Diagnosis of Mycobacterium tuberculosis infection using PCR

Organizer: Angelika Gründling (a.grundling@imperial.ac.uk)

Important:

No eating or drinking is allowed in the lab
Wear a lab coat at all times
Wear gloves when handling any reagents or equipment in the lab

Practical will be performed in groups of 8 people.

This practical introduces the molecular technique PCR and its application in clinical settings. Specifically, we will use PCR to determine how likely it is that two patients are infected with *Mycobacterium tuberculosis.* Upon completion of this practical you should understand and know more about:

o) How the PCR technique works in practice

o) How to analyse a PCR product by agarose gel electrophoresis and how to interpret the results

o) Different applications of PCR in clinical settings

o) Different applications of PCR in research settings

o) The PROS and CONS of using PCR as tool in clinical applications

The PCR-based diagnostic analysis performed in this practicum has been adapted from:

Niyaz Ahmed, Ashok Kumar Mohanty, Utpal Mukhopadhyay, Virender Kumar Batish and Sunita Grover (1998). PCR-Based Rapid Detection of *Mycobacterium tuberculosis* in Blood from Immunocompetent Patients with Pulmonary Tuberculosis. *J. Clin. Microbiol.* 1998, 36(10):3094. (See Appendix)

For a more modern version of the use of a PCR-based diagnostic test for *Mycobacterium tuberculosis* including determination of drug resistance see for instance:

M. Rathore, Girish Pai, T.K. Jayalakshmi2 and D.S. Joshi (2011). RAPID DETECTION OF MULTIDRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* BY REAL-TIME PCR BASED ASSAY IN INDIAN POPULATION. *Recent Research in Science and Technology 2011, 3(3): 58-62*

Historic fact:

The Nobel Prize in Chemistry 1993
Kary B. Mullis, Michael Smith

The Nobel Prize in Chemistry 1993

Nobel Prize Award Ceremony

Kary B. Mullis

Michael Smith

Kary B. Mullis

Michael Smith

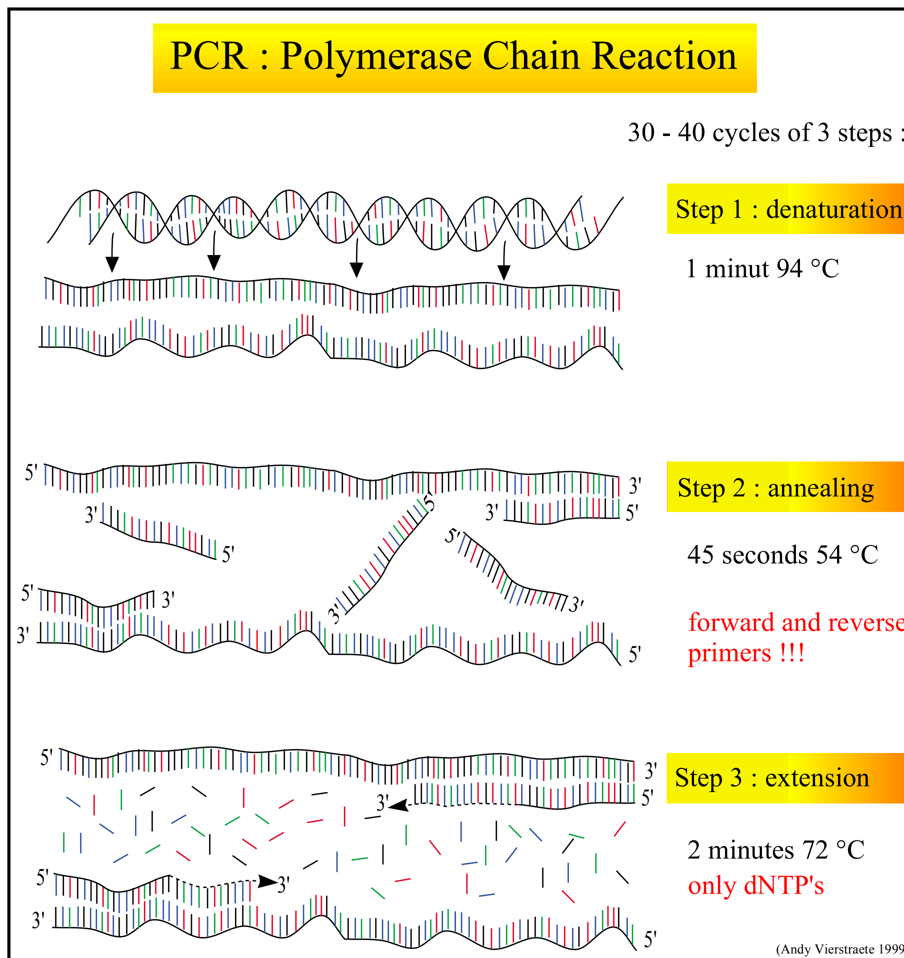
The Nobel Prize in Chemistry 1993 was awarded *"for contributions to the developments of methods within DNA-based chemistry"* jointly with one half to Kary B. Mullis *"for his invention of the polymerase chain reaction (PCR) method"* and with one half to Michael Smith *"for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies"*.

