Reviews of molecular biology of sexually transmitted diseases

Molecular techniques in the diagnosis of human infectious diseases

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The availability of clones of nucleic acid (NA) specific for genes of most viral and other microbiological organisms has made possible various approaches to search for such sequences in clinical materials using hybridisation techniques. Radioactive or otherwise labelled NA clones or sequence-specific synthetic oligonucleotides (ONs) act as molecular probes in the search for complementary NA target sequences in the clinical specimens. These procedures have complemented the more traditional diagnostic techniques such as growth in tissue culture (viruses, chlamydia) or in synthetic media (bacteria, fungi, parasites), electron microscopy, examination for antigens by immunofluorescence techniques and serological techniques such as ELISAs, RIAs and other tests looking for specific antibody as the host response to microbial infection.

Several extensive reviews¹⁻⁷ and editorials⁸ on the topic have appeared. This overview will concentrate on the use of such techniques for the diagnosis of genitourinary tract infections and intrauterine infections and also outline the scope of the technology for the diagnosis of other infectious diseases. As this field is evolving rapidly, it is to be expected that further approaches will become known in the near future.

The principles of the technology

The genetic information of microorganisms is in their nucleic acid (NA), deoxyribonucleic acid (DNA) or ribonucleic acid (RNA; for many viruses). In viral genomes consisting of DNA or RNA the NA can occur in single stranded (ss) or double stranded (ds) forms; the genomes of all higher organised microbes consist of dsDNA (fig 1a).

Inside bacterial cells there are small covalently closed circular pieces of dsDNA called plasmids that

replicate independently of chromosomal DNA. They range in size from 3-5 kilobasepairs (kbp) to 400 kbp (megaplasmids). Such plasmids can be introduced into bacterial cells in vitro by a process called "transformation". Once inside the bacterial cell they replicate using various bacterial enzymes and substrates. Many small plasmids have been used as "cloning vectors". There are numerous ways to convert pieces of genomic NA of all kinds of origin (viruses, bacteria, fungi, microbial parasites, animal or plant cells) into dsDNAs for insertion into cloning vectors and amplification in bacterial cells after transformation.⁹

DNA in its ds state is stable under physiological conditions, but strand separation (denaturation) can be achieved by heating above melting temperature, by raising the pH or by lowering the salt concentration (fig 1b). Conversely, under appropriate conditions, single strands of NA will, provided they are exactly or nearly complementary in sequence, come together ("hybridise") to form a duplex molecule via base pairing (adenine pairs with thymine and cytosine with guanine; in the case of DNA-RNA or RNA-RNA duplexes adenine pairs with uracil) (fig 1c).

Probes are pieces of NA, DNA or RNA, labelled in various ways, which will hybridise to denatured pieces of DNA or RNA present in test samples (clinical specimens of throat swab, faeces, cervical swab, lymphocytes, etc). The preparation of a probe is diagrammatically shown in fig 2. In order to enable binding of the probe to the target both must be ss, that is, denatured. After hybridisation the conditions of washing (temperature, salt concentration, pH) are chosen in a way that only perfect or nearly perfect hybrids will stay together and excess and "mismatched" probe be removed.¹⁰ Conditions of hybridisation can be chosen to result in "high stringency" or "low stringency" binding.¹⁰

Hybridisation can be carried out with the target NA either in solution or fixed to nitrocellulose or nylon filters which is then called "colony", "dot

