

Research Article

Experience of Using Pcr in the Diagnosis of Leprosy in Russia

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Abstract

Background: Early diagnosis of leprosy is still a serious problem due to the low sensitivity of standard diagnostic methods and the inability to cultivate *Mycobacterium leprae*. Identification of *M. leprae* and determination of her viability on basis of PCR-technology can assistance to solve the issues of early diagnosis and the optimal duration and effectiveness of treatment of leprosy. **Objectives:** to investigate the performance of polymerase chain reaction (PCR) in various clinical samples for leprosy diagnosis and monitoring effectiveness of treatment. **Methods:** Extraction of DNA/RNA of *Mycobacterium leprae* were performed from various clinical specimens (biopsies and SSS, scrapes from nasal pharynx, etc.) The primers and probes were designed for the 16S rRNA of *M. leprae*. The test system is adapted to DT-96 Real-time Thermal cyclers (DNA-Technology, Moscow, Russia). The test system contains an internal control (IC). The specificity and sensitivity of the PCR test system were performed on 15 strains of different species of mycobacteria and clinical samples from 32 leprosy patients and 15 healthy household contacts. **Results:** Established 100% specificity and sensitivity of detection of *Mycobacterium leprae* in skin biopsies by PCR compared with standard microscopy method (88.9%). In all other clinical samples were also determined higher sensitivity developed PCR test-system. **Conclusions:** The test system, using primers to the 16S rRNA of *M. leprae* has shown high sensitivity and specificity for fast identification and determination of viability of *Mycobacterium leprae*.

Keywords

Leprosy, 16S rRNA *M. leprae*, Real-Time Polymerase Chain Reaction

1. Introduction

Leprosy (Hansen's disease) is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*), which affects the skin, peripheral nervous system, eyes and internal organs. Despite the long history of the disease, known since biblical times, early diagnosis of leprosy is still a serious problem due

to the low sensitivity of standard diagnostic methods and the inability to cultivate *M. leprae*. Every year, more than 200,000 new cases of leprosy are registered in the world, of which the main part falls on the countries of Southeast Asia, Latin Amer-

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ica and Africa [1]. In Russia, the morbidity of leprosy is sporadic with the main endemic region in the Astrakhan region [2]. All patients are undergoing treatment or dispensary observation at the Leprosy Research Institute.

With the development of nucleic acid-based amplification assays and the completion of the genome sequencing of the leprosy bacillus the identification *M.leprae* has become possible [3]. During the past years, PCR methods have been developed to amplify different gene targets of *M.leprae*: 16S rRNA, *sodA*, the repetitive sequences (RLEP) [4, 5]. PCR amplification has been developed and used for leprosy diagnosis, and has been proven to be highly sensitive and specific, being able to detect small numbers of *M.leprae* organisms in Slit Skin Smears (SSS), skin biopsy, nasal swab and environmental samples [6]. However, also to be considered is the fact that PCR-based diagnosis has a considerable level of failure in confirmed leprosy cases after clinical or laboratory standards.

Identification and establishment of standardized procedures to provide adequate clinical material, nucleic acid extraction protocol, primer target, amplicon size, PCR inhibition, fluorescence threshold determination will assure a more reliable and reproducible diagnosis of the disease. At the same time, given that the main source of infection is patients with leprosy, especially lepromatous type, it is necessary to assess how long the viability of the causative agent of leprosy remains after a course of multidrug therapy (MDT) [7, 8]. Although the MDT regimens are generally satisfactory, there are limitations in terms of the duration of activity of the process, which determines the duration and effectiveness of the therapy.

After the introduction method of the reverse transcriptase PCR (RT-PCR), which allows the detection of RNA, became possible to determinate viability *M.leprae* [9]. Whereas nucleic acid amplification tests targeting rRNA genes give poor predictions of bacterial viability, rapidly degradable mRNA may have potential in this respect. Developing a simplified, sensitive RT-PCR method, based on *M.leprae* rRNA genes, for routine may be used in monitoring of leprosy chemotherapy.

This study was conducted in order to develop a PCR system using primers to 16S rRNA to identify *M. leprae* from various clinical samples that can be used as a diagnostic tool and monitoring leprosy chemotherapy.