Photos: Copyright © The Nobel Foundation

Q: What does PCR stand for?

Q: What is the purpose of a PCR and how does it work **(See figure 1)**

Q: What are the components needed for a PCR



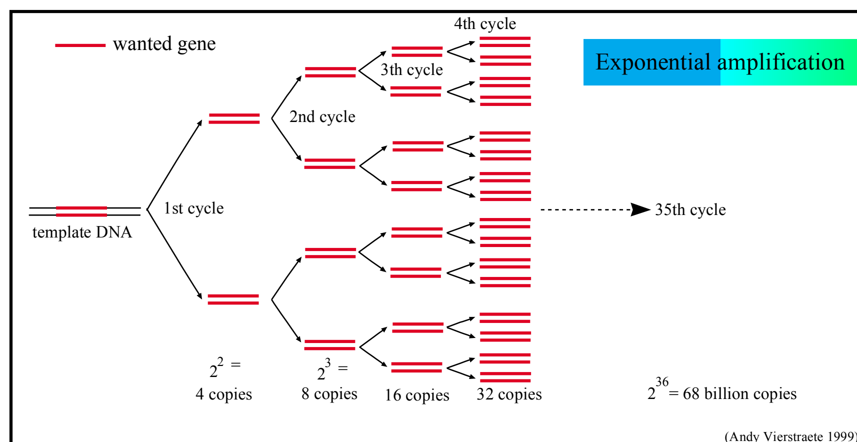


Figure 1.
Top image: Schematic representation of different steps in a PCR.
Bottom image: Exponential amplification of the gene of interest by PCR (taken from http://users.ugent.be/~avierstr/index.html)

Outline of how a PCR-based analysis is usually performed

1) Set up a PCR reaction (10 min)
2) Run PCR in thermocycler (2h)
3) Prepare agarose gel for analysis of PCR products (10 min + 30 min to solidify)
4) Agarose gel electrophoresis to separate PCR product (30- 45 min)
5) Visualize and Image PCR products (10 min)

Outline of what we will do in the practicum:

STEP 1: *Prepare agarose gel for analysis of PCR products* – one gel per group

STEP 2: *Set up a PCR reaction* - one PCR reaction per pair – each group should have 4 reactions in the end;
1st pair of students - Positive control DNA (Pos DNA)
2nd pair of students - Negative control DNA (Neg DNA)
3rd pair of students – Sample from Patient 1 (Sample 1 - DNA)
4th pair of students – Sample from Patient 2 (Sample 2 – DNA)

Please note that your reaction will be analyzed by the next class in the afternoon or two weeks time!!

STEP 3: *Set up PCR reaction in thermocycler* - this will be done for you

STEP 4: *Prepare sample for agarose gel electrophoresis* – as group

STEP 5: *Set up electrophoresis equipment and run pre-prepared PCR sample* - this will be done as group and everyone should load two samples

STEP 6: *We will discuss some more aspects about PCR and discuss the expected out come of the experiment* – Whole class

STEP 7: *Image gel to visualize PCR product*

Step 1: Prepare agarose gel for subsequent analysis of PCR products

Important: Wear lab coat and gloves

You need one gel per group

1) Set up the gel tray in the coasting unit
2) Insert 2 combs per gel
3) Obtain pre-molten 40 ml aliquot of agarose from 55oC water bath and note if you took a 1% or 2 % agarose mixture. **Caution the agarose is hot, so be sure to wear your gloves**
4) Poor 40 ml agarose mixture into mounted gel tray with combs
   (In case there are any bubbles take a pipette tip and push them to the side)
5) Let gel settle at room temperature for 30 min
   (try not to move the gel during that time)

What we have done for you:

1) weighed out and mixed the appropriate amount of agarose powder into 1 X Tris Acidic acid EDTA (TAE) electrophoresis buffer

2) heated agarose mix in microwave until agarose was molten
   CAUTION: this is very hot liquid need to wear heat resistant gloves for handling the hot mixture and wear safety glasses

3) added SYBR safe DNA stain (which is needed in the final step to visualize the DNA) – SYBR safe dye is used at a 1:20,000.

