EXPERIMENTAL GENETICS

Identification of the *M. tuberculosis-M. bovis* Complex Using the Polymerase Chain Reaction.

I. G. Mal'kov

UDC 579.873.21.083.137

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 115, № 4, pp. 404–406, April, 1993
Original article submitted November 16, 1992

Key Words: *mycobacteria; tuberculosis; DNA; hybridization; PCR*

Recently, the method of detecting and differentiating mycobacteria by means of amplification of target DNA using the polymerase chain reaction (PCR) has attracted considerable interest on the part of investigators working on the problem of tuberculosis. PCR offers the possibility of amplifying with high precision certain parts of DNA within several hours and of differentiating tuberculosis mycobacteria from atypical species [1-7]. The method of DNA amplification depends on the choice of oligonucleotide primers which are responsible for the reaction specificity. Sometimes, two or more primers have to be synthesized before the desirable ones are found, which requires additional time and expense. In view of the above we have tried to facilitate the creation of a successful method of diagnosing tuberculosis mycobacteria by choosing the appropriate specific primers flanking the site of the gene which encodes the maximally expressed antigen determinant of the protein of interest. Another crucial aspect of PCR is the preparation of samples. For mycobacteria this procedure incurs special difficulties due to the high resistance of the bacteria to the chemicals and enzymes routinely used [8].

The present study aimed at creating our own diagnostic system based on PCR, at choosing the specific primers obtained by means of the approach suggested, and at developing an effective method of mycobacterial cell preparation for the amplification reaction.

All–Russian Research Institute of Veterinary Sanitation, Hygiene, and Ecology, Moscow. (Presented by P. V. Sergeev, Member of the Russian Academy of Medical Sciences)

MATERIALS AND METHODS

The DNA sequence encoding the MbaA protein [9] was used for the selection of primers. For this purpose the protein was analyzed for determining the

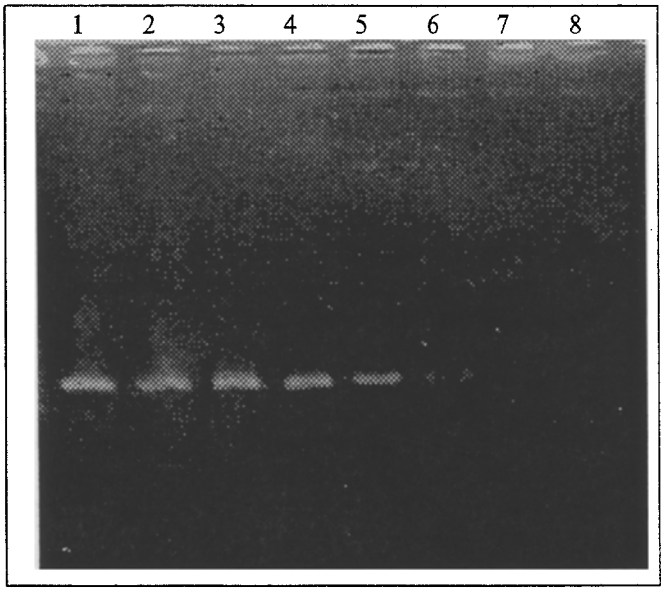

Fig. 1. Results of PCR amplification with *M. bovis* BCG in serial dilutions. Gel electrophoresis in 2% agarose with 1×TBE buffer. Number of cells per lane: 1) 10^7; 2) 10^6; 3) 10^5; 4) 10^4; 5) 10^3; 6) 10^2; 7) 10; 8) 1.

0007-4888/93/0004-0432$12.50

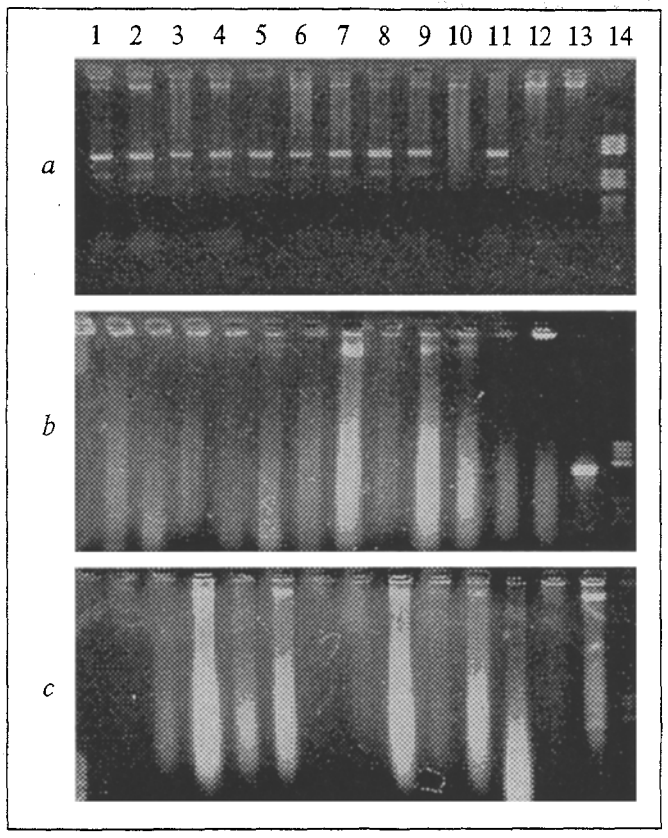

Fig. 2. Results of PCR amplification with mycobacteria lysates. Gel electrophoresis in 2% agarose with 1×TBE buffer. Lane numbers correspond as follows:

a: *1*) *M. tuberculosis* N192; *2*) *M. tuberculosis* N5281; *3*) *M. tuberculosis* dt/st; *4*) *M. bovis* ef16, *5*) *M. bovis* AN5; *6*) *M. bovis* Ravenel; *7*) *M. bovis* Bang; *8*) *M. bovis* 14; *9*) *M. bovis* Bovinus–8; *10,11*) *M. bovis* Vallee; *12*) *M. intracellulare* 15769; *13*) *M. intracellulare* tk; *14*) pBR/Hae III.

b: *1*) *M. avium* N9; *2*) *M. intracellulare* N12928; *3*) *M. kansasii* N2409; *4*) *M. fortuitum* 342; *5*) *M. intracellulare* N4937; *6*) *M. intracellulare* N14773; *7*) *M. intracellulare* tk; *8*) *M. scrofulaceum* N123; *9*) *M. intracellulare* 16E; *10*) *M. intracellulare* N290/52; *11*) *M. avium* FP; *12*) *M. avium* 14114; *13*) *M. bovis* BCG (10^6 cells); *14*) pBR/Hae III.

c: *1*) *M. phlei* L2312; *2*) *M. fortuitum* Rapid; *3*) *M. avium* N2282; *4*) *M. smegmatis*; *5*) *M. avium* 89; *6*) *M. intracellulare* N12928; *7*) *M. intracellulare* N13623; *8*) *M. intracellulare* TE; *9*) *M. intracellulare* 21E; *10*) *M. avium* N2282; *11*) *M. triviale*; *12*) *M.* B–*5*; *13*) DNA *of M. bovis* BCG (100 pg), *14*) pBR/Hae III.

region with minimal hydrophoby using "PRASIS™" software (Pharmacia LKB Biotechnology, Sweden). The gene site from 1796 to 2115 bp was taken as the target for amplification. This sequence encodes the protein region from 407 to 514 amino acid residues with hydrophoby index -0.1. The amplification reaction was performed with oligonucleotide primers $Pr64_{1,2}$ flanking this protein region and disposed from 1811 to 1830 bp (upper) and from 2203 to 2222 bp (lower).

The oligonucleotides were generated with the automatic DNA synthesizer Gene Assembler Plus (Pharmacia LKB), programmed according to the synthesis protocol for phosphoamidite chemistry.

The following mycobacteria strains were used for the experiment: 3 strains of *M. tuberculosis*, 7 strains of *M. bovis*, and atypical mycobacteria of 4 groups in Runyon's classification, among them 6 strains of *M. avium*, 10 strains of *M. intracellulare*, 2 strains of *M. fortuitum*, and 1 strain each of *M. scrofulaceum, M. kansasii, M. phlei, M. triviale* and *M.* B-5. The strains were obtained from the All-Russian State Research Control Institute of Veterinarian Preparations and the L. A. Tarasevich State Institute of the Standardization and Control of Medical and Biological Preparations, Moscow. The mycobacteria were grown in Lovenstein-Jensen medium at 37°C for 30 days. Preparation of mycobacteria for the amplification reaction was carried out as follows: single colonies were suspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) up to 10^8 cells/ml and 1 ml of the suspension was placed in a 1.5 ml Eppendorf tube. To determine the reaction sensitivity serial dilutions of *M. bovis* BCG from 10^7 to 1 cell/ml were prepared. The bacteria were pelleted by centrifugation and lysed with 0.2 M KOH in the presence of Tween 20. The tube was then shaken for 10 min with 10-15 μl glass beads (D=0.1 mm), after which 10 μl of the neutralizing solution containing 0.3 M KCl, 0.5 M Tris-HCl, and 40 mM $MgCl_2$ (pH 7.3) were added and the mixture was boiled at 95°C for 10 min. For the amplification reaction 5 μl of supernatant was taken and 10 pM of primers, 50 μM of each dNTP, 1 U *Thermus aquaticus* polymerase (Taq.) (Biopol, Russia), sterile deionized distilled water up to 50 μl and 50 μl of mineral oil were added. PCR