2. Materials and Methods

2.1. Collection and Processing of Clinical Material

This study was conducted in Leprosy Research Institute in Astrakhan, Russia. After informed consent was obtained, SSS, skin biopsy specimens, nasal swabs and scraping from the surface of trophic ulcers, as part of the routine collection of skin samples for staining of AFB and histopathologic investigations, were collected from 32 leprosy patients. 18 patients

from them had acute state of leprosy process. The patients were classified clinically, bacteriologically and histopathologically, according to the Ridley-Jopling scale [10]. 26 patients were classified as multibacillary (MB) patients (LL, BL and BB) had BI ranging from 1+ to +6,0. In addition, 6 patients were classified as paucibacillary (PB) patients (including TT, BT forms) had negative BI. SSS, nasal secretion and blood were investigated in 15 healthy household contacts. Before the study was undertaken, all individuals participating in this study were informed of the purpose of the study, and written consent was obtained from all participants. Ethical Approval: the ethical committee of Leprosy Research Institute approved this study.

SSS from six sites of each patient were obtained with sterile scalpels which were then washed in 0,5 ml of sterile water. Punch skin biopsies (6- mm³) specimens were taken from all patients. One part of biopsy was used for HPE and the other was performed for PCR. Nasal secretion was collected on a sterilized cotton swab by gently moving the swab several times against the anterior segment of the nasal cavity. Scraping from the surface of trophic ulcers was performed using sterile disposable swab.

2.2. DNA Extraction from Clinical Samples

From Skin Tissues. The biopsy samples were homogenized in a manual homogenizer with 0,5 ml sterile buffered saline to obtain suspension. It was then the DNA extraction procedure was performed using the kit of reagents for nucleic acid isolation ("DNA- technology", Russia), according to the manufactures' protocols. 50 µl of suspension was collected in a test tube, was shaken by 3-5 sec. and centrifuged at 13000x g for 1 min. It was then incubated in lyses buffer containing 170 µl 6M guanidine thiocyanate for 20 min at 50⁰ C. Then the tube was centrifuged at 13000x g for 1 min, washed thrice with 200 µl wash buffers (4,5 M guanidine thiocyanate, ethanol, methyl acetate), dried at 50⁰ C for 5 min and resuspended in 100 µl TE buffer.

DNA extraction from nasal cavity, the surface of trophic ulcers, SSS was performed following the same procedure as mentioned above.

In each reaction, in addition to the test tubes with the test samples were placed 3 tubes to test the stages of sample preparation and formulation of PCR: 1 tube – negative control sample, passed all stages of sample preparation; 2 – negative control "K- ", which was added 5 µl sterile saline; 3 – positive control "K+" - 5 µl positive control sample.

To determine the viability of *M.leprae* during MDT, RNA *M.leprae* was extracted and reverse transcription reaction (RT-PCR) was performed.

2.3. RNA Extraction

RNA *M.leprae* was extracted directly from biopsy tissues using a previously homogenization described above. The

tubes with homogenate of biopsy were added 300 µl 4,5M guanidine thiocyanate and 100 µl sterile buffered saline and mixed by vortex 3-5 sec. The tubes were incubated at room temperature 3 hr and then at 65 °C for 10 minutes, centrifuged at 13000x g for 30 sec. 160 µl chloroform/isoamyl alcohol (CIA) (24: 1) were added, mixed by 3-5 sec. and centrifuged at 13000 x g for 10 min. The aqueous phase from each tube was transferred to a new 1,5 ml tube, added 400 µl of the reagent for precipitation (isopropanol + dextran), centrifuged at 13000x g for 10 min. Supernatant was removed from each tube and added 500 µl of salt ethanol wash solution, mixed by 3-5 sec., and centrifuged at 13000x g for 5 min. The washing procedure was repeated with 300 µl methylacetate. After that, the supernatant was removed and the precipitate was dried e at 65 °C for 5 minutes and solubilized in 50 µl of RNase-free H₂O. The tubes were incubated at 65 °C for 10 min, centrifuged at 13000x g for 30 sec. The precipitated RNA was used immediately for RT-PCR.

2.4. Reverse Transcription

Reverse transcription were carried out sequentially in a single tube using Ready –To-Go-RT-PCR kits (“DNA- technology”, Russia), which content of the “RT-buffer” and “RT-RANDOM + dNTP primers”. The RT-PCR reaction mixture containing 2,0 µl of “RT-buffer”, 1.0 µl of “Primers OT-RANDOM + dNTP” and 0.5 µl of reverse transcriptase, were added the each tube. Then 16.5 µl of the precipitated RNA was introduced into each tube, vortexed for 3-5 sec and centrifuged

at 1000xg for 3-5 sec. The reverse transcription was performed at 40 °C for 30 min, followed by denaturation at 95 °C - 5 min. The condensate precipitated by centrifugation at 13000x g for 30 sec. The cDNA preparation is ready for PCR.

