Gut

Leading article

DNA probes for diagnosis of intestinal infection

The past decade has witnessed the use of molecular biological techniques as the dominant approach for the detection of fastidious microbial pathogens that would be difficult to identify by conventional culture techniques or microscopy. There have been many exciting developments in DNA diagnosis including the advent of in vitro amplification of DNA by the polymerase chain reaction (PCR)[1] and the use of strain or species specific oligonucleotides.[2] The specificity obtained with DNA probes permits discrimination among closely related species and their sensitivity enables the detection of small numbers of pathogens.[3] The success of the new techniques, combined with the rapidity, simplicity, and ease of handling large numbers of biological samples, have prompted the development of DNA probes as an alternative to microscopy or biochemical methods for the detection of a variety of human enteropathogens (Table).

DNA probes for gut pathogens

Enteropathogens	*Probe/target*	*Reference*
1 Bacteria		
Escherichia coli	Virulence plasmid sequences, genes encoding heat labile (LTh) and heat stable (STaII, STaI) enterotoxins, genes encoding Shiga-like toxin (SLTI, SLTII) and invasion plasmid antigen (ipa) genes	3, 5, 7–15, 29–33, 35
Campylobacter jejuni	Genomic DNA probes	16
Yersinia enterocolitica	Virulence plasmid sequences	27
Helicobacter pylori	Total genomic DNA probe and enterotoxin gene	18, 26
Clostridium perfringens	Enterotoxin gene	20
2 Protozoa		
Entamoeba histolytica	Highly repeated sequences and ribosomal DNA probes	21–33, 34
Giardia lamblia	Genomic DNA probes	24
3 Viruses		
Rotavirus group A Rotavirus group B	cDNA probes	25, 26
4 Helminths		
Taenia solium	Highly repeated sequences	6, 27, 28

Methodology

The ability of one strand of denatured DNA (target DNA of 'unknown' pathogen) to hybridise in a precise manner to a complementary strand of DNA (DNA probe: labelled single stranded DNA of specific sequence characteristic of one species, subspecies, strain, or isolate) forms the basis of DNA diagnosis.

The figure outlines the steps in using a DNA probe for the identification of gut pathogens in faeces. After a simple lytic processing of faecal suspension in buffer, pathogen DNA is drawn through a manifold with shaped dots (dot blots) or slots (slot blot) on to nitrocellulose or nylon membranes, denatured in mild alkali-salt solution and fixed. Faecal bacteria can be grown on stool or colony blots (non-selective or selective media), treated with the proteolytic enzyme, proteinase K, and processed as for dot blot hybridisation. Pathogens in paraffin wax embedded tissue sections (intestinal biopsy specimens) on microscopic slides can be detected by in situ hybridisation with DNA probes.

Another alternative is to partially purify total DNA from faeces and amplify target DNA by the use of PCR[4] before immobilisation on a membrane. In principle, PCR comprises an unspecified number of identical cycles each consisting of three successive steps:

(1) denaturation of target DNA;

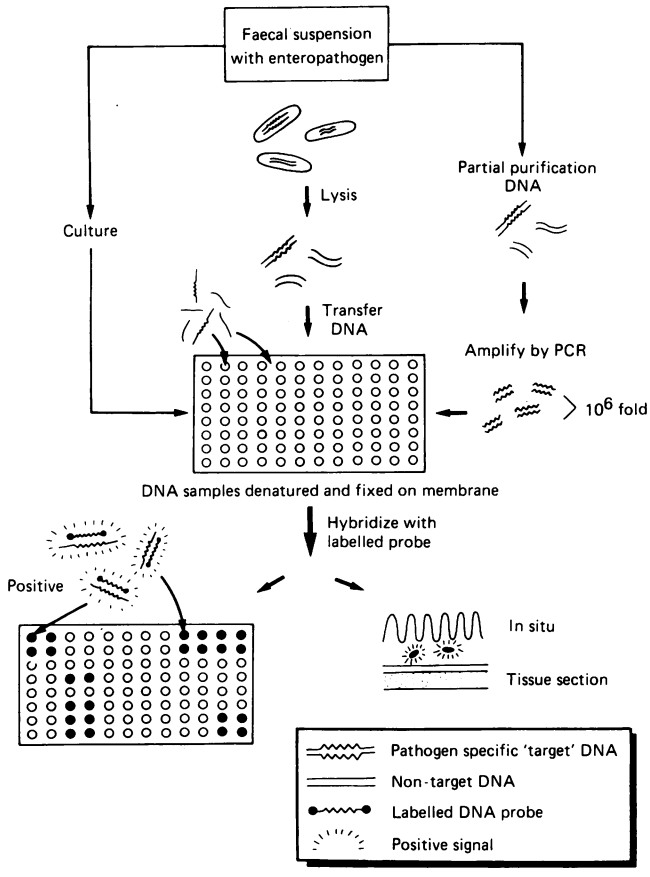
(2) annealing of two synthetic oligonucleotide primers on either side of the target sequence;

(3) extension of the primers by a thermostable DNA polymerase using denatured strands of target DNA as templates.