4) aliquoted DNA mixture into 50 ml falcon tubes and let cool to 55oC in water bath for subsequent use

Q: What does this agarose mixture contain

Q: How many g agarose are in 40 ml of a 1% agarose mixture and in 40 ml of **a 2 % agarose mixture and how much SYBR safe dye was added (1:20,000 dilution)**

Q: What difference will it make if you use a 1 or 2 % agarose gel and which one might be better for your application (306 bp PCR fragment)?

Q: Why is the agarose mixture slightly pink?

Step 2: Set up a PCR reaction (20 min)

1st pair of students - Positive control DNA (Pos DNA)
2nd pair of students - Negative control DNA (Neg DNA)
3rd pair of students – Sample from Patient 1 (Sample 1 - DNA)
4th pair of students – Sample from Patient 2 (Sample 2 – DNA)

Important: Again wear lab coat and gloves
Place all components back on ice when you are done

FYI: The reactions you prepare will be analysed by the next class in the afternoon/ two weeks time

The primer set used aims to amplify a 306-bp region of the multi-copy insertion sequence IS1081 in the *Mycobacterium tuberculosis* genome

Primer 1 (P1): 5' CGACACCGAGCAGCTTCTGGCTG 3' (same as primer BW-6 in paper)
Primer 2 (P2): 5' GTCGCCACCACGCTGGTCAGTG 3' (slightly modified primer BW-7 from paper)

DNA sequence of gene that contains the sequence which will be amplified by the primer set:

atgacctcttctcatcttatCGACACCGAGCAGCTTCTGGCTGACCAACTCGCACAGGCGAGCCCGGATCTGCTGC
GCGGGCTGCTCTCGACGTTCATCGCCGCCTTGATGGGGGCTGAAGCCGACGCCCTGTGCGGGGCGGGCT
ACCGCGAACGCAGCGATGAGCGGTCCAATCAGCGCAACGGCTACCGCCACCGTGATTTCGACACCCGTG
CCGCAACCATCGACGTCGCGATCCCCAAGCTGCGCCAGGGCAGCTATTTCCCGGACTGGCTGCTGCAGCG
CCGCAAGCGAGCTGAACGCGCACTGACCAGCGTGGTGGCGACctgctacctgctgggagtatccactcgccggatggag
cgcctggtcgaaacacttggtgtgacaaagctttccaagtcgcaagtgtcgatcatggccaaagagctcgacgaagccgtagaggcgtttcg
gacccgcccgctcgatgccggcccgtataccttcctcgccgccgacgccctggtgctcaaggtgcgcgaggcaggccgcgtcgtcggagtgc
acaccttgatcgccaccggcgtcaacgccgagggctaccgagagatcctgggcatccaggtcacctccgccgaggacggggccggctggct
ggcgttcttccgcgacctggtcgcccgcggcctgtccggggtcgcgctggtcaccagcgacgcccacgccggcctggtggccgcgatcggcg
ccaccctgcccgcagcggcctggcagcgctgcagaacccactacgcagccaatctgatggcagccaccccgaagccctcctggccgtgggt
gcgcaccctgctgcactccatctacgaccagcccgacgccgaatcagttgttgcccaatatgatcgggtactcgacgctctgaccgacaaact
ccccgcggtggccgagcacctcgacaccgcccgcaccgacctgctggcgttcaccgccttccccaagcagatctggcgccaaatctggtcca
acaacccccaggaacgcctcaaccgagaggtacgacgccgaaccgacgtcgtgggcatcttccccgaccgcgcctcgatcatccgcctcgtc
ggagccgtcctcgccgaacaacacgacgaatggatcgaaggacggcgctacctgggcctcgaggtcctcacccgagcccgagcagcactg
accagcaccgaagaacccgccaagcagcaaaccaccaacaccccagcactgaccacctag

1. Prepare a reaction mixture by adding the components in order as indicated below and mix gently afterwards. The reaction is set up in a small PCR tube and the final reaction volume will be 50 ul.