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Figure 1 Structure of native DNA (a), partially denatured DNA (b) and of target DNA probe DNA hybrids (diagrams). (a) Native dsDNA helix consisting of nucleotide bases adenine (A), guanine (G), thymine (T) and cytosine (C) covalently bonded to deoxyribose molecules which in turn are held together by a phosphodiester backbone connecting the sugar entities in 3' to 5' linkage. (In the diagrams the sugar-phosphate groups are represented by solid lines). The 5' ends of the DNA are phosphorylated, the 3' ends have free 3' hydroxyl groups. Native dsDNAs have 10 nucleotides per turn (so-called "B form"). The 2 strands (I, II) of the DNA helix are in opposite 5' to 3' orientation and are of "complementary" sequence; A is "base-pairing" with T and G with C via hydrogen bonds (G ≡ C pairs have 3 hydrogen bonds against the 2 in A = T pairs and are therefore more stable). In RNA the base T is replaced by uracil (U), which also pairs with A, and the sugar deoxyribose by ribose. (b) Partial separation ("denaturation") of DNA strands due to breakage of hydrogen bonds (by heat, high pH or low ionic strength). (c) Hybridisation of strand II of denatured DNA ("the target") with a complementary piece of DNA ("the probe"; thickened solid line; see Figure 2) under appropriate conditions (heat, salts, etc) to form a new duplex molecule. The hybridised probe is radiolabelled continuously or at the ends (detection by scintillation counting or autoradiography), covalently linked with enzymes (phosphatase or peroxidase, detected by reaction with substrates) or with biotin (detected by enzyme-labelled streptavidin). Both the target and the probe can be DNA or RNA (DNA-RNA and RNA-RNA hybrids not shown).

blot" or "slot blot hybridisation". Pieces of nucleic acid can also be separated electrophoretically on gels and subsequently blotted or electrophoresed onto membranes which are used for hybridisation ("Southern blots" for DNA, "Northern blots" for RNA).⁹ Hybrids which have formed in solution are adsorbed to hydroxyapatite and separated by centrifugation from the hybridisation mixture. Hybridisation can also be carried out in situ in order to localise certain sequences in cells or subcellular compartments.¹¹

Probes can be of various structure and be labelled

in various ways. They are either dsDNA (cloned DNA), ssDNA (obtained from M13 clones¹² or fully synthesised oligonucleotides) or ssRNA (obtained as transcripts from dsDNA vectors possessing bacteriophage transcriptional promoters for SP6 or T7 polymerases¹³ (fig 3) or from a synthetic partially ds (14) or ss (14a) oligonucleotide possessing a polymerase promoter). Probes are continuously labelled either by nick-translation (dsDNA) incorporating ³²P-dNTPs¹⁵ or biotinylated dNTPs¹⁶ using DNA polymerase I¹⁵ (fig 4), by T4 DNA polymerase¹⁷ (fig 5), or by the Klenow fragment of polymerase I (fig 6).

Figure 2 Preparation of a DNA probe. A restriction endonuclease fragment of interest (here: Hind III fragment X) is ligated ("inserted") into the unique Hind III site of a plasmid ("cloning vector"). The vector containing the insert is then transformed into E coli and amplified, leading to bacterial lysis. After purification of the plasmid its insert is liberated by Hind III digestion, isolated, labelled (here: 5' end label, for example with ^{32}P) and denatured. It is now ready as "probe" for reaction with a complementary "target" DNA in a hybridisation reaction (figure 1c).

Figure 3 The SP6/T7 polymerase system. The vector is a derivative of plasmid pBR322 into which the promoter sequences of the SP6 and T7 polymerases have been engineered. DNA sequences of interest (here: X) can be inserted between the two promoters into a multiple cloning site (not shown) using standard techniques (Ref 9 and Figure 2). Linearisation of this construct with restriction endonucleases A or B and addition of SP6 or T7 polymerases, respectively, in the presence of α - ^{32}P -labelled and unlabelled ribo-NTPs will result in the production of multiple ssRNA transcripts of high specific radioactivity as probes. The system is very efficient, yielding up to 10 μg product from 1 μg template. Template DNA is removed by treatment with RNase-free DNase I, and the transcripts purified by Sephadex chromatography. The SP6 and T7 ssRNA products are of opposite "sense" and can be used to differentiate between genomic DNA and mRNA templates (eg by *in situ* hybridisation).

Alternatively 5' end labelling with gamma- ^{32}P -ATP and T4 polynucleotide kinase is used for short DNA fragments or synthetic oligonucleotides.¹⁸

The extent of hybridisation is monitored by autoradiography of filters or of tissue sections treated *in situ*. Dots from filters can also be cut out and counted in a liquid scintillation counter.