One cycle results in a doubling of the number of copies of the target sequence. As more cycles are performed, the copy number increases exponentially, thus it is possible to amplify specific DNA sequences more than a millionfold in a few hours, which is all that is needed to complete the procedure when automated. A prerequisite for use of PCR is knowledge of the target DNA sequence.

The success of hybridisation identification is largely dependent on the correct selection of species specific probes. As there must be enough of a particular sequence present in the pathogen's genome to make a feasible target, DNA probes have been selected from repetitive, reiterated, or multicopy DNA. Ribosomal RNA sequences, sequences encoding for virulence factors such as toxins, and sequences from virulence plasmids have also been used successfully. Economic production of probe DNA and selection of specific sequences has been a direct result of development of recombinant DNA technology. Extensive DNA sequence analysis has made it possible to produce synthetic oligonucleotide probes. Methods to synthesise oligonucleotides of defined sequences have now been refined to the point where almost all chemical synthesis can be automated.

Recently, an rRNA based diagnostic system[2] has been used, in which the target molecule is ribosomal RNA rather



Methodologies for DNA diagnosis of gut infection.

than DNA. Detection of an infectious organism is achieved by hybridisation of DNA probes (synthetic oligonucleotides whose sequence is the precise complement of defined species-specific regions of rRNA) to its complement within the rRNA of the pathogen. The use of naturally abundant targets such as rRNA, as opposed to artificially amplified targets, by PCR reduces the potential for contamination. Another crucial advantage over systems using amplified targets is that signals are quantifiable, which may be useful clinically and epidemiologically. For example, most people in an endemic area may well carry a few parasites but remain asymptomatic. A positive signal with no simple means of quantification would be of little use in this situation. Quantitative data can be used for example to detect abnormally high parasite loads or progression of infection.

Traditionally, nucleic acid hybridisation has relied on radioactive labels for detection. However, cost, short half life, and safety requirements render them impractical for widespread field application. Simple detection systems that produce a visible colour change are required. There are a variety of non-radioactive labelling methods including biotin,[5 6] alkaline phosphatase,[7 8] and digoxigenin[9] available now.

Application

DNA probes are being increasingly used for the diagnosis of infectious diseases and may well replace conventional microbiological methods, particularly when the numbers of organisms in body fluids or tissues are scanty. We outline below some of the important enteropathogens for which DNA diagnosis is becoming a reality.

BACTERIA

Through the development of a series of DNA probes specific for the virulence factors produced by each class of enteropathogenic *Escherichia coli*, these groups can be detected against the extensive background of non-pathogenic, so called commensal *E coli* strains.[10] The genes encoding for heat labile and heat stable toxins have been cloned and probes developed to detect enterotoxigenic *E coli* in field studies in Asia and Africa.[9] More recently oligonucleotide probes for *E coli* toxins have been synthesised and their efficacy compared with polynucleotide probes.[11 12] The latter have the advantage that they are less susceptible to minor nucleotide changes in target sequences but are difficult to produce in large quantities and care must be taken to ensure that probes are free of vector DNA. With a modest laboratory arrangement Sommerfelt *et al*[9] produced polynucleotide gene probes in large quantities and labelled the probes with digoxigenin. With an established isotope based oligonucleotide hybridisation assay as reference, a blinded study of a large battery of enterotoxigenic *E coli* and non-enterotoxigenic *E coli* showed a satisfactory sensitivity and specificity of the non-radioactive assay. Olive[3] has used the PCR to increase the sensitivity of a hybridisation assay and could detect a single enterotoxigenic *E coli* bacterium.

