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DNA probes for the identification of microorganisms

Frank Gannon

The National Diagnostics Centre, University College, Galway, Ireland

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SUMMARY

The detection and identification of microorganisms is being carried out increasingly using DNA. Each organism has a unique DNA sequence which can be used to distinguish closely related organisms. Using PCR amplification and sequencing of ribosomal RNA genes we have developed DNA probes for a number of pathogenic bacteria and fungi. The development of DNA assays based on PCR has resulted in new questions which must be addressed including process carry-over contamination and inhibition of the PCR amplification reaction once the problems associated with the implementation of DNA assays are ironed out.

INTRODUCTION

One of the traits of humans is their need to communicate with each other. Although images are increasingly important, language remains the principal means of letting our colleagues know what we are thinking or experiencing. In some spheres of activity, adjectives are more important than nouns, but for scientists the words used must carry with them precise messages. In microbiology, where the objects of interest need magnification before visualization, it is particularly true. It follows that the words chosen to describe and indeed define a microorganism must have an international and specific meaning. It is clear therefore that one of the underpinning activities of microbiologists is the age-old skill of describing the microorganisms under study. In today's revolutionary biotechnological world, the contexts in which these basic microbiological skills are required are diverse. The most obvious role is in the identification of microorganisms which may be pathogens or cause public health concern. Clinicians or veterinary practitioners must know what organism is causing illness or disease to guide them in their choice of treatment. Such information is also required to prevent public health problems, for example by ensuring that the food we eat is free of organisms known to cause problems. Apart from the actual illness that can be caused, it is important to ensure that some organisms which can cause food spoilage are absent to avoid economic loss. In agriculture, the negative impact of some fungi or bacteria on crops again requires that they be identified and appropriate treatments initiated. Apart from those selected examples however, the knowledge of the characteristics that distinguish different microorganisms reflects an understanding of the biochemical processes which give rise to them. These can be very important features of industrial processes and the synergy of knowledge which derives from such a body of information is a further justification for the collection of data on diverse microorganisms. When a new organism is described, its relatedness to others can be a guide to the possible useful traits that it will have. For all of these reasons therefore, it is clear that the task of identifying microorganisms is one of prime and continuing importance.

Correspondence to: F. Gannon, The National Diagnostics Centre, University College, Galway, Ireland.

APPROACHES TO MICROBIAL IDENTIFICATION

The methods that are used to identify microorganisms are multiple and are the foundations on which the discipline of microbiology rests. The most obvious of these is observation of the growth of the organisms. A preliminary and informed identification of many microorganisms is possible from information on the source of the sample, the media requirements, the aerobic status of the culture, the growth or inhibition of the organism on selective media and the size, shape or color of the colonies. More information is obtained by using a microscope in conjunction with different stains to establish if the organism is Gram-positive or -negative, if it contains flagella and their distribution, its shape, its motility and whether it contains a spore. A range of biochemical tests can usually complete the process of identification, with information on the enzymatic activities and metabolic characteristics of the organism. Some of these will already have been indicated from the culture studies where a profile of carbon compound utilization may be established. Recently, a further refinement of the identification process has been the development of fatty acid profiles for microorganisms. These vary for each species and a comparison of the profile of an unknown organism with those in a data base can provide a rapid identification of the microorganism. Another rapid method is based on the

use of phage which are specific to a particular organism. The range of these are expanding and they are being tagged with marker genes which allow the rapid identification of microorganisms that they successfully infect [12].

DNA IN MICROBIAL IDENTIFICATION

Given the above array of methods with a proven ability to discriminate between and define different microorganisms it may seem excessive to present the advantages of a new method i.e. the use of DNA to identify microorganisms. In some instances this development is indeed unnecessary but, as will be indicated below, there are many situations where DNA-based identification methods are the preferred, if not the only, choice.

