and/or by colony-, dot or PCR multiplex hybridization, and 5) the lack of reference sequences that may erroneously suggest the taxonomic novelty of a clone. Elucidation of the composition of a bacterial community occurring in a natural sample was attempted by dot blot hybridization of 16S rDNA clone libraries with taxon-specific oligonucleotide probes. The composition changed significantly when the same batch of isolated DNA and the same cloning vector, but two different pairs of amplification primers were used. The distribution of taxon-specific clones was also different from that obtained previously using one of the same primer pairs but a different cloning system. The results indicate that our present knowledge of this approach allows neither the complete qualitative nor the accurate quantitative determination of microbial community compositions.

## PCR-based detection of *Mycobacterium tuberculosis* in sputum samples using a simple and reliable DNA extraction protocol

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Several procedures to release DNA from acid-fast mycobacteria for polymerase chain reaction-based amplification have been reported. Nevertheless, up to now there is no extraction method available which is simple and reliable enough to allow its application in routine clinical practice.

We present a rapid, simple and reliable protocol for the extraction of mycobacterial nucleic acids as template molecules for a subsequent polymerase chain reaction. Samples were suspended in extraction buffer and subjected to several cycles of freezing in liquid nitrogen and heating in a boiling water bath. After the treatment, the rigid cell wall of the mycobacteria was cracked and DNA could be reliably amplified from the supernatant. For the evaluation of this procedure we used serial dilutions of liquid culture. Additionally, PCR was capable of detecting mycobacteria in sputum samples from 13 out of 13 patients with clinically suspected tuberculosis which were positive by smear and culture. Amplified DNA products were characterized both by length and direct sequencing. Using PCR primers which hybridize to a conserved sequence that flanks a hypervariable region in the 16S rRNA gene of mycobacteria, we were able to distinguish even between distinct mycobacterial species by determining the nucleotide sequence of the amplification products.

In 15 smear- and culture-negative cases without suspected tuberculosis, PCR led to negative results. The routine applicability of this new extraction protocol for nucleic acid from mycobacteria will be further evaluated.

## Specific detection of *Mycobacterium avium* using DNA amplification

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Infections caused by the *Mycobacterium avium*complex (MAC) contribute substantially to morbidity and mortality in patients with AIDS, and the prevalence is increasing. There is an urgent need for methods that can detect this pathogen more rapidly and directly in body fluids.

We have evaluated a PCR amplification method based on the DNA probe sequence by Fries et al. (insert pMAv22)[1]. To improve specificity of PCR, a new primer was selected (bases 37–56) and used together with the described primer Mav 22B in order to amplify a 148 bp sequence specific for *M. avium*. A 94 bp peroxidase labelled probe produced with the primers Mav22A and B was used for Southern blot hybridization and assayed by means of enhanced chemiluminescence. The sensitivity of the assay was determined by using *M. avium* DNA in the presence of 0.5 µg human genomic DNA or blood spiked with different amounts of bacteria. While the detection limit was 5–10 fg, 10 bacteria/ml blood were detectable in spiked specimens. No bands were visible if the PCR assay was tested with 1) 23 of the most common mycobacteria other than *M. avium* (including 3 clinical isolates of *M. intracellulare*, determined by direct sequencing of a hypervariable gene region for the 16S rRNA and the strains *M. intracellulare* serovar 7, 13, 14 and 17 so far tested), 2) DNA from 10 common pathogens of the human respiratory tract and 3) human genomic DNA from 30 various clinical specimens, all culture negative for mycobacteria. We routinely use this method for the identification of *M. avium* in mycobacterial cultures, because it is rapid, specific, easy to perform and very sensitive. All cultures ($n = 30$) diagnosed as *M. avium* by conventional methods and the above-mentioned sequencing thus far (except for the above mentioned *M. intracellulare* isolates),

including the serovars 1–6 and 8–9 tested, proved to be positive with this PCR method. Further clinical studies are warranted to evaluate the applicability of this assay for the direct and early detection of this mycobacterium directly from clinical specimens.

1 Fries, J. W. U., Patel, R. J., Piessens, W. F., Wirth, D. F., Molec. Cell Probes, *4* (1990) 87–105.

## Rapid PCR detection of *Mycobacterium tuberculosis* in sputum samples

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The diagnosis of *Mycobacterium tuberculois* (MT) using conventional microbiological methods is still a time-consuming process which, particularly in regard to the rise of cases of drug-resistant mycobacteria, needs to be shortened. PCR is a suitable method, because of its independence of the growth of the organism. The aim of this study was to develop a PCR method feasible for a routine laboratory. Sputum samples were liquefied, decontaminated and concentrated by standard methods. The mycobacteria were lysed by using a combined treatment of sonication and heat incubation in presence of a detergent. The lysate containing the genomic DNA was added directly to the PCR (without prior phenol/chloroform extraction), and the PCR performed in two steps. First, for screening, we performed a genus specific PCR on 49 sputum samples (10 MT culture-positive, 39 MT culture negative) using primers published by Hance et al.[1] The 383 bp PCR products were separated on agarose gel followed by a dot-blot hybridization with a digoxigenin labelled probe. Compared to the culture, the sensitivity of this PCR was 90% (one false-negative) and the specificity 89.7%, whereby the four culture-negative but PCR-positive samples were from confirmed MT-infected patients undergoing chemotherapy. Secondly, the same samples were tested in a PCR using primers specific for MT-complex described by Eisenach et al.[2]. The 123 bp PCR products were separated on agarose gel. The sensitivity of this PCR was 60% and the specificity 97.4%. The detection level of both PCRs was $10^2$ cells/ml.
These results indicate that this two-step-PCR can be a useful adjunct to conventional methods for the rapid diagnosis of MT in sputum.

1 Hance, A. J., Grandchamp, B., Lévy-Frébault, Lecossier D., Rauzier, J., Bocart, D., Gicquel, B. Molecular Microbiology, *3* (1989) 843–849.
2 Eisenach, K. D., Cave, D. M., Bates, J. H., Crawford, J. T., J. Infect. Dis. *16* (1990) 977–981.

## Comparative 16S rRNA analysis for the identification of slow-growing and uncultivable bacteria from activated sludge

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So far, analysis of complex microbial ecosystems has been based on culture and subsequent biochemical identification. However, cultivable microorganisms represent only 10% of the entire microbiota. Molecular genetic analysis has been applied to identify and phylogenetically classify all members of a microbial community without prior cultivation.
Activated sludge samples from a wastewater treatment plant (Berlin-Marienfelde) were analyzed by culture and molecular genetic tests for the presence of nocardiform actinomycetes and mycobacteria. First we isolated bulk DNA from which 16S rRNA gene fragments were selectively amplified by using well established 16S rRNA amplification primers. Amplicons were ligated into plasmid pUC 19 and used to transform competent *E. coli* cells. About 3000 clones of the entire 16S rRNA clone library were analyzed by colony hybridization using a probe specific for 16S rRNA from mycobacteria and nocardiform actinomycetes. Positive clones were subjected to comparative sequence analysis and were classified phylogenetically. Only one sequence corresponded to known, cultivable bacteria. By using genus-specific primers for nested-PCR it was possible to amplify mycobacterial 16S rRNA genes directly from the original bulk-DNA.

## Determination of the individual efficiency of amplification by means of PCR-MIMIC's

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PCR-results may be false positive, caused by contaminations, or false negative. In the latter case inhibitors diminish an optimal amplification. False negative results can be detected using probe-inherent single-copy genes or by spiking with template DNA. Negative PCR-results are only valid in combination with proper controls. Such controls give no information about the amplification efficiency. By the use of internal standards