# Improved method for extraction of mycobacterial DNA from blood

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According to our experience and previously published data, PCR-based detection of mycobacteremia had an unsatisfactory sensitivity of <90%[2,4]. The choice of the sample preparation has to consider the following difficulties: (i) presence of PCR inhibitors such as heme or leukocyte cell debris, (ii) occurrence of low grade intermittent bacteremia, (iii) high amounts of contaminating human DNA (approx. 50 µg/ml blood), and (iv) resistance of mycobacterial cell wall to lysis. Using *M. avium* (MAV) we have developed a sample preparation method suitable for PCR amplification of mycobacterial DNA from 2 ml blood.

**Methods and results.** Blood sample preparation: after lysis of erythrocytes, the leukocyte pellet was washed twice in distilled water, pretreated for 30 min at 56 °C in 10 mM Tris buffer containing 0.1% proteinase K, 0.1% Triton X-100 in order to reduce leukocyte DNA or remove inhibitors, and finally vortexed thoroughly. After washing in 10 mM Tris mycobacterial DNA was released by sonication with glass beads and proteinase K digestion for 60 min at 56 °C. One tenth of the aqueous supernatant extracted with chloroform was directly subjected to PCR after incubation at 95 °C for 15 min. Alternatively this crude DNA sample was further purified using a commercially available spin column (QIAMP, Qiagen). PCR using specific primers for MAV and dot blot hybridization was performed as previously described[1,3]. Evaluation of the method was performed using the following approaches: (I) Effect of different lysis methods on DNA release from MAV using bacterial suspensions pretreated as follows: a) proteinase K digestion, b) sonication with glass beads, c) alkaline lysis, and d) chloroform extraction. The released DNA in the supernatant was chloroform/phenol extracted and determined spectrophotometrically (fig. 1). (II) Spiked blood samples (100, 500 CFU MAV/ml blood) were processed using different protocols (DNA release was achieved by sonication with glass beads in all protocols, except for alkaline lysis). The above mentioned blood preparation method was applied with or without proteinase K/Triton X-100 pretreatment. Sensitivity was compared using either 2 ml or 0.2 ml blood or alkaline lysis. To determine the loss of bacteria during the process of blood sample preparation pure bacterial suspensions were treated the same way. The efficiency of different methods were determined by

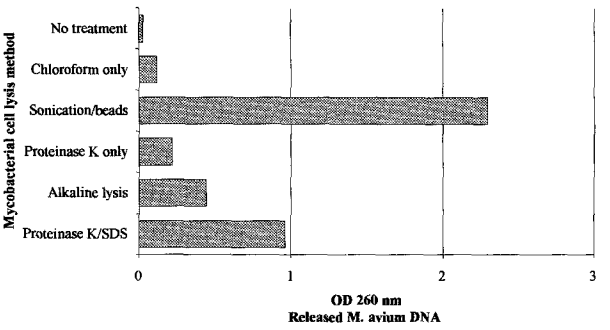


Figure 1. Comparison of different methods to lyse *M. avium* cells. Suspensions of *M. avium* (2 ml, approx. MacFarland 5) were submitted to the lysis procedure, centrifugated, and the released DNA in the supernatant measured spectrophotometrically after chloroform/phenol extraction (OD 260/OD 280 > 1.8).

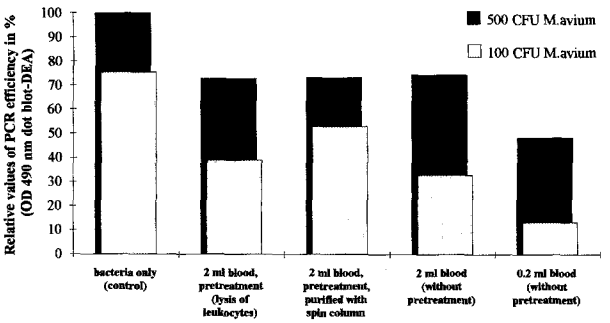


Figure 2. Influence of the pretreatment step (lysis of leukocytes with proteinase K and Triton X-100) on the loss of *M. avium* and the efficiency of PCR amplification using spiked blood samples. The data represent mean values for triplicate determinations using quantification of amplicon by dot blot-DEA. The results were compared to direct DNA extraction from bacteria not suspended in blood or manipulated by centrifugation and pretreatment (control).

quantitation of PCR amplification using the dot blot-based DNA enzyme assay described elsewhere[3].