At a philosophical level, it is easy to argue that any identification process based on the fundamental genetic material, (the ultimate source of all of the biochemical activities which by complex interplay become a microorganism) must be a superior process. At a practical level it may be an unduly sophisticated way to achieve that goal. The realities of application are that ease of the test performance, cost, speed, specificity and sensitivity will determine which test type will be used. Notwithstanding that, the technological explosion that occurred with the fusion of disparate techniques into what is now known as genetic engineering has allowed ready access to the DNA sequence of any gene from any organism of interest. The explosion of information that results opens up new possibilities for the microbiologists. These operate at different levels and are being utilized in a variety of ways. For example: DNA sequence can be the basis for diagnostic tests designed to show the presence of a given organism in a sample. Alternatively, a DNA sequence can be obtained from a novel organism with a view to classifying it by comparing this sequence with that of other known microorganisms. DNA sequences can be designed to develop species- or genus-specific tests. Finally, minor variations in DNA sequence can be used as fingerprints to follow a particular strain of a microorganism in an epidemiological study. By analogy with the process of changing the magnification used when examining a sample under a microscope, it can be considered that the lowest magnification corresponds to the study of culture, physical and biochemical characteristics. The next order of magnification will provide adequate DNA sequence data to allow the organism to be placed in a taxonomic scheme. Further analysis of the data will identify differences in the sequences, which will allow two similar organisms (e.g. different species from the same genus) to be distinguished, while the highest magnification detects the occasional random and anonymous mutation that allows two different strains of the same species of organisms to be distinguished. Viewed in this way, it is clear that DNA-based methods are much more than a complement to the more classical identification procedure.

Targets for DNA analysis

Just as it is impractical to measure every enzyme in a microorganism, it is equally impossible to determine the several million bases of DNA sequence before deciding on the identity of a microbial isolate. Instead, smaller regions of DNA are targeted for such analysis and these provide a good level of confidence for any extrapolations that are made as to the relatedness of the organism to others previously described. But which regions should be analyzed? Here it is useful to distinguish between the two operations of DNA sequence generation for identification of an unknown organism and DNA sequence generation for the purpose of detection of a known organism. In the former case the target for sequence analysis must correspond to one for which there is a well established data base. In the latter instance this is not a prerequisite. However, as will be seen below, the choice of DNA region for both purposes is frequently the same locus, i.e. the ribosomal RNA gene region.

Ribosomal RNA (of sizes 16S and 23S in bacteria) are universal and essential components of the ribosome, which of course is required by all organisms for the translation of RNA into protein. The ribosomal RNA is a complex entity with many proteins which bind to each other and to strands of ribosomal RNA (rRNA) in a very ornate architectural structure. Analysis of the rRNA sequences from some microorganisms indicated over twenty years ago that parts of the ribosomal RNA are constant and unchanged among different genera, whereas other parts can vary significantly. When the RNA molecules are presented in the secondary form they could take, the variable regions tended to be in loops and the constant regions in double stranded portions. Any alteration to the constant region would disrupt such bonds and also interfere with the interaction which may be sequence-specific, between the ribosomal proteins and the rRNA. It was soon recognized that analysis of the sequence of variable regions of the rRNA would be ideal for both the establishment for the relatedness of organisms to each other and the development of diagnostic tools [6,7,9,14]. Initially the DNA sequence was obtained by using ribosomal RNA as template for reverse transcriptase. Now it is more customary to obtain the sequence from the DNA in the ribosomal RNA gene. Faced with the prospect of an extensive gene isolation program in the context of DNA probe generation for many unrelated organisms, the emerging Polymerase Chain Reaction (PCR) approach was used in my laboratory to amplify variable regions of the 16S rRNA by selecting consensus primers which were derived from the known sequence of the constant region of other microorganisms. The approach was first illustrated by obtaining DNA sequence and ultimately a probe for the fish pathogen *Aeromonas salmonicida* [3]. Subsequently we, and others, have shown the generality of the method of deriving DNA probes in a logical manner by developing a small database of sequence for the variable region of a group of closely related organisms. However it was not always possible to distinguish between closely related species and indeed

genera in this way. Sometimes the 'variable' region is identical between closely related but distinct organisms.

