Application of nucleic acid-based technologies to the diagnosis and detection of disease

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Introduction

Before 1988, hybridization with nucleic acid probes was the only available nucleic acid-based technique for the detection and diagnosis of infectious agents. Since 1988, the widespread availability of the polymerase chain reaction (PCR) has offered a rapid and sensitive alternative. Has PCR completely superseded probing technology or is there room for both methods in today’s diagnostics? This article discusses the relative advantages and disadvantages of both methods and argues that there is still a role for both in the detection and diagnosis of disease.

In order to allow a discussion in depth of the technologies, a basic knowledge of the principles of nucleic acid hybridization and of PCR will be assumed. Those wishing to refresh their memories of these techniques can find excellent books on the subject (KELLER & MANAK, 1989; KRIJCKA, 1992; PERSING et al., 1993).

Application of nucleic acid probes

Fragments of deoxyribonucleic acid (DNA) have been cloned and sequenced from almost every conceivable human pathogen. By carefully selecting those fragments which have a species-specific sequence it is possible to generate specific probes for any human pathogen. However, specificity is not the only desirable attribute of a useful probe; they must also be sensitive, especially when used for patient diagnosis when the infectious agent is dispersed in the infected tissue. Most sensitive probes have been developed by targeting either multicycopy sequences such as ribosomal ribonucleic acid (RNA) sequences or genomic or extra-chromosomal repeats. For example, probes have been developed against the ribosomal RNAs of malaria, against the extra-chromosomal kinetoplast DNAs of Leishmania, and against the genomic repeats of both Leishmania and malaria (BARKER, 1989). Although when used at optimum efficiency on cultured material such probes can recognize as few as 100 parasites in infected material, the sensitivity is probably at least ten-fold lower.

In the late 1980s there was much optimism at the potential of DNA probes. This is reflected in the rise in number of publications on the subject throughout 1987-1989. However, very few of these numerous publications resulted in applications for general use and no DNA probe is in widespread use in the general diagnostic laboratory. Many commercially available DNA probe kits are not for direct detection in infected tissue but are intended for use with cultured organisms as an aid to a more rapid, or definitive culture confirmation. There are exceptions to this, for example chemiluminescent solution hybridization tests are commercially available for gonorrhoea and chlamydia and for some viral infections including hepatitis B, human papillomavirus and human immunodeficiency virus type 1. The success of the probes for chlamydia and gonorrhoea can be explained in part by the low sensitivity of culture, which is the current ‘gold standard’. Despite these exceptions, the interest in DNA probes for diagnosis or detection of disease has shown signs of waning.

The main stumbling block was once perceived to be the reliance on radioactive labelling of the probes for maximum sensitivity. The radioisotope commonly used, $^{32}$P, has a short half-life and cannot be incorporated into kits with a reasonable shelf life. Alternative non-radioactive labels such as biotin and digoxigenin detected with streptavidin or antibody conjugates did become available but the sensitivity of these systems when used with colorimetric substrates was at least ten-fold lower than that of radioactivity. So, although the use of DNA probes as research tools continued to blossom, their use in detection of diagnosis of disease was limited.

Chemiluminescent substrates produced a rash of publications as authors adapted their existing detection formats to chemiluminescence, but their arrival also coincided with the invention of amplification technologies. The latter were quickly perceived as sensitive rapid methods which were superior to the existing probing technologies which, after the initial excitement of chemiluminescence, have continued to decline.

This short history of DNA probes can be illustrated by referring to the history of the development of DNA probes for malaria. The more sensitive probes were directed against either ribosomal RNA sequences or the 21 base pair repeat sequence which occurs thousands of times in the genome of Plasmodium faliparum. The first probes against these targets were labelled with radioactivity. These probes were exhaustively evaluated for field use and were found to be, at best, comparable to microscopy for the detection of malaria (LARAN et al., 1989). With the advent of chemiluminescence these probes were re-evaluated, both in comparison to similar probes labelled with radioactivity and, once again, to microscopy.

