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The Principles of the Nucleic Acid Probe Assay

Introduction

The deoxyribonucleic acid (DNA) molecule consists of two complementary strands of polynucleotides that are joined by specific interactions between the bases guanine and cytosine and between adenine and thymine. The strands can be separated by heat or alkali denaturation. This is a reversible process. Under suitable conditions the complementary single strands can reanneal. Specific hybrids can also be formed between single stranded DNA and complementary ribonucleic acid (RNA).

A nucleic acid probe is a fragment of a nucleic acid (mostly DNA) that binds to complementary DNA or RNA (target nucleic acid). Probes can either be isolated from the DNA of the target organisms or synthesized as oligonucleotides in the laboratory. The reactions are highly specific so that probes will bind to their complementary sequences, even if these sequences account for only a small fraction of the target nucleic acid.

Nucleic acid probe technique consists of several steps. These steps are discussed in the following section.

Design of nucleic acid probes

At least three different approaches can be used to prepare nucleic acid probes.

Randomly cloned DNA fragments

DNA fragments with unique sequences can be obtained following digestion of DNA, isolation of fragments and removal of any cross-hybridizing fragments. The remaining DNA fragments can be cloned and used as specific probes (1, 2). These probes are usually species or even strain specific. Unfortunately, nothing is known about the biological function, the genetic variability and stability of the randomly selected DNA fragments.

Genes and gene fragments

The phenotypic properties of organisms are transcripts from specific genes (e.g. enzyme activities, toxins) or require transcripts for their formation (e.g. metabolic products). The genes involved can be directly detected using specific nucleic acid probes. This approach enables the differentiation of virulent from avirulent strains. We have designed a gene specific probe by which *Staphylococcus aureus* can be differentiated from other coagulase-positive and coagulase-negative staphylococci (3).

Probes derived from 16S and 23S rRNA

Our current knowledge on the phylogenetic relatedness of bacteria is based upon comparative sequence analysis of 16S and 23S rRNA (4, 5). These rRNA molecules contain regions of highly and less conserved sequences. Therefore, it is possible to design probes with specifities ranging from species specifity to a universal probe that will hybridize with any cellular life form (6, 7).

The great advantage of these probes is demonstrated for the differentiation of pseudomonads. Pseudomonads are a phylogenetically heterogeneous group of bacteria which are often difficult to distinguish by conventional methods. Therefore, we developed rRNA-derived nucleic acid probes with which we can clearly differentiate the genuine pseudomonads from all the other so-called pseudomonads (7). Moreover, we designed a group-specific oligonucleotide which only reacted with the 23S rRNA of *P. aeruginosa* and the closely related *P. alcaligenes* and *P. pseudoalcaligenes* (8). The results are summarized in table 1.

Using rRNA as target nucleic acid also considerably increases the sensitivity of the test since rRNAs are present in very high copy numbers in growing bacterial cells. Probes directed against rRNA allow rapid classification of unknown isolates (6). As a first step, a universal probe is applied to provide a rough estimate of the amount of target nucleic acid present in the hybridization reaction. Next the hybrids between the universal probe and the target nucleic acid are denatured, the universal probe is removed and a eubacterial or archaebacterial probe applied. By repeating this procedure using increasingly specific probes it should be possible to identify unknown isolates to the genus or even species level in just a few steps.

Labelling of nucleic acid probes

The nucleic acid probe has to be labelled to identify the hybrid molecules formed with target nucleic acid. There are two principal types of labelling; direct and indirect labelling (fig. 1). In the former, radioactively labelled nucleotides are introduced into the probes or fluorescent groups or enzymes are covalently bound

Table 1. Hybridization of genomic DNA of various bacteria with a probe specific for all genuine pseudomonads (pHF 360) and an oligonucleotide probe that reacts with *P. aeruginosa* and closely related pseudomonads (P 72)

Strain	rRNA Group	Hybrid.	
		pHF 360	P 72
Pseudomonas aeruginosa (4)	I	+	+
P. alcaligenes	I	+	+
P. aureofaciens	I	+	–
P. chlororaphis	I	+	–
P. cichorii	I	+	–
P. fluorescens	I	+	–
P. mendocina	I	+	–
P. pseudoalcaligenes	I	+	+
P. putida	I	+	–
P. stutzeri	I	+	+
P. syringae	I	+	–
Enterobacter aerogenes		–	–
Escherichia coli		–	–
Xanthomonas campestris	V	–	n.d.
X. maltophilia	V	–	n.d.
Acidovorax delafieldii	III	–	n.d.
A. facilis	III	–	n.d.
Comamonas acidovorans	III	–	–
C. testosteroni	III	–	–
Hydrogenophaga palleroni	III	–	–
Pseudomonas caryophylli	II	–	–
P. cepacia	II	–	–
P. solanacearum	II	–	–
Pseudomonas diminuta	IV	–	–
Various bacteria (10)		–	–

n.d. = not determined. Number in parenthesis indicates number of strains.

to the probe. In the latter an unlabelled reporter group attached to the probe is detected by a labelled binding protein or antibody.