Reflection on the fact that the variation in the rRNA sequences implied a limited functional role (other than one which is topographical) suggested that a more extensive degree of variability would come from an intergenic region on the DNA. This could of course be anywhere in the genome but it was particularly attractive to target the ribosomal gene cluster. Here the 16S RNA and 23S RNA genes are found adjacent to each other. As the available data banks included sequence for both of these molecules from many species it was again possible to derive primers for PCR which came from the 3′ end of the 16S rRNA and the 5′ end of the 23S rRNA genes. When DNA from different microorganisms was amplified using these primers, not only were there differences in sequence, there were great differences in the sizes of the DNA bands which were amplified. The implicit variability of this intergenic or spacer region was confirmed initially by the generation of DNA probes for Clostridia species and *C. perfringens* in particular [1]. Subsequently this method has been used to develop DNA probes for a number of bacteria including *Mycobacterium bovis* [2].

But the usefulness of the rRNA gene locus is not restricted to bacteria. Recently, in collaboration with other European laboratories we have developed identification procedures for a wide variety of fungi based on either the 18S rRNA (the 16S equivalent in fungi) or the intergenic (ITS) region. These probes are useful for the detection of species of *Aspergillus, Mucorales, Penicillium, Candida, Fusarium, Rhizoctonia, Trichoderma* and *Ustilago* (Dawson, M., Frisvad, J., Rossen, L., Rubio, V., Moens, W., Skouboe, P. and Gannon, F., unpublished results). It is reassuring, but not surprising, that the classical methods of classification find confirmation in the relatedness of the DNA sequences. These studies will provide new tools for the identification of fungi. More classical methods require time, specialist skills and, even then, are not always possible. As a result the fungi are grossly understudied despite their major roles as human pathogens, in agriculture and in the food industry (see Table 1).

TABLE 1

Organism	Disease/uses
Argronomically important fungi etc.	
Septoria sp.	Eyespot
Rhizoctonia sp.	General crop disease
Trichoderma sp.	Spoilage, biocontrol agents
Armillaria sp.	Tree rot
Verticillium sp.	Wilt diseases, glass houses, biocontrol entomopathogenic
Fusarium sp.	Cereals and seedlings, olives, vegetables
Penicillium	Spoilage of food and feed
Ichtyophonus	Farmed rainbow trout
Nectria haematococca	Pea pathogen detoxifies pea antibiotic
Ustilago maydis	Siderophore production Scavenges iron. Corn smut
Phytophtora infestans	Plant pathogen. Potato
Phanerochaete chrysosporium	White rot lignin degradation
Human fungal pathogens	
Aspergillus fumigatus	Aspergillosis in AIDS patients Mycotoxin production
Aspergillus niger	Aspergillosis in AIDS patients
Pneumocystis carinii	Immunocompromised patients
Candida albicans	Immunocompromised patients Candidemia
Candida glabrata	Dental, urinary tract, vaginal infections
Candida kruseii	Opportunistic mycoses
Cryptococcus neoformans	Dermatomycosis AIDS patients
Coccidioides immitis	Respiratory infection coccidioidomycosis
Fungi in the food industry	
Phaffia rhodozyma	Astoxanthan food color
Aspergillus oryzae	Food production Asia
Aspergillus sp.	Hydrolytic enzymes fermentation Hosts for heterologous protein secretion
Trichoderma sp.	Hydrolytic enzymes fermentation
Penicillium sp.	Starter cultures. Cheese industry