The following results were obtained: (i) for mycobacterial DNA release, sonication in combination with glass beads was superior to other procedures as shown in figure 1, (ii) in regard to the application of different protocols for spiked blood samples, satisfactory results were obtained preparing 2 ml blood with the above mentioned method; further DNA purification with spin columns did not improve the results, and (iii) no significant los of MAV DNA was noted comparing the PCR results of pure bacterial suspensions with spiked blood samples (fig. 2).

**Conclusions.** In attempting to improve the sensitivity of MAV PCR for blood, we have evaluated a new DNA extraction method exploiting the high resistance of mycobacteria to chemical and physical agents. Our experiments indicate that pretreatment of the blood sample using proteolysis and a detergent partially eliminates contaminating human DNA (up to 40%) and may con-

tribute to removing inhibitors. Although the method produces a crude DNA preparation, inclusion of a chloroform extraction step combined with the above mentioned pretreatment makes further purification unnecessary.

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## Direct sequencing versus cloned amplicon sequencing in HIV-1 diagnosis

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It is now well accepted that the HIV-1 phenotype changes during the course of infection. These changes are due to mutations occurring in the retroviral genome. Mutations in the *env* gene are observed most frequently and are selected for under the pressure of the patient's immune system. The combination of PCR and subsequent sequence analysis of the amplicons give the opportunity to define the viral genotype at each stage of infection. An exact knowledge of the viral phenotype might be a prerequisite for successful prevention and treatment strategies. We have compared two sequencing methods for determining the genotype present in several patients. On the one hand sequences were obtained by the rapid and inexpensive method of direct amplicon sequencing, and on the other hand by the more time consuming and expensive method of cloning the amplicons prior to sequencing. By direct sequencing the major type of virus present in the patients at a given point in time could be identified whereas sequencing of cloned products gave an estimate of the diversity in the whole sequence swarm of the viruses. As target sequences for amplification we selected the relatively conserved gene for the retroviral polymerase (*pol*) as well as the conserved and hypervariable regions of the gene for the outer surface glycoprotein (*env*) detectable in the PBMC DNA of HIV-1 infected patients. Both genes are important for recent therapy studies[1,2].

**Methods and results.** The *pol* genes of several patients were amplified by nested PCR and the amplicons were sequenced directly. After the PCR, the amplicons were separated on an agarose gel and subsequently extracted from the gel matrix. The amplicons were then sequenced. An evaluation of the X-ray films obtained after sequencing revealed defined *pol* genotypes from each patient investigated. For the majority of nucleotide positions a signal was obtained on the X-ray films (fig. 1A). This strongly indicates a uniform virus population with regard to these positions. Only for a few nucleotide positions was more than one single nucleotide present on the gel (fig. 1A). Hence, for these positions the virus was heterogenous. This was substantiated by sequencing amplicons cloned independently. However, the method of direct sequencing was sufficient for defining the major viral genotype present in each patient.

Similar results were obtained for the conserved regions of the *env* gene, such as the region coding for the CD4 binding. The major genotype could easily be determined by direct sequencing. As mentioned for the *pol* gene regions investigated, the conserved regions were amplified by nested PCR prior to direct sequencing. The same strategy was employed to determine the major genotype of the hypervariable regions of the *env* gene. But in contrast to the conserved regions an unequivocal determination of the major genotype was not possible. As shown in figure 1B, X-ray films obtained from the hypervariable regions could not be evaluated. Therefore the amplicons were cloned and then sequenced (fig. 1C). All clones differed from each other. Insertions and deletions led to overlapping sequences which could not be resolved by direct sequencing.

**Conclusions.** HIV-1-specific sequences could easily be amplified from the DNA of peripheral blood lymphocytes or from the viral RNA present in the plasma. A detailed characterization of the resulting amplicons requires sequencing. Here we have shown that the sequencing strategy depends on the variability of the region of interest. The higher the variability of a distinct region the more a cloning step is necessary prior to sequencing to obtain reliable information. Conversely, direct sequencing analysis is usually sufficient to record changes of conserved viral sequences,