37.6 ul	ddH_2O (autoclaved)
5.0 ul	10X reaction buffer (buffer)
2.0 ul	chromosomal DNA (100 ng/ul) depending on which pair this DNA template will be either positive control DNA or negative control DNA or Sample 1 DNA or Sample 2 DNA0
1.5 ul	Primer 1 (P1) (10 pmol/ul)
1.5 ul	Primer 2 (P2) (10 pmol/ul)
2.0 ul	40 mM dNTPs (10 mM each nucleotide)
(0.4 ul	Taq polymerase – will be added in the end with smaller pipette)

50.0 ul	total

2. Mix gently
3. Each pair needs to label their PCR tube properly depending on which DNA template they used

Positive control DNA (label with “P”)
Negative control DNA (label with “N”)
Sample from Patient 1 (label with “S1”)
Sample from Patient 2 (label with “S2”)

4. Each group should now have four labeled PCR tubes on ice

5. Add 0.4 ul Taq polymerase per reaction and mix

Step 3: Set up PCR in machine – this will be done for you

Reaction cycle:

Initial denaturation
 2 min 94 ^{o}C
Cycling steps 30X
 30 sec 94 ^{o}C
 30 sec. 53 ^{o}C
 1 min 72 ^{o}C
Final extension
 2 min 72 ^{o}C
Final cooling step
 Cool to 10 ^{o}C

Step 4: Prepare samples for gel electrophoresis

This will be done as group

Each group should have received a tube with a blue 5 x concentrated DNA loading buffer (labelled – 5X Buffer) and the following tubes

1) **add 12.5 ul 5x concentrated DNA loading buffer to each of the PCR samples with a 50 ul PCR reaction**

   1) PCR- positive control ("P ")
   2) PCR – negative control ("N")
   3) PCR – sample 1 ("S1)
   4) PCR – sample 2 ("S2)

Q: How did the magic number 12.5 ul DNA loading buffer come about?

Q: What is the purpose of adding the DNA loading dye to your sample?

Step 5: Set up Electrophoresis apparatus and run DNA gel

This will be done as a group and everyone should at least load one samples

Important:
Wear lab coat and gloves
Check that electrophoresis box does not have any cracks and obvious defects- if you see any defects don't use this electrophoresis unit ask for a new one

1) Place electrophoresis box on the bench with the black electrode away from you and the red electrode pointing towards you

2) Place gel tray with agarose gel and combs in the middle of the electrophoresis box (make sure the top comb is closer to the black electrode)

3) Slowly fill box with 1x TAE electrophoresis buffer until all chambers are filled and the agarose gel is covered

4) Slowly remove the combs from the gel

5) Load samples into the wells of your gel
    a. First four people top part of the gel
    b. Second four people bottom part of the gel
    c. Everyone should load two samples and first person needs to load also a DNA ladder in the first lane

Lane 1 top: 10 ul DNA ladder (premixed with sample buffer)
Lane 2 top: 10 ul positive control
Lane 3 top: 10 ul negative control
Lane 4 top: 10 ul sample 1
Lane 5 top: 10 ul sample 2

Lane 1 bottom: 10 ul DNA ladder
Lane 2 bottom: 10 ul positive control
Lane 3 bottom: 10 ul negative control
Lane 4 bottom: 10 ul sample 1
Lane 5 bottom: 10 ul sample 2

6) Connect black (minus electrode) and red
(plus electrode) with power supply and run gel for 30 min at 100V.

Q: Which direction does the DNA move and why?

Q: Do smaller or bigger DNA fragments move faster?

Q: How will you know if your DNA fragment has the correct size?

Q: What do you expect to see on the gel if patient one is infected with TB and patient 2 is not infected with Mtb?

Q: What do you expect if patient 1 is infected with Mtb and patient 2 is infected with Mycobacterium bovis?

Q: What are some of the limits of this PCR test?

Q: How can you make PCR more specific for Mtb?

Q: what is the advantage of using PCR compared to culture based methods?

Q: Can PCR-derived methods be used to determine resistance of Mtb to specific drugs?