Radioactively labelled probes have preferably been used in the research laboratories since they show a higher sensitivity. In the meantime there are also non-radioactively labelled probes with the same sensitivity and the latter can be also used in the routine laboratory.

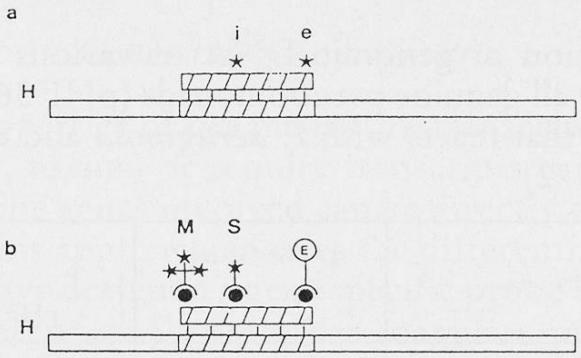

Fig. 1. Labelling of nucleic acid probes and hybrid (H) formation.
(a) Direct label; e, end label; i, internal label;
(b) Indirect label. A hapten or reporter group (black dot) is covalently linked to the probe. Binding proteins are labelled with an enzyme (E), a single (S) label or multiple (M) labels

Sample preparation

Small amounts of target nucleic acids are sufficient for hybridization assays with specific probes. An extensive purification of the target nucleic acid is not necessary. Cell lysis is the first step followed by the extraction of nucleic acids. Gram-negative bacteria are readily lysed with detergent (sodium dodecylsulfate), alkali (only for DNA preparation) or a combination of both. For preparation of RNA, RNase inhibitors such as guanidinium thiocyanate should be added. In general, the lysis of Gram-positive bacteria is more difficult. Pretreatment of cells with acetone and addition of cell wall lytic enzymes to the detergent can facilitate lysis of cells. If sufficient lysis cannot be achieved by using such techniques, physical methods have to be applied such as sonication or shaking in a cell mill.

For in situ cell hybridization the nucleic acid must be released from cells grown on a solid support or the cells must be made permeable for the probe to react with the target nucleic acid within the cell. In the case of colony hybridization nucleic acid is transferred onto a filter membrane (nylon, nitrocellulose or teflon membranes), whereas during the hybridization of fixed single cells the solid support is formed by the biological sample itself.

Colony hybridization is a convenient, rapid, and simple method for testing, in a few steps, mixed cultures (7). Gram-negative cells can be lysed on filters using simple alkali treatment (9) whereas Gram-positive bacteria require an additional heating step (10).

Fixed whole cells of Gram-negative bacteria are permeable to oligonucleotide probes and specific hybridization to rRNA takes place in situ. Permeabilization of Gram-positive bacteria is more difficult and requires additional treatment with cell wall lytic enzymes. Recent work has shown that it is possible to detect and identify single cells using fluorescently labelled, rRNA targeted oligonucleotide probes (11, 12). Flow cytometry can be used to resolve individual target and non-target bacteria hybridized in solution with fluorescently labelled oligonucleotide probes (13).

Hybridization methods and detection

The association and dissociation of complementary nucleic acid strands are determined by physicochemical parameters such as temperature, ionic strength of the milieu, denaturation agents, base composition and chain length of the strands and number of mismatches.

The specific hybridization of probes involves two distinct steps, the binding (hybridization) of the probe to the potential target and the discrimination between specific hybrids and non-specifically bound or unbound probe (washing). In both processes the specificity can be controlled by changing the stringency of the conditions applied.

There are two different formats for the hybridization; solid phase and solution hybridization. In the solid phase hybridization target nucleic acid is denatured and immobilized on filter membranes. The labelled probe is added and hybridization carried out under predetermined conditions (fig. 2). Unbound or non-specifically bound probe is removed by washing the membrane. Target nucleic acids can be applied directly to the solid support by spotting aliquots of the nucleic acids as so-called dot-blots onto the membrane. Using this technique, nucleic acids from pure and mixed cultures can be analyzed qualitatively for the presence of specific target sequences. Autoradiograms or coloured hybridization products can be easily evaluated using a densitometer (fig. 3).