2.5. Polymerase Chain Reaction (PCR)

The primers for PCR were selected on the basis DNA *M. leprae* sequences obtained from the NCBI database ([http // www.ncbi.nlm.nih.gov /](http://www.ncbi.nlm.nih.gov/)), as well as using literature data [11, 12]. Primers were synthesized taking into account the GC composition of the matrix, the estimated temperature of hybridization, the presence of homologous regions of DNA sequences in other organisms. Oligo 6.0 software was used to select primers and oligonucleotide samples.

The synthesis of primers and oligonucleotide fluorescently labeled probes for real-time PCR was carried out on DNA synthesizers ASM-800 and ASM-102 (Russia, Novosibirsk), allowing to synthesize both experimental (20 nmol) and preparative amounts (0.5 µmol). Fluorophores (FAM, HEX) and fluorescence quenchers (BHQ1) synthesized in the laboratory of isotopic methods of analysis of the Institute of Bioorganic Chemistry. Oligonucleotides were purified by high-performance liquid chromatography using reversed-phase columns Diaspher-110-C18 (Russia). The purity of the obtained oligonucleotides was controlled by the method of analytical vertical gel electrophoresis in polyacrylamide gel (Table 1).

Table 1. Characterization of primers, probe and amplicon size for detection of *M. leprae*.

Gene Name	Nucleotide sequence of primers and probes	PCR product size (b.p.)
16s-U	5'- CGA ACG GAA AGG TCT CTA AA -3'	290
16s-U	5'- GTC GAA CGG AAA GGT CTC TAA A -3'	
16s-P	(FAM)- 5'- CTT CAA GGC GCA TGT CTT GTG GTG GAA -3'-(BHQ1)	

Polymerase chain reaction was performed in 35 µl of the reaction mixture containing 5 µl of the DNA sample and the following components: 10 pM of each set of primers; 1.1 mM dNTP mix; 64 mM Tris-HCl (pH 8.6); 25 mM (NH₄)₂SO₄; 2.5 mM – MgCl₂ as well as 5 U of Taq polymerase. To prevent changes in the concentration of the components, the reaction mixture was covered with 20 µl of mineral oil. To implement the “hot start” and prevent nonspecific annealing primers used paraffin layer separating the components of the reaction mixture.

Amplification was done as follows: 80 °C - 30 sec, 94 °C - 1 min 30 sec -1 cycle; 5 cycles of 94 °C for 30 sec and 64 °C for 15 sec; and 45 cycles 94 °C - 10 sec, 64 °C - 15 sec using a DT-96 real-time PCR thermocycler (“DNA- technology”, Russia).

To control the course of the PCR reaction, an internal control system was introduced — IC (cloned fragment of the human growth factor receptor gene, a pair of primers and probe complementary to it). The criterion for the suitability of the IC system was the absence of inhibition of a specific reaction, while IC should work in the absence of a specific reaction.

2.6. Specificity and Sensitivity of the Test

We used 15 types of Mycobacteria to analyze the specificity of the test: *M.kansasii*, *M.marinum*, *M.scrofulaceum*, *M.vaccae*, *M.intracellulare*, *M.clegg*, *M.duvalii*, *M.phlei*, *M.avium*, *M.lufu*, *M.gastri*, *M.gordanae*, *M.bovis*, *M.smegmatis*,

M. paratuberculosis. All mycobacteria were cultured on Lowenstein-Jensen medium, except *M. leprae*. The specificity of the test was also analyzed using skin tissues collected from 8 patients who had skin diseases other than leprosy. The sensitivity of the test was determined using serial 10-fold dilutions of the suspensions of *M. leprae*, which it were prepared from skin biopsy specimens from leprosy patients.

3. Results

Initially, the amplicons obtained from PCR were analyzed for electrophoresis using DNA from different types of microorganisms, DNA from leprosy patients and experimental animals (Shepard model). The 290-bp fragment specific amplification was observed only with DNA *M. leprae*. As a result of

the PCR and subsequent electrophoresis were recorded synthesis of amplicon size 290 bp only in clinical isolates from patients with leprosy. In all cases, the results were consistent with standard bacterioscopic and histological methods.

The sensitivity of PCR was determined by testing serially diluted samples of a stock DNA extracted from human-derived leprosy bacilli. Reproducible and unequivocal bands were detectable up to a lower limit, which was equivalent to 100 bacilli in ethidium bromide stained agarose gels.

Sensitivity of test system for detecting *M. leprae* by Real-time PCR with primers of 16S rRNA *M. leprae* was performed in leprosy patients and healthy household contacts. All samples concomitantly were tested by PCR and bacterioscopic method (AFB). Conventional BI scores AFB counts from extracted material were compared with the PCR results. The sensitivity of the method depended on the nature of the clinical isolate (Table 2).

