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16S RNA PCR and gene sequencing: Applications in bacteriology

Since 1985, the use of molecular biology techniques in the diagnosis of infectious disease has grown. These techniques allow for the identification of a bacterial strain in a pathological product or culture. The principle behind these techniques is the amplification of an entire gene or parts of a gene using specific primers, which can be revealed by gel or capillary electrophoresis, by hybridisation, or even by sequencing and comparison with sequence data in the data banks such as EMBL, NCVI, BiBi, and Genebank.

The polymerase chain reaction or PCR uses two main approaches:

- The targeted microorganism approach, which uses specific primers ("we know what we are looking for").
- The large spectrum approach, that targets a common gene in all the bacteria i.e. 16S rRNA ("we don't know what we are looking for"), followed by sequencing and comparison to the data banks to identify the bacteria in question.

The advantages of the molecular biology techniques in comparison to classic bacteriology techniques are the increase in sensitivity (twice as sensitive for genital Chlamydia for example), less time-consuming (the time required is halved for mycobacteria identification from culture) and/or an increase in specificity for the identification of atypical strains from culture.

The 16S gene: Why

This gene codes for the 16S sub-unit of ribosomal RNA (rRNA) and is used essentially due to its structure, which is well conserved in all bacteria. It is composed of a succession of: conserved domains, complementary sites (used for the gene's sequencing by universal primers), and other sequences which are specific to that particular group of bacteria that are known as signature sequences (species, genre, family).
The reason behind the choice of 16S RNA rather than 23S or 5S is technical (gene size, amount of information) and also due to the fact that today our sequence data banks for the 16S gene are highly developed. Finally, the identification is reliable: the gene sequencing results obtained for 16S are similar to those obtained with the entire genome.

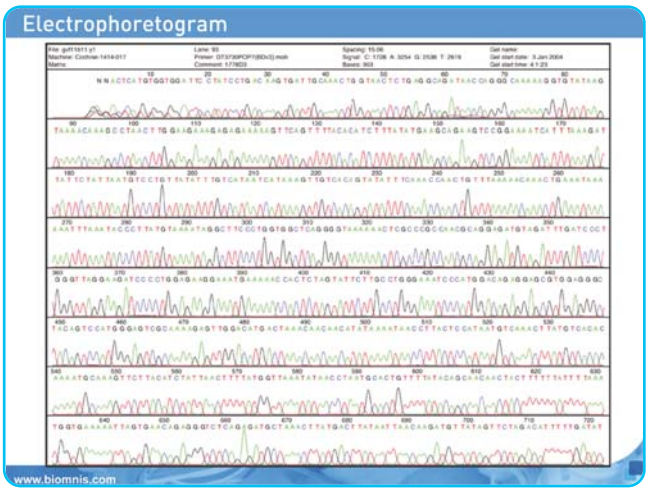
The 16S gene: Limitations

It is not possible to quantify the bacteria present as the coding genes of rRNA can be present in numerous copies. The number of copies varies in function to the size of the genome and the growth rate of the bacteria. For quantification, other genes are used that are present in the same number of copies in all the bacteria (generally one copy). Other genes are used to differentiate the similar bacterial species for which the 16S RNA is not discriminate i.e. MLSA for Neisseria meningitidis.

The PCR-sequencing technique

A universal primer is chosen that is a short nucleotide chain of 15 to 25 nucleotides, which is complementary to the known DNA sequence to be amplified and is situated up-stream of the sequencing zone. This primer is necessary for the binding of DNA polymerase. The sequencing of the DNA depends upon the sequence of nucleotides making up the DNA. The first step is the synthesis of a complementary DNA strand by the DNA polymerase (from the primer) by the adding of deoxyribonucleotide triphosphates: dNTP (dATP, dCTP, dGTP, dTTP). One peculiar phenomenon in sequencing is the use of the slightly different nucleotides, dideoxyribonucleotide triphosphates ddNTP (ddATP, ddCTP, ddGTP, ddTTP). During the reaction, the dNTPs are available in large quantity and the ddNTPs in small quantity. As soon as the DNA polymerase uses a ddNTP instead of a dNTP the strand synthesis stops. The random incorporation of ddNTP allows for the creation of variable sized DNA fragments. The integrated ddNTPs are tagged with 4 different fluorochromes. The DNA fragments obtained are of different lengths; the sequencer separates the DNA fragments according to their size using chromatography.