Fig. 2. Scheme of filter hybridization. H, hybrid; M, membrane; P, probe (asterisks indicate label); I, immobilized target nucleic acid

Hybridization in solution has some advantages over the membrane techniques. The target is not fixed and is completely accessible for the probe making the rate of hybridization considerably higher. Disadvantages are the possible self-reassociation of target DNA and the some-what more complicated separation of probe-target hybrids from unbound probe.

The hybridization products can be removed from the solution in various ways. One possibility is the capture probe technique (fig. 4). This technique requires a capture probe (C) and the labelled detector (reporter) probe (D). Both probes hybridize to the same target nucleic acid. The capture probe immobilizes the hybridization products by binding them to the surface of tubes, plastic sticks or magnetic beads. Unhybridized detector probe and non-specifically bound contaminations are removed by washing (14).

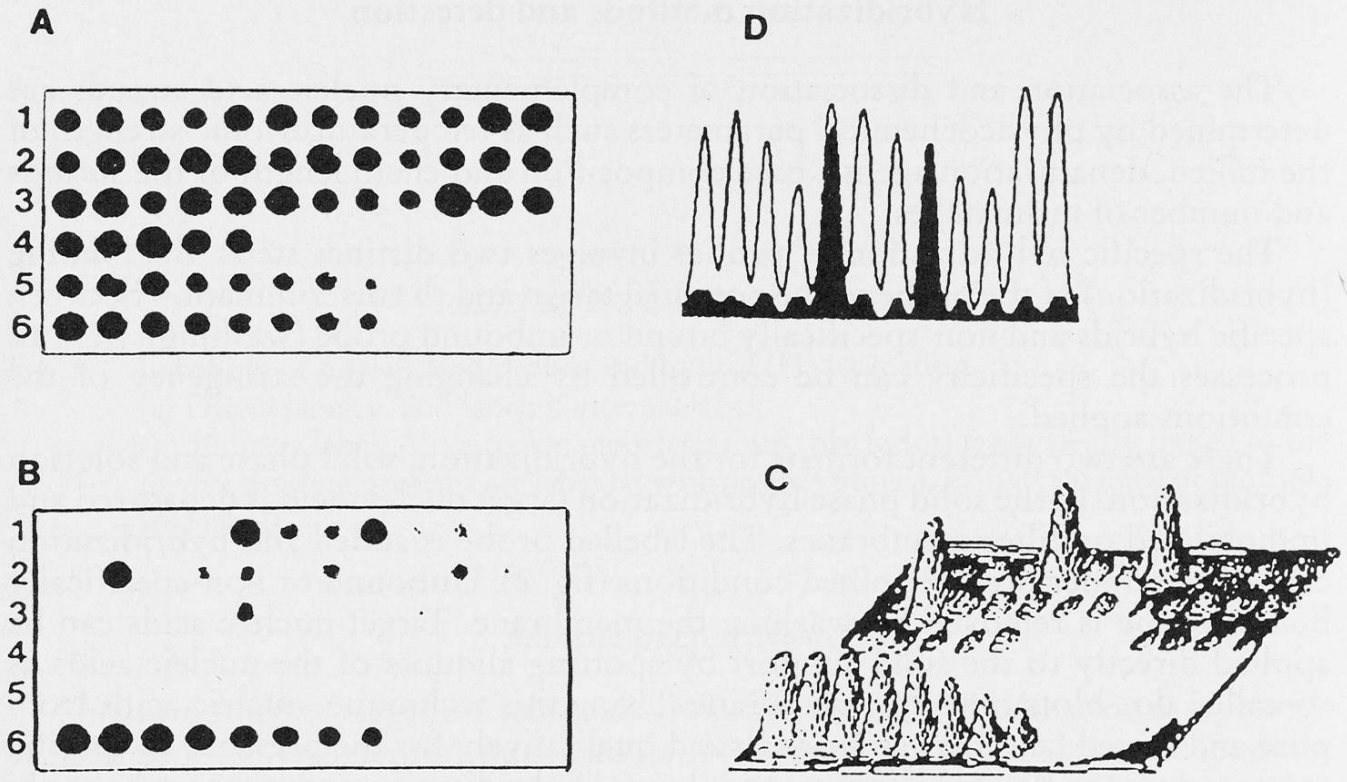

Fig. 3. Dot blot hybridization of DNAs of various eubacteria to ^{32}P-labelled DNA probes derived from 23S rRNA. The same filter was hybridized first with a universal probe (A) and after removing the universal probe with a group specific probe (B) which reacts with *Bacillus subtilis* and closely related bacilli.