For various reasons (safety, costs, stability), non-radioactive methods of labelling probes have been developed.^{16 19 20} The most frequently used is the incorporation of biotinylated nucleotides (such as biotin-11-dUTP, figure 7) into probes.^{16 19 20} Hybridised biotinylated NA is detected by an avidin-

Figure 4 Labelling of probes by "nick-translation" of dsDNA with DNase I and E. coli DNA polymerase I. dNTPs can carry a radioactive label (^{32}P , ^{14}C , ^3H) or can be biotinylated.

Figure 5 Labelling of DNA probes using T4 DNA polymerase.

Figure 6 Labelling of DNA probes using the large fragment (Klenow) of *E. coli* DNA polymerase I.

enzyme complex, wherein avidin binds with high affinity to biotin. The enzymes most frequently used in the complex are alkaline phosphatase, horseradish peroxidase and beta-D-galactosidase which convert colourless substrates into coloured products; these are detected either by eye or quantitated photometrically.¹⁹ Biotinylation of oligonucleotides has also been accomplished at both the 3' and 5' ends.²¹ NA probes have been directly end-labelled with enzymes.^{22,23} This does not seem to affect the hybridisation kinetics.

An elegant NA sandwich hybridisation was introduced by Virtanen *et al.*²⁴ Here the target NA is immobilised to a filter by a capture NA and then probed by a labelled NA complementary to a part of the target, which does not interact with the capture NA.

Figure 7 Structure of biotin-11-dUTP.

Figure 8 The polymerase chain reaction (PCR). The starting material for PCR, the "target sequence" of dsDNA is denatured by heating (typically 95°C, initially 60 seconds, subsequently 15 seconds), then primers complementary to opposite strands in the desired distance of target DNA are annealed (55°C, 30 seconds) and then extended with *Thermus aquaticus* (Taq) polymerase (72°C, 90–240 seconds). This cycle is repeated 20–30 times leading to amplification of the target sequence by up to 10⁶-fold. Many modifications of the original PCR have been described. RNA can also be amplified by PCR after an initial reverse transcriptase step.

Various modifications and extensions of these procedures for hybridisation reactions have been reviewed by Jablonski.²⁵

Advantages and disadvantages of DNA probes

Before discussing some applications of the technology to specific pathogens, the relative advantages and disadvantages of hybridisation techniques compared to more classical techniques should be considered.

A major advantage of the hybridisation technology is in the detection of genomes of microorganisms which are present in a latent state (members of the herpesvirus family) or for which cultivation *in vitro* has proven to be difficult (for example enteradenoviruses) or, as yet not possible (human papillomaviruses (HPV), human parvovirus B19 (HParV), hepatitis B viruses (HBV) and other hepatotropic viruses). For several organisms the use of a probe

provides an earlier diagnosis than growth in tissue culture (such as human cytomegalovirus, HCMV) or cultivation on cell-free substrates (mycobacteria). The technique can also be used in situ in formalin-fixed tissues and exfoliated cells.

Disadvantages can be that only a limited number of microorganisms is detected in a specimen, depending on the composition of the probe; that detection of the genome of a microorganism is open to interpretation as to its activity (latent or productive infection); that there is the possibility of cross-reactivity of the probe with cellular (chromosomal and ribosomal) sequences present in the specimen; that the quantity of microorganisms in the specimen may be low and below the limit of detection by a probe (giving rise to false negative results); that there are no established external quality controls for the procedures yet; that the initial costs are high; and that ^{32}P - or ^{125}I -labelled probes have a relatively short shelf life, and their use requires special safety precautions.

However, most of the disadvantages mentioned may become less significant in the near future. There is work ongoing to incorporate several probes into one hybridisation assay and to develop probes which are highly cross-reactive within a group of microorganisms.²⁶ Probing for mRNA as opposed to genomic NA and for such mRNAs preferentially made in productive infections allows some statements to be made about the degree of replicative activity of the microorganism.²⁷ The problem of false-positivity of the probe ("spurious" cross-reactivity with cellular NA sequences) is well recognised and can be minimised.²⁸ There are several and some very powerful procedures to amplify genomic NA before hybridisation. The polymerase chain reaction (PCR)²⁹⁻³¹ (diagrammatically shown in fig 8) amplifies specific DNA in vitro based on repeated cycles of denaturation by heat ($> 90^\circ\text{C}$), annealing of ON primers (at 50°C) and primer extension at $60-70^\circ\text{C}$ using the heat-stable DNA polymerase of *Thermus aquaticus* (*Taq* polymerase). Twenty to thirty such cycles can result in 100,000-fold to 1 million-fold amplification of the target sequence. The procedure is so sensitive (detection of 1 target sequence in the DNA of 10^6 cells) that contamination can be a major problem.