Q: Is PCR used in diagnostics labs?

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Q: What could be other application of PCR?

Q: When, how and why is PCR used in molecular biology labs?

Q: What comes next

Diagnostics and epidemiology: Sequencing projects to determine what type of infection and obtain some information on antibiotic resistance and how infections spread.

Science 22 January 2010:
Vol. 327 no. 5964 pp. 469-474
DOI: 10.1126/science.1182395

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REPORT

Evolution of MRSA During Hospital Transmission and Intercontinental Spread

Simon R. Harris[1,*], Edward J. Feil[2,*], Matthew T. G. Holden[1], Michael A. Quail[1], Emma K. Nickerson[3,4], Narisara Chantratita[3], Susana Gardete[5,6], Ana Tavares[5], Nick Day[3,7], Jodi A. Lindsay[8], Jonathan D. Edgeworth[9,10], Hermínia de Lencastre[5,6], Julian Parkhill[1], Sharon J. Peacock[3,4] and Stephen D. Bentley[1,†]

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ABSTRACT

Current methods for differentiating isolates of predominant lineages of pathogenic bacteria often do not provide sufficient resolution to define precise relationships. Here, we describe a high-throughput genomics approach that provides a high-resolution view of the epidemiology and microevolution of a dominant strain of methicillin-resistant *Staphylococcus aureus* (MRSA). This approach reveals the global geographic structure within the lineage, its intercontinental transmission through four decades, and the potential to trace person-to-person transmission within a hospital environment. The ability to interrogate and resolve bacterial populations is applicable to a range of infectious diseases, as well as microbial ecology.

Molecular biology: Synthesized genes

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DOI: 10.1126/science.1190719

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RESEARCH ARTICLE

Creation of a Bacterial Cell Controlled by a Chemically Synthesized Genome

Daniel G. Gibson[1], John I. Glass[1], Carole Lartigue[1], Vladimir N. Noskov[1], Ray-Yuan Chuang[1], Mikkel A. Algire[1], Gwynedd A. Benders[2], Michael G. Montague[1], Li Ma[1], Monzia M. Moodie[1], Chuck Merryman[1], Sanjay Vashee[1], Radha Krishnakumar[1], Nacyra Assad-Garcia[1], Cynthia Andrews-Pfannkoch[1], Evgeniya A. Denisova[1], Lei Young[1], Zhi-Qing Qi[1], Thomas H. Segall-Shapiro[1], Christopher H. Calvey[1], Prashanth P. Parmar[1], Clyde A. Hutchison III[2], Hamilton O. Smith[2] and J. Craig Venter[1,2,*]

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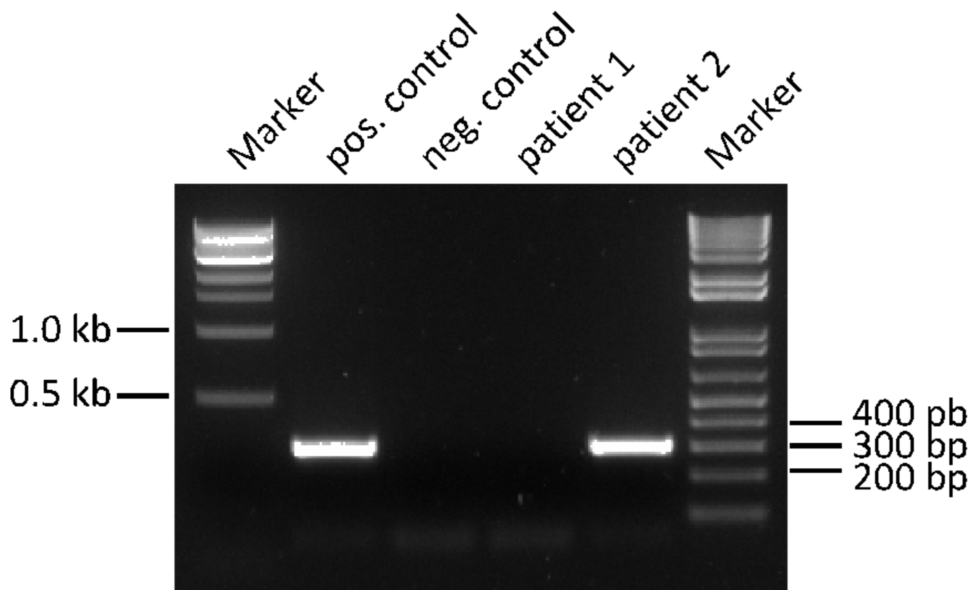
ABSTRACT