(A) Universal probe. Lanes 1 to 4 DNAs from various eubacteria. Lane 5, serial dilutions of DNA from *Bacillus globisporus*. Lane 6, serial dilutions of DNA from *B. subtilis*

(B) Group specific probe reacting with *B. subtilis, B. licheniformis* and *B. pumilus*

(C) Densitometric scan of panel B

(D) Overlay of densitometric scans of lanes 1 of panel A (white) and panel B (black)

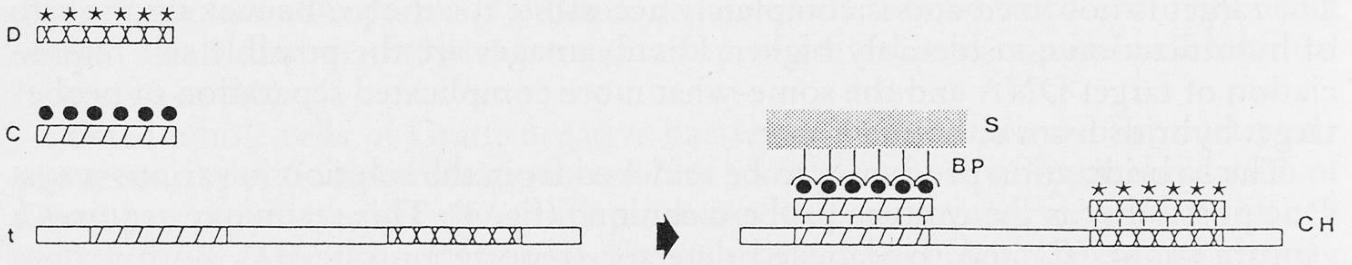

Fig. 4. Capture probe technique (hybridization in solution). BP, binding protein; C, unlabelled capture probe; CH, captured hyrid consisting of capture probe and detector probe hybridized to target nucleic acid (+); D, labelled detector (reporter) group; S, solid support to which the binding protein is linked

In vitro amplification of target nucleic acids

The low sensitivity of hybridization assays does currently not allow to detect less than 10^4 bacteria with the usual test systems. This obstacle can be overcome using in vitro amplification techniques such as the polymerase chain reaction (PCR).

PCR requires a set of two short oligonucleotides (primers) flanking the sequence to be amplified. One primer is complementary to the (+) strand, the other complementary to the (–) strand (fig. 5). The primers anneal specifically to denatured DNA and after addition of DNA polymerase, buffer and deoxynucleotides, new strand fragments are synthesized starting from the primers. After denaturation the old and newly synthesized DNA can serve as template for more synthesis. Each cycle of denaturation, annealing, and extension results in an approximate doubling of target molecules; 25 to 30 cycles routinely amplify the target by 10^5 to 10^7 fold. Thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (Taq polymerase) allows the primer annealing and extension to be performed at high temperature, thereby assuring specific primer attachment and less secondary structure in the template. DNA as well as RNA sequences may be amplified by PCR. The latter requires the synthesis of cDNA using reverse transcriptase prior to the PCR reaction.

PCR adds a new dimension to the detection of specific nucleotide sequences. One attomol of target molecules can be amplified in 30 cycles to an amount of DNA that can be detected by agarose gel electrophoresis (> 0.1 µg). The combination of PCR amplification of targets and subsequent detection by hybridization with sequence specific oligonucleotides has already produced stunning results. Recent reports demonstrated the specific detection of less than 100 cells of several other species. By the PCR method, even a single bacterium can be detected (16).

