Using the WAVE® HS System in Clinical Microbiology RISA Analysis of Infectious Pseudomonas Species

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Classic microbiological analysis of clinical samples has involved the isolation and culture of microorganisms using defined culture media. Both phenotypic (biochemical and physiological characteristics, serotyping, antibiotic resistance, etc.) and genotypic criteria are then used to identify the isolates. However, the culture approach can have limitations as the physiological status of the microorganisms may hamper their growth; additionally, the culture media or growth conditions may be too selective. Furthermore, the culture and identification steps are labor-intensive and time-consuming, often limiting the rapid analysis of a high number of samples. The development of culture-independent techniques based on genetic fingerprinting, that imply the analysis of nucleic acids, i.e., DNA and RNA directly extracted from the samples, can be used to analyze microbial community composition. Specific PCR amplification of phylogenetic marker genes such as the genes encoding the small subunit (SSU) and the large subunit (LSU) of ribosomal RNA (rRNA) followed by genetic fingerprinting has been developed to describe diverse microbial communities in various environments. Genetic fingerprinting methods such as denaturing or thermal gradient gel electrophoresis (DGGE or TGGE), single strand conformation polymorphism (SSCP) analysis, terminal restriction fragment length polymorphism (T-RFLP) or ribosomal intergenic spacer analysis (RISA) have been successfully used to differentiate bacterial isolates and to characterize complex bacterial communities. These methods provide fast, inexpensive and reproducible estimates of community composition in the form of DNA fragment profiles.

RISA exploits the length heterogeneity of the prokaryotic intergenic transcribed spacer (ITS) regions between SSU and LSU rRNA genes. PCR amplification across the ITS region produces DNA fragment lengths characteristic of the taxa present in the sample.

We investigated the potential of the WAVE System (Transgenomic Ltd., Paris) to resolve the mixed rDNA amplified fragments of sizes ranging from 300 bp to more than 1000 bp. Tests were performed on individual bacterial species DNA as well as on DNA extracted from sputum samples obtained from children with cystic fibrosis (CF).

DNA Extraction

Genomic DNA of bacterial cultures was extracted using Qiagen® Genomic-tips, according to the manufacturer’s instructions (Qiagen, Courtaboeuf, France). Bacterial DNA was extracted from sputum samples with the FastDNA® SPIN Kit (MB Biomedicals, Irvine, CA) after triplicate washes in DigestEur solution.

T-Taq™ Polymerase PCR Conditions

Bacterial DNA was amplified using the primers ITSF (5’-GTCGTAA CAA GGTAGCCCGTA-3’) and ITSReub (5’-GCCAAGGCGATCCACC-3’) (Cardinale, 2004). The 5’ and 3’ ends of primers ITSF and ITSReub were, respectively, complementary to positions 1423 and 1443 of the 16S rRNA gene and 38 and 23 of the 23S rRNA gene of E. coli. Reaction mixtures (50 µL) for PCR contained 5 µL of 10X T-Taq buffer, 0.5 µM of primers, 200 µM dNTPs, 2.0 U of T-Taq Polymerase (Transgenomic Ltd., Paris) and 50 ng of purified DNA. Amplification was performed in a PerkinElmer® Cetus 2400 thermocycler using the following program: 94 °C for 3 min; 25 cycles consisting of 94 °C for 1 min, 55 °C for 30s, and 72 °C for 1 min; and extension of incomplete products for 5 min at 72 °C.

WAVE System Conditions

PCR products of amplified ITS were separated by DHPLC on a DNASep® HT Cartridge and analyzed with the DNA fragment sizing method. Separation was achieved by application of a flow rate of 0.35 mL/min, at an oven temperature of 50 °C or 40 °C and a gradient formed with WAVE Optimized® Buffer A, consisting of 0.1 M TEAA and WAVE Optimized Buffer B consisting of 0.1 M TEAA in 25% (v/v) acetonitrile. Separated PCR products were detected and visualized as peaks with an HSX-3500 Fluorescence Detector using the HS Staining Solution I.
Results

Preliminary tests performed on the 100-bp ladder allowed us to define the most appropriate gradient enabling the separation of ITS fragments over a size range of 300 bp to 900 bp with the oven temperature at 50 °C. Lowering the oven temperature at 40 °C with the same gradient conditions allowed the increase of the size range separation of fragments up to 1300 bp (Figure 1). DHPLC proved to be efficient for the differentiation of bacterial species based on the size and number of the ITS amplicons as demonstrated in Figure 2. The various Pseudomonas species tested were efficiently characterized by a unique and specific profile.

The RISA method was then used to characterize the bacterial diversity within sputum samples obtained from children with CF. DHPLC profiles exhibited various numbers of peaks depending on the patient and the level of infection. The presence of a peak corresponding to an infection by the species P. aeruginosa was detected (Figure 3) and results were compared to microbiological data obtained with conventional culture methods. Concordance was always observed between DHPLC and the microbiological data.

The RISA approach coupled with the WAVE HS System proved to be efficient in characterizing bacterial species and for microbial diversity analysis of clinical samples. This technique could be of great interest to clinicians as it allows the rapid and sensitive detection of pathogens without culturing.

Reference


Figure 1. DHPLC profiles at 50 °C (black) and 40 °C (red) of the 100-bp ladder. The size increment between peaks is 100-bp.

Figure 2. DHPLC profiles at 50 °C of the ITS of (A) *Pseudomonas fluorescens*, (B) *P. chlororaphis*, and (C) *P. fragi*. Each species profile is overlaid with the 100-bp ladder profile.

Figure 3. DHPLC profiles at 50 °C of the ITS of (A) *Pseudomonas aeruginosa*; (B) overlay of *P. aeruginosa* and a sputum sample from a 12-year old child infected with *P. aeruginosa*; (C) overlay of *P. aeruginosa* and a sputum sample from a 9-month old child not infected with *P. aeruginosa*.