Table 2. Sensitivity of AFB and 16S rRNA gene based PCR methods as diagnostic tools in detecting *M. leprae*.

Clinical samples	Number of samples	AFB test		PCR	
		abs	%	abs.	%
SSS	54	32	59,3	36	66,7
Biopsies	18	16	88,9	18	100
Nasal swabs	32	20	62,5	22	68,8
Scraping of trophic ulcers	15	0	0	3	20,0
Blood	29	0	0	0	0
Total	148	68	45,9	79	53,4

DNA *M. leprae* was identified most often in biopsies of MB patients. In PCR analysis, scrapings from the nasal mucosa of *M. leprae* were detected more often in 6.3% of cases and in 7.4% of cases of SSS than in bacterioscopic examination for MB patients. In addition, the results of the PCR analysis showed that *M. leprae* was found in two bacterioscopically negative scarifications of PB patients. All samples from trophic ulcers of MB patients were bacterioscopically negative, whereas *M. leprae* DNA was detected in 20% of cases. DNA *M. leprae* was not detected in all blood serum samples. The sensitivity of the test was 95.2%. The specificity of the developed test was tested on samples from healthy individuals (skin scarifications, scrapes from the nasal mucosa, blood serum) and was 100%.

Thus, PCR method for identification of the DNA *M. leprae* was introduced as a more sensitive and specific method than the AFB examination. A comparative analysis of the results of standard *M. leprae* detection methods in different samples with the PCR method using a primer for 16S rRNA *M. leprae*

showed a higher sensitivity of the latter. The test system allows us to identify *M. leprae* in any clinical material in a short time and confirm the diagnosis. However, using this test system, it is impossible to distinguish between live from the dead of *M. leprae*, that is, this method is relevant only for the diagnosis of leprosy, but not for monitoring anti-leprosy treatment. In this regard, the next stage of the study was the development of a test system for determining the viability of *M. leprae* based on RT-PCR to assess the effectiveness of anti-leprosy treatment. Therefore, we use ribosomal genes, in particular 16S rRNA for determine the viability of *M. leprae* during MDT, since mRNA is present only in living pathogens and rapidly degrades in dead bacteria [8, 9]. Methods based on a quantitative estimation of RNA levels in the tissues have been useful for monitoring treatment [13].

To study the possibility of using our test system in evaluating the effectiveness of anti-leprosy treatment SSS and biopsies from MB patients in the active stage of the disease were examined using the proposed method and the standard bacterioscopic method (Table 3).

Table 3. Results of detection of *M. leprae* (%) depending on the duration of the treatment.

The duration of the treatment	Number of SSS	Methods	
		AFB test	PCR of 16S rRNA
Before the treatment	42	90,5%	100%
After 6 month of MDT	42	27,3%	45,5%
After 9 month of MDT	42	7,1%	14,3%
After 12 month of MDT	42	2,4%	7,1%

As shows in Table 3 there was a decrease of BI and the percentage of positive samples in the PCR during treatment, which indicates the effectiveness of the MDT. However, DNA *M. leprae* was detected after 6, 9 months and the end of the course for MDT more often ($p < 0.05$) on the RT-PCR than bacterioscopy of some patients. This provides a basis for continuing specific therapy of these patients.

4. Discussion

During the last years, PCR assays have been developed to amplify different gene targets of *M. leprae*. Many works indicated that Ag85B, RLEP and 16S rRNA were higher specificity and sensitivity [14-17]. However, special attention is paid to the primer flanking a section of the 16S rRNA gene, which is a component of mycobacterial ribosomes and is expressed in a large number of copies (from 10^3 to 10^4 per cell). The 16S rRNA bacterial gene contains not only sequences common to all bacteria, but also specific to each species [12]. So it is most widely used for identifying bacteria. Research using this gene has greatly contributed to the discovery of new *Mycobacterium* species and continues to serve as an important tool as an alternative to phenotypic identification methods. The same time using this gene in the RT-PCR can determinate of bacterial viability.

