Detection of viral nucleic acids at low concentrations in plasma pools

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In the past, contamination of blood products with different viruses including HIV, HBV and HCV has led to numerous infections of recipients. Therefore, each blood donation has to be screened for viral markers (HIV and HCV antibodies, HBs antigen). Nevertheless, transmissions still occasionally occur, as shown recently when more than 30 individuals were infected with HBV via a coagulation factor concentrate.

Like other blood products, the preparation of such a concentrate starts with a plasma pool composed of several thousand individual donations. Viruses present in such a pool pose a potential risk which could be estimated if the virus load could be determined. The examination for serological viral markers of the plasma pools used for the production of the aforementioned coagulation factor concentrate, including markers for HBV, gave negative results as expected when screening was correctly performed. Therefore, the level of contamination with HBV in the affected plasma pool must be very low. As PCR is the most sensitive method available we generated a protocol that allows us to detect less than 10 HBV genome equivalents per ml plasma.

Nested PCR assays were performed under standardized conditions by amplification of a conserved sequence of the gene coding for the surface protein. Special care was taken to exclude contamination. The efficacy of the procedure used for extraction of nucleic acids plays an important role if virus concentrations are at the limit of detection. The extraction of nucleic acids in a single-step procedure with the chaotropic reagent sodium iodide was very effective[1]. Plasma spiked with decreasing amounts of purified recombinant HBV DNA (Chiron Corp.) was used as a control. In these controls one HBV molecule per assay could be detected by nested PCR with a statistical probability following the Poisson distribution. The investigation of the incriminated plasma pool resulted in two positive PCR assays out of 11 performed. Altogether, pool equivalents of 1.0 ml were tested. According to the Poisson equation, given an error probability for one percent, the contamination of the plasma pool with HBV amounts to at least four genome equivalents per ml. Therefore, in order to detect a viral load of five genome equivalents in a plasma pool or other material, equivalents of at least 0.92 ml of the sample have to be tested in the PCR assays. Under these conditions at least *one* positive reaction can be expected with a probability of 99 percent.

Thus, it has been demonstrated that one HBV genome equivalent per assay can be detected by nested PCR. According to the Poisson distribution, under the assumption of a very low virus load of the material examined and with a given probability, it is possible to calculate the sample volume necessary to obtain at least one positive reaction as well as the number of assays needed. Current methods of virus concentration will have to be further validated, especially if the viral concentration is very low (<10 viral particles per ml). These methods will also have to be investigated for the potential accumulation of factors, which may inhibit the enzymatic reactions in subsequent steps.

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Effect of genome size and *rrn* gene copy numbers on PCR amplification products of 16S rRNA genes from mixed bacterial species

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Phylogenetic diversity of microorganisms, underestimated in the past by classical enrichment and isolation techniques, is beginning to be more realistically assessed by the detection of so far uncultured forms. Gene amplification, molecular cloning and sequence analysis of 16S ribosomal RNA have been used to determine the phylogenetic position of a fraction of naturally occurring organisms in relation to their culturable neighbours. Each step in community analysis is open to error or bias, including cell lysis, DNA extraction and quality, choice of primers, PCR conditions, and cloning, as well as the application of nonspecific probes. Some of these errors can be detected, for example the formation of chimeric PCR products, others can be minimized by improved PCR conditions, while others remain problematic with the present knowledge. As it is one of our ultimate goals to quantify microbial populations it appears important to place more emphasis on the identification of factors that might influence individual steps in molecular ecology studies. In order to assess the effect of genome size and number of 16S rRNA genes on the quantities of PCR-generated partial 16S rRNA fragments, equimolar mixtures of DNA from pairs of different species for which these parameters are known, i.e. *Bacillus subtilis* DSM 402, *Escherichia coli* DSM 498, *Pseudomonas aeuroginosa* DSM 1707 and '*Thermus thermophilus*' DSM 579, were subjected to gene amplification.