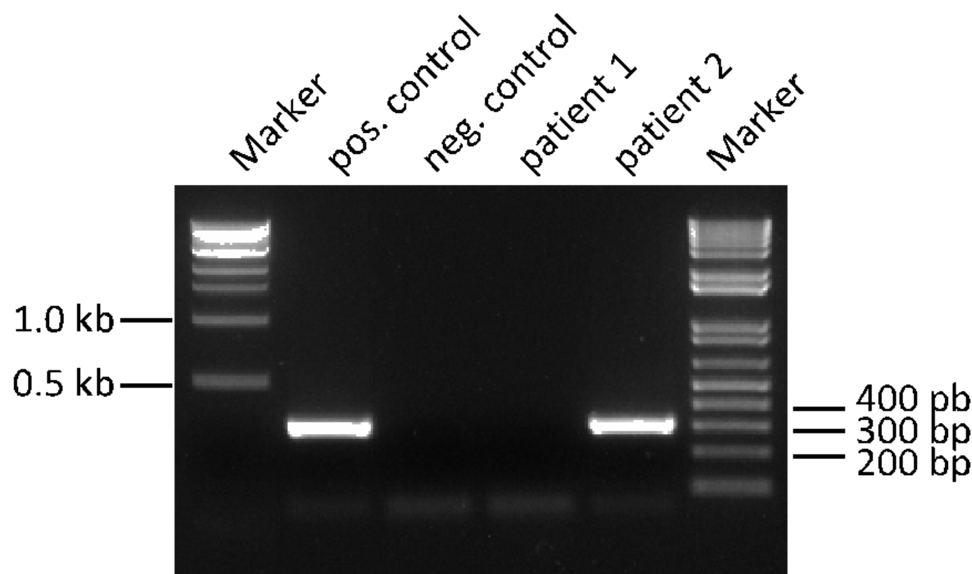
We report the design, synthesis, and assembly of the 1.08-mega-base pair *Mycoplasma mycoides* JCVI-syn1.0 genome starting from digitized genome sequence information and its transplantation into a *M. capricolum* recipient cell to create new *M. mycoides* cells that are controlled only by the synthetic chromosome. The only DNA in the cells is the designed synthetic DNA sequence, including "watermark" sequences and other designed gene deletions and polymorphisms, and mutations acquired during the building process. The new cells have expected phenotypic properties and are capable of continuous self-replication.

Step 6: Take image of GEL and document result

Need 1 volunteer per group to take the picture and send the result out to everyone in the group

Document result: Please prepare a picture as shown below and describe your result – each group should email me a file with their results and description of their data.





Positive control yielded 300 bp PCR fragment

Negative control did no yield a PCR product

Patient 1: No PCR product – therefore this patient is most likely not infected with Mycobacterium

Patient 2: 300 bp PCR product – therefore this patient is likely infected with Mycobacterium

Journal of
Clinical Microbiology

PCR-Based Rapid Detection of *Mycobacterium tuberculosis* in Blood from Immunocompetent Patients with Pulmonary Tuberculosis

Niyaz Ahmed, Ashok Kumar Mohanty, Utpal Mukhopadhyay, Virender Kumar Batish and Sunita Grover
***J. Clin. Microbiol.* 1998, 36(10):3094.**

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PCR-Based Rapid Detection of *Mycobacterium tuberculosis* in Blood from Immunocompetent Patients with Pulmonary Tuberculosis

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A PCR test based on insertion sequence IS1081 was developed to detect *Mycobacterium tuberculosis* complex organisms in the peripheral blood. The method was applied to blood samples from immunocompetent individuals with localized pulmonary tuberculosis. Seven of 16 (43.75%) blood samples were found to be positive for the circulating DNA copies of *M. tuberculosis* complex.