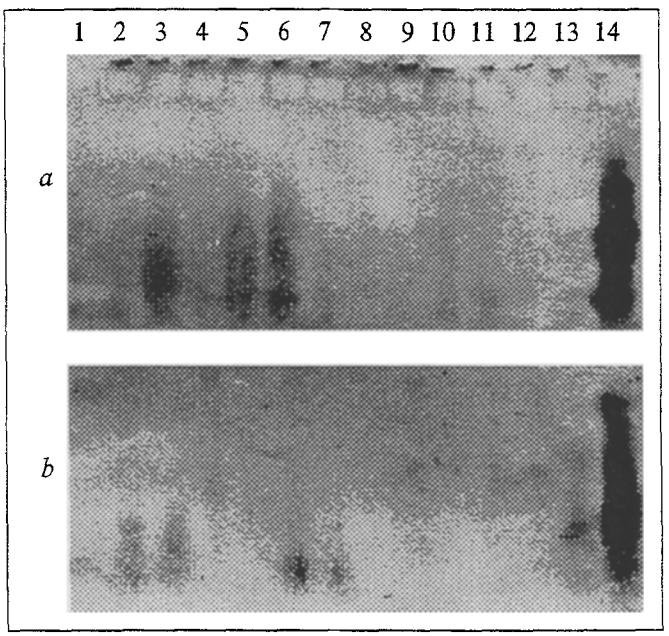

Fig.3. Hybridization of PCR products transferred from gel (Fig.2, *b, c*) to nitrocellulose with digoxygenin–labeled purified product of amplification reaction.

was carried out in a DNA thermal cycler (Biotherm'91) using a thermocycle for 45 rounds of amplification. The annealing temperature was 61°C for 1 min, primer extension occurred at 72°C during 2 min, and denaturation was accomplished at 93°C during 1.5 min. After the amplification was completed the mineral oil was removed. One fifth of the final reaction volume was loaded on top of 2% agarose gel and run at 120 V for 1.5 h. Plasmid pBR322 restricted with Hae III was used as a molecular weight marker. The gels were stained, photographed, transferred to nitrocellulose, and probed using the purified synthesized product of DNA *M. tuberculosis-M. bovis* as a DNA probe. Labeling of DNA with the digoxygenin labeling kit and hybridization were performed according to manufacture protocol (Boehringer Mannheim).

RESULTS

DNA bands of the expected size (412 bp) were detected in the agarose gel starting from 100 cells of *M. bovis* BCG per sample. (Fig. 1). The test for primer specificity in the amplification reaction revealed that all strains of the *M. tuberculosis-M. bovis* complex yielded a bright band (412 bp) and an additional weaker band of about 270 bp (Fig. 2, *a*).

Of the 23 atypical mycobacteria strains not one yielded visible bands in agarose gel after PCR (Fig. 2, *b, c*).

No bands were observed either in DNA-DNA hybridization of the products of amplification of atypical mycobacteria transferred to nitrocellulose with the product of amplification of *M. tubercolosis-M. bovis* as a DNA-probe.

Thus, due to the approach used for choosing the target for amplification, the specific gene site which made it possible to differentiate reliably *M. tuberculosis-M. bovis* from atypical mycobacteria was identified. Such an approach could be useful for the development of new diagnostic systems on the base of PCR for a variety of infections.

Another positive result of our investigation is the proposed method for mycobacteria cell destruction, which could be successfully used in the practical domain, e.g., for the examination of pathological material.

REFERENCES

1. A. Brisson-Noel, B. Gicquel, D. Lecossier, V. Levy-Frebault, X. Nassif, and A.J. Hance, *Lancet*, **2**, 1069 (1989).
2. C. C. Pao, T. S. B. Yen, J.-B. You, J. S. Moa, E. H. Fiss, and C.-H. Cang, *J. Clin. Microbiol.*, **28**, 1877 (1990).
3. U. Sjobring, M. Mecklenburg, A. B. Andersen, and H. Miorner, *J. Clin. Microbiol.*, **28**, 2200 (1990).
4. P. Shankar, N. Manjunath, R. Lakshmi, *et al.*, *Lancet*, **335**, 423 (1990).
5. P. W. M. Hermans, A. R. J. Schuitema, D. V. Soolingen, *et al. J. Clin. Microbiol.*, **28**, 1204 (1990).
6. R. J. Patel, J. W. U. Fries, W. F. Piessens, and D. F. Wirth, *J. Clin. Microbiol.*, **28**, 513 (1990).
7. J. W. U. Fries, R. J. Patel, W. F. Piessens, and D. F. Wirth, *Molec. Cell. Probes*, № 4, 87-105 (1990).
8. K. Fukushi, *Nucl. Cell Wall*, № 11, 523-526 (1979).
9. J. E. R. Thole, W. J. Keulen, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema, and J. D. A. van Embden, *J. Infect. Immun.* № 6, 1466 (1987).