The problems of quality control and relative costs of DNA probes need to be more precisely defined.

APPLICATIONS OF DNA PROBES TO DIAGNOSIS OF INFECTIOUS DISEASES

Herpes simplex virus (HSV)

Infection with HSV type 1 and type 2 (large DNA viruses) are very common in man. After initial infection a latent virus-host cell relationship is established, mainly in sensory ganglion cells from which frequent reactivations can occur. HSV causes keratitis, encephalitis, recurrent cold sores and recurrent genital herpes. In the immunocom-

promised host, HSV infection often causes severe disease. (For details of the virology and epidemiology of HSV and the viruses discussed below see ref 32).

Although HSV DNA-specific hybridisation techniques are unlikely to supersede culturing and immunofluorescence techniques, they have been applied to viral diagnosis. The choice of appropriate probes has allowed differentiation between HSV1 and HSV2 infections,³³⁻³⁵ recognition of genital HSV transmission³⁶ and recognition of mRNA transcripts during latency.^{37 38}

It should be mentioned that large HSV-"specific" DNA probes shown cross-reactivity with mammalian cellular sequences;^{39 40} therefore appropriate controls are essential and shorter probes preferable.

Papillomaviruses (HPV)

Papillomaviruses are small DNA viruses replicating in various mammalian species (cattle, rabbits) and in man. At present, there have been 60 types of human papillomaviruses (HPVs) described which are differentiated on the basis of less than 50% cross-hybridisation of their NAs under stringent conditions.^{32 41} As the HPVs cannot be grown in tissue culture, hybridisation techniques have been essential for their classification. HPVs have been found to be associated with a number of cutaneous and mucosal benign lesions (common warts, plantar warts, epidermodysplasia verruciformis, condylomata acuminata) but special interest has arisen from finding an association of certain HPV types (mainly types 16 and 18, but also 6 and 11 and many others⁴¹) with precancerous lesions (cervical intraepithelial neoplasia (CIN)) and with cancer of the cervix and vulva.⁴²⁻⁴⁴

On the other hand, HPV DNA has been found in normal tissue adjacent to anogenital condylomata and only in half of CIN and carcinomatous lesions.^{45 46} Sixty percent of cervical lesions with abnormal cytology contain HPV types other than 6, 11, 16 or 18.⁴⁷ Recent findings indicate that HPV DNA is present frequently in completely healthy carriers.⁴⁸⁻⁵² Clear epidemiological evidence for the aetiological role of human papillomaviruses in cervical neoplasia is still lacking.^{53 54} It is speculated that the amount of HPV DNA can decrease dramatically as lesions develop and that HPV is one of several factors involved in tumour development.^{55 56}

Human Cytomegalovirus (HCMV)

HCMV, a genus of the herpesvirus family, is a frequent viral cause of intrauterine infection resulting in congenital malformation or later sequelae.⁵⁷ In the immunocompromised patient HCMV can cause a wide range of clinical symptoms (pneumonia, retinitis, hepatitis, encephalitis, colitis). Diagnosis by growth in tissue culture may take several weeks,

although testing for an immediate early antigen using monoclonal antibody allows a diagnosis after 24 hours in many cases.⁵⁸ Great care has to be taken in the selection of the probe as wide parts of the CMV genome cross-react with human DNA.⁵⁹ With these precautions, latent or reactivated HCMV can be detected in lymphocytes and other tissues.²⁷⁻⁶⁰ Recently, the polymerase chain reaction (PCR) has been used to amplify HCMV-specific sequences; a high degree of sensitivity and concordance with viral isolation data from different sources (infected fibroblasts, peripheral leucocytes and urines) was achieved.⁶¹