Tuberculosis is a persistent problem in the developing world and the biggest cause of mortality. The advent of AIDS has made the disease a major public health problem which has recently been exacerbated by increasing numbers of high-risk patients. A rapid and timely diagnosis of tuberculosis is thus essential to combat this disease. The tests based on PCR have shown promise for the detection of mycobacteria in clinical samples (2, 5, 12). However, several different PCR systems that have been described for the diagnosis of tuberculosis have produced widely differing results with regard to the sensitivity of the assay with different types of clinical samples (7, 8, 11). Peripheral blood appears to be the clinical material of choice for PCR, especially in cases of disseminated and extrapulmonary forms of the disease (6, 10). Earlier studies with blood-based PCR assays in humans suggested that PCR with peripheral blood mononuclear cells for the diagnosis of tuberculosis may be useful only in those who are substantially immunocompromised (3, 6, 9, 10), due either to AIDS or to conditions such as alcohol abuse, renal disorders, diabetes mellitus, etc. It appears that more data are required to determine the effectiveness of the blood-based PCR assay for the diagnosis of tuberculosis, especially in immunocompetent patients (1). In this study, we prospectively used the PCR technique for the detection of *Mycobacterium tuberculosis* complex in the peripheral blood of immunocompetent, HIV-negative individuals with localized pulmonary lesions of tuberculosis.

Blood samples from 16 patients reporting at the District TB Hospital, Karnal, India, with localized pulmonary tuberculosis were collected. All 16 patients were HIV negative with no history of any immunosuppressive condition, such as renal transplantation, diabetes mellitus, alcoholism, radiotherapy, and cancer. These patients were not hospitalized and had been receiving antitubercular therapy beginning 2 weeks after a confirmatory diagnosis based on acid-fast-bacillus-positive sputum smears and chest X-ray findings. Ten blood samples were collected from healthy individuals of the same age group as that of the tuberculosis-positive individuals. Five milliliters of peripheral blood was collected in EDTA-anticoagulated tubes from each of the patients. Leukocytes were pelleted after the lysis of erythrocytes by a hypotonic erythrocyte lysis buffer under chilled conditions (1). These leukocytes were resuspended in 1 ml of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA [pH 8.0]), and a 400-μl aliquot of this was processed for PCR. The leukocyte samples were subjected to supercooling in a liquid nitrogen container for about 15 min and then thawed abruptly in a boiling water bath for 10 min. This was followed by lysis with lysozyme, digestion with proteinase K, and extraction with hexadecyltrimethylammonium bromide and sodium chloride essentially as described previously (13, 14). DNA was precipitated with 0.6 volume of isopropanol in the aqueous phase and separated by chloroform treatment and extraction. The DNA was then dissolved in 50 μl of Tris-EDTA buffer containing RNase A (Sigma) at the rate of 20 μg/ml. A 10-μl aliquot of total DNA was used for a 25-μl PCR mixture.

PCR protocols were thoroughly standardized with respect to sensitivity and specificity by an extensive series of spiking studies with pure genomic DNA and cells of *M. tuberculosis* for seeding into cattle and goat blood. Primers (BW-6 [5′ CGA CAC CGA GCA GCT TCT GGC TG 3′] and BW-7 [5′ GTC GGC ACC ACG CTG GCT AGT G 3′]) aimed at the 306-bp region of the multicopy insertion sequence IS1081 (14) were used to amplify mycobacterial DNA in the blood leukocyte samples. The amplification parameters included an initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 68°C for 1.5 min, and extension at 72°C for 2 min. The extension step in the 35th cycle was held for 10 min before the samples were shifted to 4°C for storage. The PCR products were identified by agarose gel electrophoresis and combined ethidium bromide staining followed by Southern blot hybridization to an α-^{32}P-labeled 306-bp PCR product from the genomic DNA of *M. tuberculosis* H_{37}Ra with the BW6 and BW7 primers directed against the IS1081 sequence. Positive and negative controls with or without the genomic DNA of *M. tuberculosis* complex were included in each run. Of a total of 16 blood samples, 7 (43.75%) were found to be clearly positive for the presence of circulating DNA copies of *M. tuberculosis* complex in blood. PCR product or signal could not be detected on PCR and probe assays performed with blood donated by healthy individuals with no history of tuberculosis. The results were confirmed by retesting the samples by PCR assay. In all the samples, PCR products were clearly visible on