In early study using patient's biopsies confirmed [18], that *M. leprae* is rapidly killed after one month of MDT. However, Santos A. R. et al. tested a PCR assay targeted a RLEP on different samples of leprosy patients after MDT and described viable *M. leprae* in 3.3% of cases in patients with high BI after 12 months of treatment [19]. A RT-PCR assay for monitoring bacterial clearance in leprosy patients during chemotherapy based on *M. leprae* 16SrRNA gene expression found that 44% MB patients and 4% of PB patients had been viable bacilli after 6 month of MDT [20]. Martinez N. A. and coworkers propose a real-time PCR integrated approach based on RNA/DNA ratios for viability determination, i.e. decrease of *M. leprae* specific RNA is evaluated as a function of total *M. leprae* DNA content [9]. Lini N. et al. tested hsp 18 as the gene target for monitoring the effectiveness chemotherapy

and observed that hsp 18mRNA could not be detected in patients who underwent 2 years of MDT [21]. WHO recommended shortening the treatment to 12 month, although according to our results and literature data a considerable amount of *M. leprae* DNA remained in the skin after 12 month of MDT. Determination of the relapse rate after 12-month MDT is highly relevant, and should be considered as a top priority for research. Generally, 6-months and 1-year MDT courses are recommended for paucibacillary (PB) and multi-bacillary (MB) cases respectively. Nevertheless, no effective BI change was observed in some leprosy cases, even if MDT was prescribed for more than 1-year [22]. On the one hand, the lack of treatment effectiveness after 12 months may be associated with the appearance of drug-resistant strains. Generally, a lack of BI decline is an indicator of drug resistance, but the study demonstrated that drug resistance might not be the main factor for lack of BI decline [23]. In our opinion, the treatment of leprosy patients should be approached as an individual with the use of molecular methods, in particular RT-PCR using primers to 16S rRNA, which allows detecting viable *M. leprae*. The presence of viable *M. leprae* dictates the need for a further course of specific chemotherapy, which will allow to interrupt the transmission of *M. leprae*.

5. Conclusion

In leprosy, a rapid and early diagnostic method is still needed. The higher sensitivity of the PCR technique makes it a more robust tool not only for leprosy diagnosis, and for monitoring of effectiveness MDT. Using primers to 16S rRNA of *M. leprae* allows solving both of these problems. The use of PCR-positivity in patients after MDT would help in the development of individual strategies length treatment of leprosy patients.

Abbreviations

PCR	Polymerase Chain Reaction
MDT	Multidrug Therapy

MB	Multibacillary Patients
PB	Paucibacillary Patients
BI	Bacterioscopic Index
AFB	Acid-Fast Bacteria
SSS	Slit Skin Smears
HPE	Histological Punch Examination

Author Contributions

Lyudmila Valentinovna Saroyants: Conceptualization, Resources, Supervision, Writing – review & editing

Kristina Shotaevna Arnaudova: Data curation, Methodology

Andrey Valentinovich Naumov: Writing – original draft

Data Availability Statement

The data supporting the outcome of this research work has been reported in this manuscript.

Conflicts of Interest

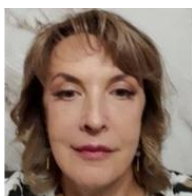
The authors declare no conflicts of interest.

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Biography



Lyudmila Valentinovna Saroyants is a professor in the Department of Clinical Pharmacology at Astrakhan State Medical University. She completed her PhD in Medicine from the Institute of Immunology (Moscow) in 1991 and her Doctor of Medical Sciences degree from the same institute in 2012. She holds certifications in dermatovenereology, immunology and in clinical and laboratory diagnostics. In addition, she has been awarded the title of "Excellence in Healthcare." She has participated in numerous international research projects, served as a keynote speaker at international congresses on leprosy, and acted as a scientific editor for numerous publications.



Kristina Shotaevna Arnaudova is an associate professor at the Department of Clinical Immunology at Astrakhan State Medical University. In 2019 she completed her PhD in Medicine from the Medical University (Moscow). In recent years, she has participated in a number of research projects.



Andrey Valentinovich Naumov is an assistant at the Department of Preventive Medicine and Healthy Lifestyle at Astrakhan State Medical University. He completed his PhD in Medicine from the Astrakhan State Medical University in 2022, and his Master of Genetic from the Astrakhan State University in the same year.

Research Field

Lyudmila Valentinovna Saroyants: development of immunological and molecular-genetic methods for identifying disease pathogens; immunogenetic markers of disease susceptibility; innate and acquired immunity in diseases; preclinical and clinical studies of biologically active substances; undesirable adverse reactions, pharmacogenetics; population genetic studies, personalized and predictive medicine

Kristina Shotaevna Arnaudova: molecular genetic methods serological methods, rapid DNA extraction methods, polymerase chain reaction, test systems for the diagnosis of leprosy

Andrey Valentinovich Naumov: comorbid pathology, immunological aspects of diseases, the impact of various factors on the health of the population, preventive aspects in internal medicine, immunogenetics, personalized and predictive medicine