Examples of the uses of DNA probes for the identification of bacteria

The use of the rRNA region is the best example of the targeting of a gene locus as the source of DNA probes or data for species classification. As the data banks become more extensive a new sequence from this region can be used with increasing confidence to identify a novel organism. A recent example will illustrate this fact. A fish disease with a new pathology emerged on an Atlantic salmon farm on the west coast of Ireland. Standard microbiological assays identified the organism isolated from these fish as either a *Neisseriacrae, Pasteurellaceae*, C.F.B. related bacterium (*Bacteroidse, Flavobacterium* or *Cytophaga*) or *Fusobacterium*. The refinement of the identification came from the sequencing of the 16S rRNA gene followed by homology alignments. In this way many of the previous possibilities were excluded and the organism was shown to be a *Fusobacterium* species (Maher, M., Smith, T., Palmer, R. and Gannon, F., unpublished). In this example, the DNA sequence was obtained to allow the organism to be characterized, but with the availability of the DNA sequence a beneficial consequence is the fact that DNA probes and specific primers for PCR are also generated.

The deep ocean provides another example of the use of DNA sequence for the identification of microorganisms. Colleagues at University College Galway, R. Powell and J. Patching, have recently analyzed the gut content of the sea cucumber by PCR using DNA primers derived from the ribosomal RNA. When the amplified material was sequenced and analyzed, it was found that archaebacteria related to

thermophilic species were present. While, as yet, there are no reports of the culture of these archaebacteria, this molecular approach has shown the presence of these organisms throughout the oceans [5].

This example reminds us of the great ignorance that we have about the microorganisms that abound in the universe. If organisms cannot be cultured, even though they are viable, it is probable that they have escaped analysis and in some cases detection. DNA-based methods can overcome this and projects are underway in laboratories around the world to take a gram of soil, a milliliter of water or other samples for analysis. This will involve the preparation of total DNA, amplification of a ribosomal RNA gene, subcloning and sequencing of the resulting fragments and analysis of the outcome for complexity, identity of microorganisms, their relative amounts and variations in their levels which arise in response to environmental or other challenges. In this way a new body of knowledge and an insight into relatively unexplored biological diversity present at the microbial level will be obtained.

Other targets for DNA probes

In the examples cited above, the ribosomal genes that were analyzed were preselected because of their known variable regions. Other genes could be targeted if one is attempting to design a DNA probe specific for the organism. Typically this target gene will encode an activity which is known to be distinctive for that organism. Examples would include some of the antigens of *Mycobacteria* [15], the hemolysin gene of *Listeria monocytogenes* [13] or the A layer protein of *A. salmonicida* [8]. Obviously, the sequence for any of these genes, while being a promising source of species-specific probe, may not obligatorily be unique to that organism. For example, the gene may be present but inactive in related organisms. This possibility is quickly excluded by hybridizing the probe to the DNA from a range of related organisms.

Anonymous DNA probe targets

Although it is comforting to know the name of the gene from which the DNA probe has been derived, it is not necessary to plan to isolate a specific target gene. The requirement for a DNA probe is that it should contain a sequence unique to the organism of interest. This does not imply a need for knowledge of the function (if any) of the gene. When there is not a satisfactory target, the most frequently used device to obtain the DNA probe is by the procedure known as differential hybridization. When the steps outlined in Table 2 are followed, a colony that includes a fragment of DNA which apparently is present in the organism of interest but absent from the most closely related organism is isolated. However, to prove that this is the case and that the result is not due, for example, to the reiteration of a sequence in one organism as compared to the other, the putative probe is isolated and used to screen a larger panel of organisms. A functional weakness of the approach is that multicopy genes, genes with closely related sequences or plasmid DNA (which is frequently multicopy) will be preferentially selected by this procedure. As a given plasmid is not necessarily present in all isolates of an organism, it is necessary to extend the characterization of the probe to include as many isolates from as many different locations as is possible—and even then the possibility of a loss of the plasmid in some environments is not excluded.