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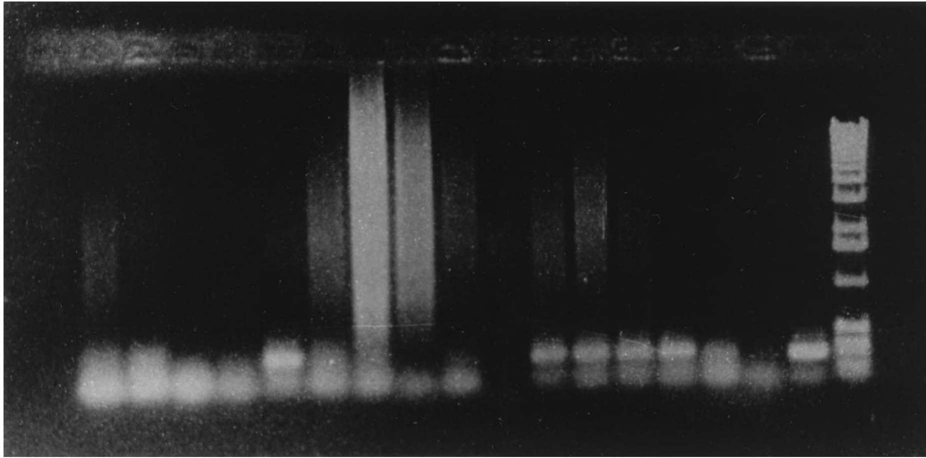

FIG. 1. Screening of human blood samples from immunocompetent tuberculosis patients by PCR with BW6 and BW7 primers. From left to right, the first 16 lanes contain PCR products from blood samples; the rightmost 2 lanes show PCR product from *M. bovis* DNA (positive control) and a 1-kb DNA ladder (marker), respectively.

the gel without any confusion (Fig. 1). Our results in this regard are contrary to those of Schluger et al. (10), Kolk et al. (6), Rolfs et al. (9), and Folgueira et al. (3), who recorded a much lower rate of positive PCR with blood samples. We do not believe that the higher rate of positive PCR results in our study were due to PCR-generated contamination, since we retested the samples and routinely included positive and negative controls during extractions, PCR mixture preparation, and electrophoresis. Separate lab spaces were dedicated to the processes of DNA extraction, PCR mixture preparation, and cycling to rule out the problem of artifactual contamination. Comparison of our data with those of all the earlier reports indicates that we used a fairly sensitive assay which could detect less than 10 copies of *M. tuberculosis* DNA per 5 ml of EDTA-anticoagulated blood. The test also could detect other species of the *M. tuberculosis* complex, particularly *Mycobacterium bovis*, and hence could not be taken as a species-specific assay for *M. tuberculosis*.

All the patients in our study had been undergoing antitubercular therapy for approximately 2 weeks. This could be a reason for the low sensitivity (<50%) of the assay. However, the PCR results conflicted with earlier findings which suggested that CD4 cells play a critical role in the containment of hematogenous dissemination, and thus blood-based PCR and culture methods were of less significance in case of HIV-negative patients and the HIV-positive individuals having mean CD4 counts of more than 200 cells/μl (3). The observation of *M. tuberculosis* DNA in the blood of patients included in this study underlines the facts that the incidence of hematogenous dissemination is underestimated in HIV-negative patients and that it is worthwhile to depend on the blood-based PCR assays based on multicopy target sequences for rapid diagnosis of tuberculosis by using blood as a convenient clinical specimen. In our opinion, the PCR technique evaluated in this study could be a good candidate for use in routine diagnosis of tuberculosis from blood samples, once standardization is accomplished. This, however, will require a thorough investigation regarding the sensitivity of the assay with a large number of patient populations. Furthermore, modifications in the DNA extraction process may yield better sensitivity. Multiplex PCR with multicopy sequences (like IS1081) along with highly specific target regions such as those based on 16S/23S ribosomal RNA sequences (4) may eliminate the need for Southern hybridization with ^{32}P, making the assay cost effective for field purposes.

In conclusion, this study has demonstrated the reliability of the blood-based PCR assay, which was more sensitive than the previous PCR protocols for the diagnosis of tuberculosis in immunocompetent patients. These findings support the hypothesis that the escape of tubercle bacilli from alveolar spaces to the bloodstream may be more frequent in case of immunocompetent patients than previously thought.

We are thankful to Kuldeep Singh, Incharge, TB Hospital, Karnal, for providing patient data and blood samples for this study and to the Director of NDRI, Karnal, for providing necessary facilities.

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