Despite all this work on DNA probes, microscopy remains the method of choice for diagnosis in the field. Why is this? It is because DNA probe technology involves a whole new level of complexity with no gain in sensitivity or specificity. A simple membrane-based chemiluminescent probing protocol from sample preparation to result may involve as many as 16 different steps (10 for radioactive and enzyme-labelled probes) carried out over 24 h, with each step requiring different solutions and different pieces of equipment such as water baths, ovens, slot-blotters, bag sealers, autoradiography cassettes etc. Although the procedures at each step are not complicated, in all it is a lengthy procedure requiring training and sustained concentration for optimum results.

Despite these limitations it is unlikely that the use of DNA probes for diagnosis will become extinct. There are niches to which probes are well suited, particularly when conventional methods of diagnosis are inadequate. For example, culture is used for the detection of chlamydia and gonorrhoea but is not ideal. Further important roles for DNA probes include hybridization in situ for species confirmation after culture or for use in large scale epi-
demioiological screening when hundreds or perhaps even thousands of samples can be fixed to a membrane and processed simultaneously at low cost. It has been esti-
mated that the reagent cost for chemiluminescent mem-
brane-based hybridization can be less than 10 pence (£0.10) per sample if 100 samples are processed simulta-
neously. A comparative technique—for example, micro-
scopy—would be extremely tedious on such a large scale. Similarly, if vectors of disease such as sandflies or mos-
quitoes are being screened, sensitivity is maintained be-
cause the parasites are amplified and concentrated within the
vector.

If DNA probes are to be useful even in these well-
defined niches, it is essential that they should be readily available. Yet there is not one commercially available probe for a tropical disease. The reason is one of econom-
ics. The demand for any one probe is likely to be limited. This, coupled to the tropical emphasis, means that there is no money to be made in this field. One way around this is to consider the collective demand for DNA probes. The demand for any one probe might be small but if a bank of tropical disease DNA probes were estab-
lished, the collective demand could be high. If each probe were designed to be used with the same basic detection format, a chemiluminescent detection system for example, then it would be easy to supply the generic de-
tection kit with the specific probe requested. Such a bank would promote the cause of probe technol-
ogy as a research tool in the tropics.

Application of the polymerase chain reaction

Conceived in 1985 and made practicable by the intro-
duction of thermostable polymerase in 1988, PCR tech-
ology has undergone a boom and become an invaluable tool in the research laboratory. Once again, mirroring the plight of DNA probes, there was much optimism that this technology would revolutionize diagnostics. And, similar to recent years this optimism waned as practi-
cal problems of application came to the fore. The most significant problems of the method were its complexity, unreliability and cost.

Although PCR is intrinsically sensitive, its sensitivity in clinical detection depends on the amount of sample which can be added to the reaction without causing in-
hibition. For example, more than 1/10 μL of blood added to a 100 μL reaction causes complete inhibition. Samples therefore must be concentrated and freed of inhibitors for maximum sensitivity and the methods employed are often complex. For maximum sensitivity and specificity reactions are often performed in 2 steps (so-called ‘nesting’) which increases costs and manipulations. PCR products are commonly analysed by agarose gel electrophoresis or hybridization. The former method is not conducive to large-scale routine analysis and the latter involves a whole new level of complexity and cost. Now, to a large extent, these problems have been addressed. Reagents for simple sample preparation even from blood are commercially available. Nesting, if required, can be done automatically within the same re-
action and the PCR products can be detected colorimet-
rically on microtitre plates. Similarly, post-PCR hybridiza-
tions can be performed rapidly and efficiently in solution and the hybrids formed can be captured and detected on microtitre plates or magnetic beads, for example. Once time and effort has been invested in adapting a given PCR assay to a simplified format, the test is not complex and lends itself to automation.