The current florescence detection systems detect the fluorescence from the 4 nucleotides of the same column. The result is presented in the form of an electrophoretogram. This electrophoretogram then undergoes either graphical or textual alignment i.e. the written line sequences are sent to the internet and compared to the sequences within the data banks. BLAST results can also be obtained; these are comparison results for the closest sequence matches and corresponding identifications that are provided with a homology percentage result.



Clinical microbiology significance: Bacterial identification

The PCR sequencing of 16S RNA is of significant interest thanks to the precise identification of strains that are rarely documented, rarely isolated, or phenotypically difficult to identify (i.e. few known biochemical characteristics, or unusual biochemistry profile). This technique is also of interest for the identification of the rarely documented gram negative bacillus as it is not typically detected in clinical samples, and for coryneform bacteria and gram positive bacillus for which the identification criteria is unreliable or does not provide satisfying results. In the situations previously mentioned, identification by sequencing of the 16S gene is the fastest and most reliable method.

Another benefit of significance is the identification of slow growing bacteria such as mycobacteria. Results can therefore be released more quickly after culture, avoiding the often long and not very reliable biochemistry tests.

This technique has also permitted the discovery and description of new pathogen agents and contributed to the reclassification of bacteria.

Finally the technique is useful for the identification of bacteria that cannot be cultured or are difficult to culture such as Bartonella Quintana or Coxiella burnetii. This has a clinical impact for endocardial fibroelastosis diagnosis with negative blood culture results.

Bacteria identification by 16S gene sequencing: The limitations

Sequencing of 16S RNA has a weak species resolving power for certain types of bacteria. For example the following bacteria cannot be distinguished from one another: Neisseria cinerea and N.meningitdis, or Streptococcus pneumoniae, S. mitis and S. Oralis or even the bacterial group of Campylobacter that are not jejuni nor coli (same 16S RNA). Certain species show levels of similarity of greater than 99.5% but have clear phenotypic differences (i.e. three species of Edwardsella). Ambiguous sequences have been added to certain public data banks in the past.

Recommendations for the use of 16S RNA sequencing for bacterial identification

(Drancourt et al. 2000 J. Clin. Microbiol)

Sequence at least 500 to 525bp, or ideally 1300 to 1500bp. The results are expressed in % homology: the identification criteria of the species are greater than 99% sequence homology, or ideally greater than 99.5% and a standard strain reference sequence comparison that is identified by DNA-DNA hybridisation (standard technique).

Clinical microbiology interest for bacterial detection directly from samples.

- In the case of prior antibiotic treatment hindering bacterial culture, which has been described in cases of endocardial fibroelastosis, for Streptococcus salivarius and Capnocytophaga canimorsus by 16S PCR sequencing (on patient valves already treated and in blood culture).
- In the presence of biofilm, in osteitis and osteomyelitis, the biofilm hinders successful culturing.
- In cases of endocardial fibroelastosis with a negative culture, with prior antibiotic treatment, fastidious microorganisms, or microorganisms that cannot be routinely cultured (i.e. C.burnetii, B. Quintana).16S PCR is therefore directly performed on the blood culture sample.
- Mycobacterium sp. detection in respiratory and CSF samples.
- Direct detection on biopsies for Tropheryma whipleii, Bartonella henselae.
- Direct detection in joint/bone samples.
- Environmental bacteria detection.

Non-targeted direct bacterial detection: The limitations

16S PCR is possible on samples from areas that are normally sterile, (such as biopsies, blood cultures, and aspirates) or areas of low levels of microbes. However, one of the main limiting factors if the risk of amplification of contaminants: i.e. contaminated commensal microbe reagents or contamination during the testing process (DNA extraction or PCR). In addition, the turn-around time is only longer with species specific PCR and the 16S PCR technique is more demanding: 1.5 days of work on average to obtain a sequence from a colony or directly from a sample.

Carole Emile, from a communication by Véronique Jacomo, Biomnis Paris.