DNA probes have been developed to identify enteroinvasive *E coli*.[10 13–15] These bacteria, like *Shigella* possess a 120–140Md plasmid which encodes for invasiveness. There is very close sequence homology between the enteroinvasive *E coli* and *Shigella* plasmid which has enabled specific fragments of this plasmid to be used to detect enteroinvasive *E coli* and *Shigella*. Venkatesan *et al*[15] have developed a DNA probe derived from a multiple copy element found on the chromosome and invasion plasmid of *Shigella flexneri* to detect enteroinvasive *E coli*, which overcomes the restriction imposed by the plasmid coded probes in cases where there is spontaneous loss of invasion plasmid. Shiga-like toxin producing *E coli* (such as *E coli* 0157:H7) which cause haemorrhagic colitis and haemolytic uraemic syndrome, can also be detected by DNA probes which represent cloned fragments of the genes encoding for one or other of the two Shiga-like toxins (SLT I, SLT II]. Recently Karch and Meyer[10] have developed oligonucleotides designed to amplify a segment of the Shiga-like toxin genes by PCR.

Campylobacter jejuni is now one of the most common enteric infections both in the developed and developing world. The organism grows slowly and culture results may not be available for several days. The use of genomic DNA probes has not only permitted the rapid identification of *C jejuni* but it is now possible using non-radioactive DNA probes to distinguish and classify a variety of *Campylobacter* strains.[16] Similarly, *Yersinia enterocolitica* requires special culture conditions and grows slowly. In addition, *Yersinia* serology is not always widely available and the results often appear after the patient has recovered. However, preparation of DNA probes from the virulence plasmid of *Y enterocolitica* and from chromosomal loci responsible for invasiveness are now available for the detection of this organism and for the identification of virulent species.[17] DNA probes have been used to detect *Helicobacter pylori* in gastric mucosa by in situ hybridisation.[18] An rRNA based diagnostic system has also been developed for the detection of *H pylori* in gastric mucosa homogenates and compared well with the urease test.[19] Oligonucleotide probes derived from the enterotoxin gene of *Clostridium perfringens* have been used to test strains isolated from confirmed outbreaks of food poisoning.[20]

PROTOZOA

DNA probes accurately identified *Entamoeba histolytica* in faecal samples, which compared favourably with microscopy for trophozoites or cysts.[21] In addition, with DNA probe as many as 96 specimens $\times 10$ or 20 sheets can be hybridised in

parallel. DNA probes are an attractive alternative to microscopy, which is time consuming and requires extensive experience to assure accuracy of morphological identification. DNA probes that distinguish between pathogenic and non-pathogenic *E histolytica* zymodemes are also available.[22 23] Since non-pathogenic zymodemes are almost 10 times more common than pathogenic zymodemes the availability of these DNA probes promises to eliminate the potentially unnecessary treatment of individuals colonised with non-pathogenic *E histolytica*. DNA diagnosis should be useful for extensive field surveys of prevalence of *E histolytica* infection and to evaluate the effects of control measures.

Genomic DNA probes have been produced from *Giardia lamblia* and can detect *Giardia* cysts from faecal specimens after concentration techniques. However, there are difficulties in liberating DNA from cysts and as such the approach is not sensitive enough to be useful clinically.[24] Incorporation of PCR and oligonucleotide probes should increase the sensitivity considerably.

VIRUSES

Cloned cDNA probes for the detection of group A[25] and group B[26] rotavirus in faecal samples are a sensitive and specific alternative to immunoelectron microscopy or ELISA.

HELMINTHS

The morphological similarity observed between stage specific forms is one of the problems encompassing many pathogens. One example is that of the tapeworms *Taenia solium* and *T saginata*. DNA probes that differentiate between them are now available.[6 27 28]

Conclusions

DNA probes are an attractive diagnostic option as they do not rely on growth of the organism. Commonly, there are several steps in collection, transportation, and culture of the organism that may compromise viability. There are many instances in the diagnosis of infectious diseases in which it is desirable to detect a pathogen directly. For example, opportunistic infection of patients with AIDS which cannot be diagnosed reliably by serology because of severe immune system dysfunction. There are many other gut infectious diseases such as intestinal coccidiosis, isosporiasis, cryptosporidiosis, strongyloidiasis, and mycobacterial infections for which diagnosis in chronic stages may be hampered by the scarcity of detectable organisms. In many cases these diseases are self limiting but they can be life threatening in immunocompromised individuals. DNA diagnosis should prove useful in these infections. DNA probes can be expected to make a major impact as specific reagents for the identification of different strains or geographical isolates of pathogens. The potential for increasing the sensitivity of DNA probes by choosing naturally amplified targets (ribosomal RNA) and by PCR are especially promising. The rapidity, sensitivity, and specificity should encourage us to continue refining these techniques and search for new applications.

S CHAR
M J G FARTHING

Department of Gastroenterology,
St Bartholomew's Hospital,
West Smithfield,
London EC1A 7BE

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