Materials and methods

DNA extraction and amplification. Extracted DNA was purified by Prep-a-Gene kit. Conserved 16S rDNA primers used were 10-30f (5′-GAGTTTGATCCTG-

GCTCAG-3′) and 530r (5′-GA/TATTACCGCGGCG-GCTG-3′). PCR amplification was performed in a total volume of 100 µl, containing 0.5 µg of each primer, 10 µl of 10 × reaction buffer (Boehringer Mannheim), 1 mM (each) dATP, dGTP, dTTP and dCTP, and 100 ng of each bacterial DNA. The reaction mixtures were incubated in a thermal cycler for 3 min at 98 °C. Two units of Taq Polymerase (Boehringer Mannheim, Germany) were added and the reaction was performed for 28 cycles at 55 °C for 1 min, 72 °C for 2 min, and 93 °C for 1 min. The final cycle was an elongation step at 72 °C for 5 min.

Image analysis. 5 µl of the PCR solutions were loaded onto a vertical gel, consisting of 3% Metaphor agarose (FMC, Rockland, Maine). Gels were run at 5 V/cm for 14–16 h at 4 °C, and stained with SYBR green (Molecular Probes, Ca.). Gel images were converted to digitized files and analysed by Image Quant 3.3 (Molecular Dynamics).

Results and conclusion

As determined by image analysis of SYBR green-stained amplification products the experimentally determined ratio corresponded well with the expected ratio calculated from the number of *rrn* genes per equimolar amount of DNA in mixtures containing DNA of *Escherichia coli* and '*Thermus thermophilus*' and DNA of *Pseudomonas aeruginosa* and '*T. thermophilus*'. The values for the pair *Bacillus subtilis* and '*T. thermophilus*' showed higher deviation from the predicted value. The dependence of the amount of 16S rDNA amplification products on these two parameters makes it impossible to quantify the number of species present in 16S rDNA clone library of an environmental sample, as long as these two parameters are unknown for these species.

1 Farrelly, V., Rainey, F. A., and Stackebrandt, E., Environ. appl. Microbiol. *61* (1995) 2798.

Simple 'universal' DNA extraction procedure compatible with direct PCR amplification

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Large scale use of amplification methods in diagnostic laboratories requires efficient but simple DNA extraction procedures compatible with various types of clinical specimens. A combination of SDS and proteinase K has been widely used for solubilizing tissues as well as for the extraction of DNA from microorganisms. However, SDS is inhibitory for *Taq* polymerase at any useful concentration[1] and, therefore, does not allow direct amplification without extraction with organic solvents and ethanol precipitation. We have used Tween 20 to neutralize the activity of SDS on *Taq* polymerase in the amplification mix, resulting in a simple and rapid extraction procedure applicable to a variety of clinical specimens which does not require further purification steps.

Tissues (disintegrated mechanically), sputum (liquefied and centrifuged), liquid specimens (centrifuged) or bacterial cultures were incubated with digestion buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% SDS, 200 µg/ml proteinase K) at 55 °C for 3 h or overnight (tissue) followed by heating at 95 °C for 10 min and transferring a maximum of 10 µl directly into the amplification tube. Amplification was in a final volume of 100 µl containing 2% (v/v) Tween 20. This extraction procedure has successfully been used in conjunction with various PCR systems including amplification of *Rochalimaea (Bartonella) henselae* and *R. quintana*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*.

The sensitivity of the procedure was determined using a eubacterial amplification system[2]. Ten µl of the amplified material were analyzed by gel electrophoresis and ethidium bromide staining without hybridization: the equivalent of between 7 (gram-negative bacteria) and 600 (gram-positive bacteria, *Mycobacterium fortuitum*) organisms per amplification was reproducibly visible on the agarose gels. We conclude that DNA extraction with SDS and proteinase K is simple, rapid, applicable to large numbers of specimens, and allows direct amplification without further purification if Tween 20 is included in the amplification mix. It is also compatible with the use of uracil-N-glycosylase (UNG) for the prevention of false positives due to amplicon carry-over (contamination control).

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Detection of *Aspergillus fumigatus* by polymerase chain reaction (PCR)

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Invasive aspergilloses (IA) have a poor prognosis, but it is known that early diagnosis and treatment result in a more favourable outcome. PCR could contribute to a rapid diagnosis. In this study we developed a PCR to detect *Aspergillus fumigatus* and assessed the application to clinical specimens.

The primers were designed based on the published cDNA sequences of the cytotoxin Asp f1, because