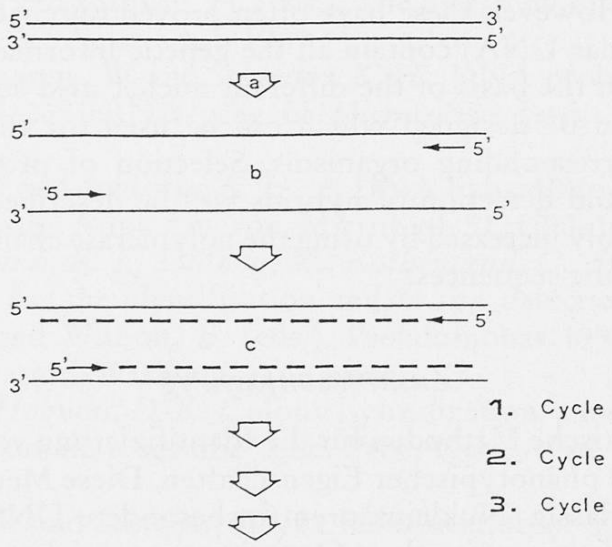

Fig. 5. In vitro amplification of DNA by the polymerase chain reaction technique; a, denaturation of DNA; b, hybridization (annealing) of primers; c, synthesis of new DNA strands

Conclusion

Nucleic acid probes offer a fast and specific method for identifying bacteria at different taxonomic levels. It is not necessary to perform time-consuming metabolic tests or to isolate the organism in pure culture. In situ colony or single cell hybridizations permit the analysis of mixed cultures. Probes are especially useful for the identification of slow-growing and fastidious bacteria. They are also ideal for detecting the presence of a particular gene (e.g. coding for a toxin) in a mixture of organisms.

However, nucleic acid probes cannot only be used for the specific detection and identification of bacteria but also for crop and livestock disease diagnosis, in particular for the detection and identification of viruses and viroids. They are also applied to select the most suitable progeny for further crossing and propagation in plant and animal breeding and they have also been used for embryo selection or sex determination in cattle embryos. DNA probe based methods have also been applied for food related authenticity testing. It is possible to distinguish meat from chicken, pig or ruminants in commercially canned products by a DNA probe test using as little as 50 mg of the product. Moreover, individual cultivars of, for example, rice and barley can be identified by probe based methods. For barley, it is possible to distinguish, even in the malted state, between varieties which have different brewing qualities. Probe based typing of yeasts is rather important for the wine, beer and baking industries. The advantage of being able to use the same basic technique to tackle diverse problems makes it likely that the nucleic acid probe based assays will find a wide application in food science and industry.

Summary

Conventional diagnostic methods for identifying microorganisms rely on differences in phenotypic properties. However, these have often proved unreliable and time-consuming. Nucleic acids (in particular DNA) contain all the genetic information which characterizes each living organism. On the basis of the different nucleic acid sequences complementary hybridization probes can be designed which can be used for the specific detection and identification of the corresponding organisms. Selection of probe, sample preparation, hybridization methods and detection of hybrids will be described. The sensitivity of the method can be considerably increased by using the polymerase chain reaction for the in vitro amplification of the specific sequences.

Zusammenfassung

Traditionelle diagnostische Methoden für die Identifizierung von Mikroorganismen beruhen auf dem Nachweis phänotypischer Eigenschaften. Diese Methoden sind sehr zeitaufwendig und oft unzuverlässig. Nukleinsäuren (insbesondere DNS) enthalten die gesamte genetische Information, die einen einzelnen Organismus auszeichnen. Auf der Grundlage der verschiedenen Nukleinsäuresequenzen können komplementäre Hybridisierungssonden entworfen werden, die sich zum Nachweis und der Identifizierung der entsprechenden Organismen eignen. Die Auswahl der Sonden, die Herstellung der Proben, die Hybridisierungs-

methoden und der Nachweis der Hybride werden beschrieben. Die Empfindlichkeit der Methode kann durch In-vitro-Amplifikation der spezifischen Sequenzen mit Hilfe der Polymerase-Kettenreaktion beträchtlich erhöht werden.

Résumé

La détermination des propriétés phénotypiques est à la base des méthodes diagnostiques classiques pour l'identification de microorganismes. Or, ces méthodes ne sont souvent pas fiables et il faut beaucoup de temps pour leur exécution. Les acides nucléiques, la DNS en particulier, contiennent toute l'information génétique qui caractérise chaque organisme vivant. Sur la base des différentes séquences d'acides nucléiques, des sondes d'hybridation complémentaires peuvent être définies et appliquées lors de la détection et identification des organismes correspondants. La présente publication décrit la sélection des sondes, la préparation des échantillons, les méthodes d'hybridation et la détermination d'hybrides. Il est possible d'augmenter considérablement la sensibilité de la méthode par une amplification in vitro des séquences spécifiques à l'aide de la polymérase, une réaction en chaîne.

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