Human Immunodeficiency Virus (HIV)

By the end of 1989 approximately 200,000 cases of acquired immunodeficiency syndrome (AIDS) have been recorded worldwide (of whom more than half have died), and worldwide several million people are thought to be infected with HIV, the causative agent of AIDS. The virus, a member of the retroviridae family, is transmitted by blood and sexual contact, and homosexuals, intravenous drug abusers and haemophiliacs (before universal screening of blood donations in 1985) have been the major risk groups so far. The rate of heterosexual transmission is increasing at an alarming rate. The infection is diagnosed by testing for HIV-specific antibody (particle agglutination tests, ELISAs, Western blots) or by culturing the virus. Although culture is carried out in many laboratories, the method is cumbersome and needs special safety precautions.⁶²⁻⁶⁴ Hybridisation techniques have been used to detect HIV genomes in lymphocytes⁶⁵⁻⁶⁶ and nerve tissue.⁶⁷ Recently the PCR has been applied in order to detect HIV-specific sequences in lymphocytes and tissues and has done so with a high degree of sensitivity.^{63,68,69} Such a test meets a special need as the HIV-specific seroconversion occurs several months or more after the initial infection⁷⁰ and the virus has in some cases been transmitted by blood which was screened negative for HIV-specific antibody.⁷¹ Several groups have detected HIV infection by PCR in seronegative individuals,⁷²⁻⁷⁴ mainly in the high risk seronegative sexual partners of HIV-infected people and in infants born to seropositive mothers.^{73,76} From such data it was estimated that 95% of HIV-infected individuals do seroconvert within 5-8 months.⁷⁷ Once PCR is sufficiently standardised and properly controlled, it may become more widely applied as a direct test for HIV infection. Besides these applied aspects it is clear that hybridisation and PCR techniques have greatly augmented our knowledge of HIV pathogenesis and variation.^{78,79}

Hepatitis viruses

Infectious hepatitis can be distinguished into at least five forms: hepatitis A to E. Hepatitis A and B viruses

(HAV, HBV) are well characterised and a number of serological tests for specific antibodies (HBsAb, HBeAb, HBcAb; HAVAb) and antigens (HBsAg, HBeAg; HAVAg) are available. HBV, a small DNA virus, is very fastidious, and there is no easy way to grow it in tissue culture. In approximately 5% of human infections a chronic infection ("carrier state") results, and there are an estimated 200 million HBV carriers worldwide who act as a reservoir for transmission. HBV carriers have a 200-fold increased risk of developing hepatocellular carcinoma. HAV is a small RNA virus. Infection is mostly by the faecal-oral route, no chronic carrier state or disease results. Infectious forms of hepatitis not caused by HAV or HBV were called nonA,nonB hepatitis until very recently when a serological test for antibody against a parenterally transmitted virus called hepatitis C virus (HCV) became available;⁸⁰ but the significance of positive results from this test remains to be defined.

HBV infections are mainly transmitted sexually and by infected blood. A further major route of transmission is from carrier mother to child during birth. The inability to grow HBV led investigators to search for the presence of the HBV genome in serum and tissues. HBV possesses a genome of partially dsDNA which has been cloned and sequenced for several subtypes (for example ref 81). Molecular probes derived from these sequences were used to show the presence of HBV DNA in serum,⁸² hepatocytes⁸³ and various other organs and peripheral leucocytes.⁸⁴⁻⁹¹ Cloned DNA probes were successfully replaced by ON probes.^{92,93}

Hybridisation techniques have shown the presence of HBV DNA in serum of HBsAg- and HBeAg-positive individuals and also in sera of some patients testing negative for these markers. The technology has also revealed the presence of HBV in non-hepatocytes including leucocytes. These findings provide very important criteria for infectivity of the individual. Furthermore, measuring HBV DNA is an important tool in evaluating treatment of chronically HBV-infected individuals, for example by interferon.⁹⁴

The delta agent (also named HDV) consists of closed circles of naked RNA which is intensely internally base-paired. Infection with HDV is only found in association (co- or superinfection) with HBV infection; HDV parasitises capsid and envelope of HBV. The only HDV-specific expressed antigen is delta antigen which is incorporated into the capsid. Delta antigen and specific antibody can be measured. In addition, an HDV-specific RNA:RNA hybridisation procedure has been published⁹⁵ which will shed more light on mechanisms of chronicity and latency.