TABLE 2

Steps in the preparation of anonymous sequence of probes by differential hybridization

1.	Prepare a DNA library for organism A
2.	Screen the library in parallel with probes from total DNA of organism A and closely related organism B
3.	Select for further analysis colonies which hybridize with probe from organism A but *not* from organism B

Although, as indicated above, the ribosomal RNA region had yielded a DNA probe for *A. salmonicida* [3], the difference in the sequence of this from *A. hydrophila* was merely two nucleotides and was therefore very sensitive to minor variations in temperature during hybridization and washing. To obtain a more robust assay for *A. salmonicida*, the differential hybridization approach was used [11]. The resulting DNA probe has subsequently been used successfully by R. Powell and his colleagues to identify the pathogen in the environment and as a practical tool to assist decisions by fish farm management (Powell, R. and Smith, P., unpublished data).

DNA probe based assays

As can be seen from the above, the problem of obtaining a DNA sequence which can be used as a probe can be resolved by either searching for a target gene or one that is anonymous. Today it is correct to say that it is possible to get a DNA probe for any organism of interest. The coupling of PCR amplification with DNA sequencing as outlined above [1,3] has been particularly effective in this context. However, the availability of a probe is not the end of the work that is needed to provide a useful diagnostic system. Four further aspects, namely: sample preparation, amplification, hybridization and signal development must be considered and optimized.

Aspects of sample preparation of importance are indicated in Table 3(a). The way in which the DNA is made available for probing will depend on the origin and number of organisms in the sample and on the context in which the test is performed. A specialist laboratory seeking to identify a rarely occurring microorganism in a sample will be able to use different methods from those appropriate to a high through-put service laboratory. Similarly the choice of whether or not to amplify the target DNA and the consequences of this will be informed by the points outlined in Table 3(b). Of particular importance here is the fact that DNA probes detect DNA irrespective of whether the

TABLE 3

Points to consider

(a) Sample preparation
- Nature of sample
- Expected number of organisms
- Possibility of contamination generation
- Cost
- Throughput
- Skill needs
- Safety

(b) Amplification
- Sensitivity level needed
- PCR, LCR, 3SR or other system
- DNA sequence required
- Specificity
- Direct costs
- Royalty/Licence costs
- Positive and negative controls
- Optimization of reaction
- Quantification
- DNA contamination avoidance
- 'Clinical' relevance
- Dead or alive?

(c) Hybridization
- Denaturation
- Temperature
- Time
- Washing conditions

(d) Signal development
- Radioactive?
- Alternative non-radioactive probes
- Membrane bound
  - Southern
  - Dot blot
- Microtiter plate
- Bead assays
- Avoidance of non-specific hybridization

organism is dead or alive. The clinical or practical importance of a positive result therefore must be very carefully considered, particularly when the powerful amplification techniques are used in conjunction with DNA probes to detect very small numbers of the organism in biological, food or field samples.

The choices related to hybridization and washing conditions (Table 3(c)) are essentially defined by the sequence and length of the probe, but there are a number of options in terms of the way in which a positive signal is detected (Table 3(d)). Currently the most common end point is the detection by autoradiography of a radioactive probe bound to the target DNA which had been retained on a membrane. By common consensus, this approach, while acceptable for a research laboratory in the short term, has no future in an industrial or clinical context. The direction that most are taking is towards assay systems similar to those used by the immunodiagnostic sector. By succeeding in the conversion of DNA probes assays to a microtiter-based, enzyme-amplified signal, the comfort of familiarity and the savings in terms of new equipment required would accelerate the uptake of the DNA assays for routine use.

