Nocardioform actinomycetes in activated sludge: phylogenetic classification and in situ identification on the basis of 16S rRNS analysis

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The analysis of complex microbiota present in activated sludge is of utmost importance for the understanding and possible control of severe separation problems in sewage treatment, such as sludge bulking or sludge scumming. Previous studies indicated that nocardioform actinomycetes are frequently responsible for these conditions which not only affect the efficiency of sewage treatment but also represent a threat to public health due to spread of pathogens[1]. Among these are ubiquitous mycobacteria or nocardioform actinomycetes such as *Rhodococcus equi*, *Nocardia asteroides* or *Gordona bronchialis*, i.e. organisms that may be part of the microbial flora of the scum[2]. However, isolation and identification of these filamentous, nocardioform actinomycetes is hampered by their fastidious nature. Most species are still uncultivable and their taxonomy is by and large unclear.

To study the ecology of these microorganisms at the molecular level, we carried out a comparative sequence analysis of nocardioform 16S rDNA sequences which derived from a clone library of 16S rRNA gene fragments amplified from bulk sludge DNA[3]. The DNA was extracted from an activated sludge sample of the waste water treatment plant Marienfelde (Berlin). 16S rRNA gene fragments (approx. 500 bp) were selectively amplified by using 16S rDNA amplification primers specific for eubacteria. Amplicons were ligated in pUC 19 and used to transform competent *E. coli* cells. Colony hybridization with oligodeoxynucleotide probe MNP1 (specific for nocardioform actinomycetes and mycobacteria) detected 27 out of 3000 recombinant clones in our 16S rDNA clone library. The sequence data from the MNP1 probe-positive clones were used for comparative sequence analysis and construction of dendrograms. Although all sequences differed from hitherto sequenced species in databases, comparative sequence analysis allowed the phylogenetic classification of the bacteria from which the 16S rDNA fragments derived.

In parallel, part of the respective sludge sample was spread on agar plates with modified Czapek-Dox medium to isolate nocardioform actinomycetes. The characterization of the culture isolates revealed a high discrepancy between the two approaches. Only one 16S rDNA sequence of a culture isolate was represented in the clone library. This indicates that culture conditions select species, which represent only a small fraction of the organisms in the activated sludge and therefore were not represented in the clone library. However, conventional culture methods failed to grow bacterial species most abundant in the clone library.

Based on the nocardioform 16S rDNA sequences from the clone library we synthesized specific, fluorescent oligonucleotide probes. In situ hybridization of activated sludge from the same waste water treatment plant using these specific probes identified the respective organisms in the sludge.

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PCR of DNA from dried blood spots on filter paper coupled to a simple DNA enzyme immunoassay for rapid detection of HIV-1

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For the HIV PCR technique to be implemented as a tool in large scale studies it is essential that the sample preparation and the hybridization procedures are easy to perform and ensure the specificity of the amplified DNA. Therefore, we developed a method for the detection of proviral HIV-1 DNA from blood samples dried on filter paper appropriate for long-term storage. To simplify the analysis of amplified products we adapted the previously described colorimetric microtitre plate hybridization assay[1] to the detection of HIV-1 specific sequences.

Amplification of DNA from blood spot materials. Blood spots were prepared dropwise on filter paper and allowed to dry down at room temperature. The filters

were suspended in lysis buffer, DNA was isolated by a standard column procedure and quantified for direct use in PCR. HIV-1-specific sequences were amplified using primers complementary to three different regions of the genome. The amplification of HIV DNA was possible without loss of sensitivity from dried blood samples stored for about 6 months at room temperature. To validate the sensitivity of the method, cells containing a single integrated provirus were titrated with HIV negative donor blood. HIV-1 positive patients were investigated to evaluate the specificity of the method. We could show that our procedure was comparable in sensitivity and specificity to other methods such as phenol/chloroform extraction from isolated peripheral blood mononuclear cells or a whole blood DNA purification procedure. Compared to another procedure of DNA extraction from dried blood spots[2] our method yields more reproducible results with respect to amount and amplification of the isolated DNA.

Detection of PCR products by DNA enzyme immunoassay (DEIA). HIV-1-specific sequences were detected by hybridization with biotin-labelled probes immobilized on streptavidin-coated microtitre wells. Measurement of the amount of hybridized DNA was done by a sandwich ELISA including a monoclonal antibody against double stranded DNA. The absorbancy was read on a spectrophotometer at 450 nm. The results obtained were compared to Southern blot analysis of PCR products using a chemiluminescent detection system. The colorimetric signal obtained by the amplification of increasing numbers of genome copies clearly reveals a positve result for one amplified copy, demonstrating the sensitivity of the assay. The specificity of DEIA was determined by running 26 patient samples in parallel for each of the three different genomic regions. All samples that did not hybridize in Southern blot analysis showed an optical density value below the cutoff. In contrast, the optical density of those samples with a positive signal in blot hybridization varied in general between an OD of 0.3 and 2.1.

Conclusions. The application of PCR and techniques confirming the specificity of PCR products in routine diagnostic laboratories, requires that the procedures are simple and reproducible. The microtitre plate assay we described for detection of HIV-1 is sensitive, simple, rapid and reproducible. The DEIA test is perfectly compatible with standard enzyme linked immunosorbent assay equipment, permitting the processing of a large number of samples. Moreover, the ability to analyze DNA extracted from dried blood specimens, as demonstrated in this study, allows the long-term storage of blood samples even at elevated temperatures and after transport over long distances.

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Detection of hantaviruses by polymerase chain reaction

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Hemorrhagic fever with renal syndrome (HFRS) refers to a group of human diseases with similar clinical symptoms. Members of the genus hantavirus, which belongs to the family Bunyaviridae, were identified as etiologic agents of HFRS[3]. They possess a single stranded, negative sense RNA genome, consisting of three segments, termed small (S), medium (M), and large (L), encoding a nucleocapsid protein, two glycoproteins (G1, G2), and a polymerase, respectively. Four serotypes causing HFRS have been described: Hantaan (HTN), Puumala (PUU), Seoul (SEO), and Dobrava/Belgrade (DOB/BEL). Recently, an additional serotype causing severe disease with high lethality has been found in the 'Four Corners Region' in the USA and described as human pulmonary syndrome (HPS). In contrast to other members of the genera of Bunyaviridae which are transmitted by arthropod vectors, rodents are the reservoir of hantaviruses. In Germany a seroprevalence of anti-hanta antibodies of about 1–2% within the normal population was determined. Although in southern Germany antibodies to both major antigens (PUU/HTN) were detected in patient sera, in western Germany antibodies to the PUU antigen and in Berlin–Brandenburg antibodies against the HTN antigen were predominantly identified[7]. RT-PCR for the detection of hantaviruses was described by several authors[4,5]. We have established RT-PCR for the detection of hantaviruses to allow molecular diagnosis and genetic characterization of new hantaviruses prevalent in eastern Germany and eastern Europe.

From the RNA extraction methods tested, the best results were obtained with a slightly modified acid guani-