prolonged the detection procedure and increased its complexity and costs. Pathogen DNA can be isolated by several techniques [9-11]. Complication of this stage can result in loss of the test material [12].

We constructed external primers and developed stage-by-stage protocol of amplification of more sensitive N-PCR technique.

The specificity of primers was assessed using DNA of tuberculosis and nontuberculosis mycobacteria and microorganisms (isolates of museum samples) presumably presented in clinical samples from patients with mixed infections. All these isolates, except MBT were negative in the N-PCR system.

We also performed an Internet search for the homologues of the constructed primers among the partially sequenced genoms of pathogenic bacteria.

The efficiency of MBT detection with our test-system and standard Cobas Amplicor system was compared in different biological samples collected from patients with different forms of tuberculosis (Table 1).

In sputum and exudate, N-PCR showed a higher efficiency than Cobas system and indices of growth on solid media (Table 1). The Cobas system failed to produce amplification (as indicated by internal standard) in more than 20% sputum and exudate samples, which could be explained by the presence of amplification inhibitors. According to manufacturer's instruction, 50 μl sample and 50 μl amplification mixture should be taken. Such dilution probably modulates activity of amplification inhibitors and is responsible for false-negative results. This suggestion is confirmed by the fact that further purification of samples improves the efficiency of MBT detection by Cobas test-system.

In patients with tuberculous meningitis, the pathogen can be detected in CSF. Our data showed that Cobas amplification with CSF samples from patients with presumed tuberculous meningitis was successful in all (100%) cases (according to internal control), and, therefore, nothing interfered amplification. Probably the content of pathogenic cells is these samples is not enough for detection with this test-system. N-PCR exhibited much higher efficiency, but there is a possibility of false-positive reactions. To test this suggestion, the amplicons obtained after N-PCR with CSF samples were hybridized with an oligonucleotide probe to IS 6110 (DNA/DNA hybridization) (Fig. 1).

Hybridization confirmed that CSF samples *3-5, 7, 9, 11, 12* contained pathogenic DNA. Samples *6, 8,* and *9* contained nonspecific material and produced no hybridization signal.

The specificity of the obtained amplicons was also confirmed by restriction analysis (Fig. 2).

Thus, the proposed N-PCR test-system is characterized by high efficiency. A simple procedure of sample treatment accelerates the detection of pathogen. Its application for the diagnosis of tuberculosis and the control of its therapy is very important for medical practice. The great advantage of this system is that the procedure of pathogen detection is one order of magnitude less expensive than of commercially available test systems.

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