NEW QUESTIONS

With the universal acceptance now that DNA has a major and ancillary role in the detection and identification of microorganisms it is seductive for those of us active in the area to overlook some of the flaws in the practice of a blind reliance on this approach. As indicated above, and to restate the obvious, the detection of a positive DNA signal does not equate with a positively pathogenic organism. We have indicated that it is possible to detect the organism that causes bovine T.B. in the blood of infected cattle [3]. Equally we (Glennon, M., Smith, T., Noone, D. and Gannon, F., unpublished) and others [4,10] have shown that *M. tuberculosis* can be detected in clinical samples including sputum and BALF (broncho alveolar lavage). However, on more extensive analysis we find that in an effort to increase the sensitivity of the method we eventually reach a situation where the background or randomly positive samples obscure the analysis. In an ideal system, the DNA amplification systems will not amplify DNA that is not in the sample under test. But in the reality of clinical samples, the possibility of carry-over contamination from a previous amplification experiment or the presence of the microorganisms in the micronuclei generated in the clinical environment when the BALF or sputum sample is provided cannot be ignored. The incorporation of Uridine into amplified DNA and the use of uracil N glycosylase (UNG) in the pretreatment of samples can be very effective to reduce carry-over contamination from previous PCR reactions but its successful use presumes that the enzyme which degrades these UNG-containing products is efficient in all situations including the imperfect environment of all clinical samples. Absolute proof of this can be tedious if not impossible.

Even if contamination is not a problem, the difficulties of achieving equivalent amplification of limiting amounts of DNA in every sample are enormous. If the efficiency varies, then the validity of the end-point of the analysis is under question. Because of our work on trying to convert specialist laboratory assays to those which will have practical and widespread use we have become concerned with the lack of standards that are being applied to results that are reported. Is a single positive sample adequate, or should duplicates (or triplicates) be required? Is a DNA band detected by a radioactive probe following a long exposure autoradiography deemed to be positive, or should the end-point be a visible DNA band on a gel? Is a sample considered to be PCRable if an endogenous genomic DNA fragment, which is usually present at high copy numbers, is amplified, or should the test for the amplification be based on the detection of an exogenous (and usually much more pure than the natural sample) fragment added at a 'correct' level to the sample.

The need for standardization of these emerging tests is obvious. Equally, it is clear that methods are required to distinguish between live and dead organisms. The approach

to do this may lie in the use of RNA (which may be more ephemeral than DNA and therefore be degraded in 'dead' samples) to detect live organisms. However this does not constitute a proof of death (as opposed to viable but not growing) and introduces a new degree of complexity in the delivery of the test.

Although the above comments stem from concerns that have arisen during our studies of human T.B., they are even more valid when one considers the food industry. The starting material frequently or inevitably contains viable organisms which cannot be tolerated in the final product. The range of treatments applied to the food reduce or eliminate this risk. However, the *Salmonella enteritidis, Listeria monocytogenes* or *Staphylococcus aureus* DNA will still be present. A positive DNA signal is not useful as a management tool in that case but could be very damaging if the meaning of the test is not understood.

Finally, when standards are established and the real pathogen distinguished from the pasteurized, or neutralized organism, the question of the meaning, in practical terms, of a positive result remains. In some tests a single fragment of target DNA can give a glowingly positive and genuine signal. But is that one organism a danger to the host in which it is found or the food sample in which it has been detected? A new calibration of signal-to-effect is required and a greater quantitative aspect to DNA probe tests must be developed.

CONCLUSION

In the previous section, some of the concerns facing those active in DNA probe development are presented. These concerns should not, however, blind us to the wonderful opportunities which come with this new methodology. Rapid, efficient, sensitive, specific and ultimately cheap assays based on DNA probes will become the gold standard for the detection of many microorganisms. An interplay between these and the culture of microorganisms will be required for a period before fully valid DNA tests are available. This transition phase will also help to reassure the more classical microbiologists and those in regulatory bodies faced with the availability of a new diagnostic system.

Less controversial will be the use of DNA sequences to *characterize* microorganisms, including fungi. A flood of new sequence data is coming from this work. Happily, the corresponding analytic tools have been developed at the same time. The steps from the availability of a microorganism through to the placing of its sequence into a computer-driven taxonomic context has been completely streamlined. When this is coupled with the absence of the need to grow the organism, it is a strong prediction that a greatly expanded world of microorganisms will be revealed. This will of course be of scientific interest, but in the context of this paper the new question may be: what will industrial microbiologists be able to do with this potential?

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