Hepatitis C virus genome has recently been detected by PCR in the sera of patients with chronic nonA-nonB hepatitis.⁹⁶

Human parvovirus B19 (HParV B19)

HParV B19 is recognised as the causative agent of erythema infectiosum or fifth disease.⁹⁷ Most people are infected during childhood, but a significant number (20–40%) are still susceptible as adults. In adulthood the infection may be asymptomatic or present with fever, headache, myalgia and often arthritis/arthralgia,⁹⁸ particularly in adult women. Individuals with sickle cell anaemia or other inherited disorders of erythrocytes develop a severe aplastic crisis after infection with HParV B19.^{99–100} HParV B19 is not sexually transmitted, but infections during pregnancy have been associated with hydrops fetalis, stillbirth and spontaneous abortion.^{101–105}

The virus, a small ssDNA virus, does not easily grow in tissue culture. Therefore, normally the diagnosis of acute infection is made by testing for IgM antibody specific for HParV B19 in a RIA. However, there is a need to test for the presence of this virus in cases of spontaneous abortions, and dot hybridisation tests using labelled fragments of cloned HParV B19 DNA or ssRNA transcripts as probes have been developed.^{106–109} Recently, application of the PCR technology has increased the sensitivity of testing for the presence of parvovirus DNA in clinical specimens enormously.^{110–101}

Chlamydia

Chlamydia trachomatis is among the most frequent causes of sexually transmitted diseases.^{112–114} There are 15 serotypes causing primary urethritis and cervicitis with the complications of epididymitis, proctitis, endometritis and salpingitis. In newborns they can cause severe conjunctivitis and pneumonia.

Chlamydiae are obligate intracellular parasites. Laboratory diagnosis is mainly by cell culture, but also by IFT and ELISA. Several groups have described hybridisation tests to identify chlamydial DNA.^{115–121} The probes are complementary to chromosomal DNA,¹¹⁵ cryptic plasmids^{118–120} or ribosomal RNA.¹²¹ All probes were cross-reactive throughout serotypes but otherwise specific.¹²² The DNA hybridisation assay is slightly less sensitive but as specific as cell culture.^{120–122} False negative results with DNA hybridisation were from specimens with low infectivity (less than 100 inclusion-forming units; ref 122).

Gonococci

In cases of male urethritis gonococci have been detected by hybridisation using a probe specific for a gonococcal plasmid.¹²³ Comparison of such results with those of culture of the microorganism must be carried out with caution as culture itself may not detect 100% of the infections.¹²⁴

Diagnostic nucleic acid probes for other infectious agents

Molecular techniques have been used for the detection in clinical specimens of various other microorganisms: Viruses (adenoviruses,^{24 125–128} varicella-zoster virus,^{129 130} Epstein-Barr virus,¹³¹ rotaviruses¹³²), bacteria (enteropathogenic *E coli*, shigella, salmonella, campylobacter,¹³³ vibrio,¹³⁴ legionella, mycobacterium, mycoplasma¹³⁵). The probes against the pathogenic gut bacteria are mainly based on reaction with toxin or virulence plasmid genes, whereas probes against the respiratory pathogens are based on sequences found in ribosomal RNA of these microorganisms. The probes are highly specific and in many cases highly convergent with the results from cultures which normally take much more time. Infections with parasitic agents (plasmodium, babesia,¹³⁶ leishmania¹³⁷) can also be diagnosed by DNA probes which are preferentially based on so-called repetitive sequences in the chromosomes or mitochondria of these parasites.

Summary and outlook

Nucleic acid probes of different structure and labelled in various ways are increasingly used for the diagnosis of viral, bacterial and parasitic infections. The technology complements rather than replaces more classical diagnostic techniques (culture and serology) and has provided new insight into the pathogenesis and epidemiology of infectious diseases. Its usefulness for other purposes, such as susceptibility and resistance testing of microorganisms¹³⁸ is being explored.

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