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Terminal Restriction Fragment Length Polymorphism – Principle, Profit, Problems and Progress

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Abstract: Terminal restriction fragment length polymorphism (T-RFLP) is a method for identification of prokaryotes, based mostly on the variability of 16S rDNA sequences between species. In this article, the basic principle, advantages and disadvantages of T-RFLP are summarized. Special attention is given to the potential bias related to T-RFLP. Currently, evolutionary microbiology, biotechnology, molecular taxonomy, phylogenetic analysis, and microbiological diagnostics belong to research areas where T-RFLP is used. This technique can greatly improve the diagnostics and the research on the pathogenesis of oral, gastrointestinal and gynecological diseases.

Keywords: Molecular analysis, Oral microflora, Microbial community, 16S rDNA

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1. Introduction

Oral microflora consists of a large number of various microbial species. Many of those are involved in the pathogenesis of oral diseases. Standard microbiological methods can diagnose only a small part of the variety of these microorganisms. The need for determination of structure, composition and dynamics of microbial communities in biological samples has led to development of new molecular methods that are cultivation independent. These strategies overcome the limitations of cultivation methods, as many microbial organisms are very difficult to cultivate or are uncultivable. Molecular techniques providing the definite profile of microbial community are mostly PCR-based and evaluate directly the environmental sample. Procedures such as thermal gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) [1], amplified length heterogenity analysis (ALH) [2] and others have been developed to improve the formerly used phenotype-based methods. Terminal restriction fragment length polymorphism analysis (T-RFLP) represents a culture independent tool for genetic fingerprinting of microbial communities [3].

2. Terminal Restriction Fragment Length Polymorphism

DNA from environmental or clinical samples is amplified by polymerase chain reaction (PCR) with primers homologous to conservative sequences (genes for 16S rRNA or other). The main factor used for discrimination in T-RFLP is based on the variability of 16S rRNA genes in prokaryotes [4]. The target gene is present in all the microbial species and so the amplified DNA contains the genes of every species in the sample. One of the primers is fluorescently labeled. 5' end provides a higher discriminative power because of considerable length polymorphisms in this region. Amplified DNA products (amplicons) are digested with either one or with a combination of two or more different restriction enzymes and then loaded into electrophoresis (usually capillary gel electrophoresis or polyacryl amid gel electrophoresis). Afterwards, the restriction fragments are separated by electrophoresis and consequently visualized by the fluorescence detection [5]. The result of this process is a pattern or profile consisting of terminal restriction fragment (TRF) peaks. Every TRF represents virtually one specific species. Most of T-RFLP studies are focused on composition and diversity of microbial communities, identification of specific organism or comparison of microbial abundance and structure. The basic principle of T-RFLP is shown in Fig. 1.

The variability of 16S rRNA derived amplicons is determined by the variable regions of the gene located between the conservative regions. A great amount of known 16S rRNA sequences is presented in the Ribosomal database project (RDP). It is possible to predict specific terminal restriction fragments of many species according to sequence data [6]. The observed TRF profiles can be easily identified by comparison with predicted profiles. Specific sets of primers were designed to amplify the target gene specific for a microbial community or group. 16S rRNA primers were developed to amplify only the Eubacteriaceae-specific or archaebacterial-specific 16S rRNA, 18S rRNA primers are available specific for fungal communities, some studies even use primers homologous to conservative sequences of functional genes (merR- mercury resistance, nifH- N2 fixation ...) [7].

There are several technological aspects affecting the resulting TRF profile that have to be taken into account [8]. The differences between the Gram-positive and Gram-negative cellular wall can influence the relative amounts of DNA in the sample during the process of DNA extraction. Therefore, it is needed to standardize cell lysis and DNA extraction. The rRNA genes markedly vary in number of copies between different organisms (1 to 13 copies per genome in group of Eubacteria). This means that TRF patterns have to be properly calibrated for each species before they can be used to determine the abundance of organisms in a sample. Concentration of template DNA used for PCR and the number of PCR cycles play a key role in T-RFLP analysis. Low number of PCR cycles often generates limited and weak profile. On the other hand, the high number of cycles can cause the formation of nonspecific and chimeric amplicons. To reduce all possible biases in PCR, running several parallel PCR reactions from the same sample can often dramatically eliminate random bias and artifacts.