PCR may not be reliable for diagnosis because of the possibility of contamination (Kow & Higuchi, 1989). DNA which has been amplified by PCR is released into the laboratory environment, possibly as invisible micro-
aerosols formed as tubes are opened and closed. With time this DNA builds up in the environment, coating floors and walls and borne through the air on dust par-
ticles. Laboratory workers pick up this DNA on hair, clothes or fingers. If even a few molecules of this DNA manage to get into a new amplification reaction they will
be further amplified and a negative reaction will become positive. This gives rise to false positive reactions and can lead to misdiagnosis. To illustrate the scale of the prob-
lem, one drop of product from a PCR reaction mixed with the water filling an Olympic-size swimming pool could be amplified by a subsequent PCR, even after such a homoeopathic dilution. Expensive kits are available which specifically degrade previously amplified DNA, so removing any contaminating DNA from new reactions (Thornton et al., 1992), but I have found these to be unnecessary. To a large extent contamination can be con-
trolled by separating work areas so that reactions are pre-
pared in a different room or with different equipment from those involved with product analysis.

PCR is often thought of as expensive, but after an ini-
tial outlay of about £3000–£7000 for a thermal cycler (the range of prices reflects the range of machines avail-
able) nearly all the additional cost is associated with the provision of reagents. For maximum sensitivity and the sample preparation, the reaction and analysis of product can be as little as 50 pence (£0.50). The real cost is for the licence agreement from La Roche, which is needed if the test is to be offered commercially. At present PCR kits are available commercially as real-time PCR detection kits. A comparative techniaue—for examnle, micro-
titre plates or magnetic beads, for example. Once

One important application of PCR is in the detection
of drug resistance. For example, the genetic basis of the resistance of malarial parasites to pyrimethamine has been elucidated (Wellem, 1991). Resistance is defined
by a localized specific mutation in the dihydrofolate re-
ducase gene which can be identified by PCR. This assay can be performed on samples without culturing, and the result can be available within a day. This has immediate implications for determining therapy regimes. Chloro-
quine is an important antimalarial drug but the mechan-
ism of resistance to it is yet to be determined. It would be lucky if one defined mutation were responsible, but this is rarely the case. Often several distinct mutations can re-
sult in the same phenotypic expression. Even pyrimeth-
amine resistance is not as straightforward as might first appear because, although there is one major mutation re-
sponsible for the development of resistance, the degree of resistance is governed by several loci. So, to gain an ac-
curate profile of resistance, several PCRs would have to be performed, each with different sets of primers and this would be impractical for large numbers of samples.

Other amplification technologies have been developed and these include the ligase chain reaction (LCR), the QF replicase system with self-sustained sequence replication (3SR) and nucleic acid sequence-based amplification (NASBA). A kit for the rapid and sensitive detection of tuberculosis based on 3SR is soon to be marketed by GenProbe but at a cost of about US $30 per test it is likely to be some time before this is used in the tropics.
This cost, however, compares favourably to that of PCR because, although the actual cost of a PCR kit if purchased from La Roche is less, a licence is needed if the kit is to be used commercially and this adds to the cost. No licence is required for use of the GenProbe system.

Nucleic acid probes versus PCR
DNA hybridization is, on the whole, insensitive and repeated elements within organisms must be targeted for maximum sensitivity. It is a relatively cheap technique, however, and in membrane-based systems allows hundreds or even thousands of samples to be screened simultaneously, which is useful for epidemiological studies.

PCR is a much more expensive technique, particularly if the reagents are not assembled in-house and expensive kits have to be purchased. It need not be complex but is not easily applied to large scale screening. The technique can be very sensitive, but that sensitivity is dependent on adequate sample preparation. PCR is the preferred technique when sensitivity is the overriding requirement and is particularly suited to individual patient diagnosis. In the future PCR is also likely to find increasing application in the rapid detection of drug resistance.

References

Announcement

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