Important aspects in the T-RFLP analysis include the choice of primers DNA and enzyme concentrations used in digestion [9]. The relative amounts have to be exactly determined to avoid the formation of nonspecific restriction fragments. If the amount of restriction enzyme is too low, formation of longer and partially digested fragments can be

observed. The addition of larger amount of fluorescently labeled DNA to reaction mixture appears to induce the star activity (an enzymatic activity with low specificity for the recognized sequence). Many studies use two or more restriction enzymes in one reaction to acquire higher resolution of fragments. The most common are the restriction enzymes HhaI, MspI and RsaI as well as their combination [5].
Another issue is the detection of fluorescently labeled terminal restriction fragments. TRF patterns containing a high number of peaks of a similar size require larger amount of loaded DNA to achieve sufficient separation of individual peaks. It is also needed to purify and desalt the restriction fragments to avoid the interference with electrophoresis detection. Usually, 50 200 ng of digested DNA is loaded into the capillary electrophoresis, while injection time and voltage can be used to regulate DNA uptake [10].

Bioinformatic analysis of T-RFLP data includes identification of peaks and organisms corresponding to particular peaks. There are several mathematical strategies providing different algorithms to solve this problem. It is needed to determine the detection threshold of peaks as well as their size according to comparison with a DNA standard. Interpretation of data varies between studies as different statistical approaches are used. Most studies focused on 16S rRNA patterns use the rRNA sequence database for identification of TRF peaks, where observed and in silico predicted TRF peaks are compared [11]. The observed TRF sizes can fit to several different species and so it is useful to perform multi-enzyme digestions.

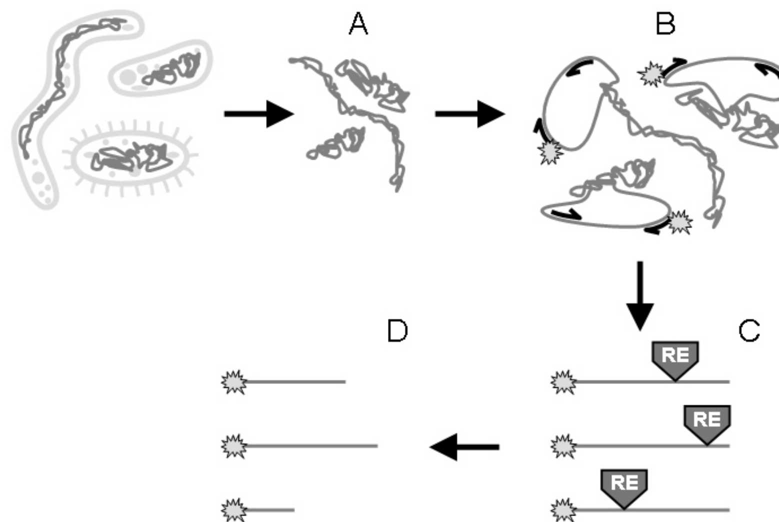

Fig. 1. The principle of molecular analysis based on terminal restriction fragment length polymorphism (T-RFLP). Environmental samples with mixture of bacterial species are collected for DNA extraction (A). Genes encoding 16S-RNA are amplified using primers homologous to conservative sequences; 5´-primer is fluorescently labeled (B). Population of heterogeneous PCR products is digested with one or more restriction enzymes (C). Restriction fragments are separated on capillary gel electrophoresis; the terminal restriction fragments are visualized (D). Each of the fragments represents a bacterial species.

The main advantages of TRFLP include the rapid data generation, possibility of automation overcoming the time consumptive manual examination as well as high discriminative power and accuracy. On the contrary, potential bias coming from DNA extraction, PCR and electrophoresis as well as the incomplete Ribosomal database project are the main disadvantages in comparison to other molecular techniques for the characterization of microbial communities.
The main area of research where T-RFLP is used is the comparative microbial analysis. Researchers use T-RFLP for comparison of endangered or exotic ecological niches, for phylogenetic research that sheds light on various aspects of gene flow between species and individual organism, but also for systematic biology [12, 13]. The future of T-RFLP is tightly connected to molecular biomedicine. Microbiological high throughput analysis especially in gastrointestinal and fecal flora has been proved to be useful in gastroeneterology [14]. The qualitative and quantitative analysis of oral microflora is of a great importance for the research on periodontitis and caries pathogenesis [15, 16]. The value for clinical medicine should, however, be evaluated in larger clinical studies.

3. Conclusion

T-RFLP is a high-throughput method for characterization of microbial communities. Difficulties with reproducibility and specificity of T-RFLP still narrow the wide application field. But the perspective is promising. Potential applications of T-RFLP include the detection of microbial biosensors of environmental pollution, biomining of previously unknown organisms that can be used in biotechnology, but most importantly, T-RFLP will further improve and potentially uncover new perspectives in biomedical diagnostics, especially in dental medicine and gynecology.

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