Revista da Sociedade Brasileira de Medicina Tropical
25(1):59-71, jan-mar, 1992

ARTIGO DE REVISÃO

MOLECULAR BIOLOGICAL TECHNIQUES FOR THE DIAGNOSIS OF INFECTIOUS DISEASES

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Infectious agents have traditionally been detected in clinical samples by microscopic identification, by isolation after culturing or by detection of a specific host immune response[31]. Microscopy is useful in identifying bacteria, fungi and parasites, but is generally not able to recognize viruses. Although considered the most sensitive diagnostic method, culture cannot be used routinely in all cases, especially in viral diseases. The progress immunology has achieved in recent years has made possible the detection of many micro-organisms through immunocytochemistry and by different serologic reactions using ELISA and radioimmunoassay. However, some infections cannot be identified due to low amount or absence of relevant antigens or antibodies.

In the last few years molecular biology experienced a tremendous advance and provided powerful tools to diagnose many infectious diseases. Today, Desoxyribose Nucleic Acid (DNA) and Ribose Nucleic Acid (RNA) technology is becoming increasingly used as effective options for the detection of infectious agents. All microbes have their own individuality expressed at the genetic level in their sequences of nucleic acids. The pinpointing of these specific sequences by the procedures described below permits accurate, sensitive and effective diagnosis of a large number of infectious diseases.

FUNDAMENTALS OF MOLECULAR HYBRIDIZATION

The rationale for diagnostic molecular biological techiniques is based on nucleic acid structure. DNA is chemically monotonous and structurally very simple. Its primary structure is made by long chains of only the four nucleotides of adenosine (A), cytosine (C), guanidine (G) and thymidine (T), linked by phosphodiester bonds. Secondary strucutre is formed by a double helix stabilized by hydrogen bonds betwen adenine and thymidine (two bonds) and quanine and cytosine (tree bonds). DNA is structurally very stable, but the two chains can be separated by chemicals or by heat, a process called denaturation is irreversible, the two DNA strands rejoin as soon as the denaturing agent is removed. This renaturation is both fast and specific. The speed comes from the positive cooperativity in the formation of multiple hydrogen bonds which give enormous stability to the doubel helix. The specificity derives from the unique pairing of A with T and G with C. For the same reason, only exactly complementary sequences form stabel duplexes. Since the system has no memory, a single DNA strand can associate with the strand from which it came apart or with any other strand having a complementary sequence. The latter is generally called hybridization. The ability of DNA to hybridise with great specificity to known complementary sequences has disclosed new and important perspectives for medical diagnosis. DNA probes, wich are being used with increasing frequency worldwide, have made possible the rapid identification of many micro-organisms previously detectable only by tedious and time-consuming methods. More recently, another technique, the Polymerase Chain Reactions (PCR), has had profound impact on medical diagnosis, by apmplifying *in vitro* specific nucleic acids sequences of different infectious agents.

DNA PROBES

A DNA probe is a known DNA sequence obtained by molecular colning (polynucleotides) or by chemical synthesis (oligonucleotides). The probe is complementary to a DNA or RNA sequence of

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Recebido para publicação em 02/02/91.

interest (target sequence) and has a label that makes possible its selective identification. When the target is in its native place (in tissues of a histologic section, in cells of a smear or in a chromosomal preparation), the method is called *in situ* hibridization. If the DNA is solubilized and boun to filters of nytrocelulose or nylon prior to hibridization, it is called a blot or filter hibridization.

In diagnostic procedures, DNA probes behave much like antibodies, in the sense that they bind a specific target and carry a label. However, the use of DNA probes has many advantages over immunodiagnosis. First, DNA is much more stabel than the majority of proteins. Second, hybridization with DNA probes is independent of genetic expression in the specimen under study. In this way, one can diagnose a viral infection by finding the viral genome itself instead of seraching for proteins that represent products of viral gene expression. Thus, it is possible to detect a virus even when it is in a latent infection, in the form of a provirus, as occurs with the retrovirus HIV 1.

Probes types

There are DNA and RNA probes. The former are used much more frequently because of their greater stability. DNA probes are obtained by techniques of genetic engineerring and can be originated from the genome of an organism (genomic probes) or made through the reverse transcription of RNA (complementary or cDNA probes). Oligonucleotide probes (20-100 nucleotides long) are made by chemical systhesis in solid phase. The technical achievements in this latter fiel have been great and these probes are progressively replacing cloned probes - for many hibridization systems they are preferred.

Labelling

The most commoly used type of probe labelling is the incorporation of radioctive isotopes, especially Phosphorus $(P)^{32}$, which provides high sensitivity. However, the drawbacks of high costs, short half-live, health risks and the logistics of handling radioctive materials in a clinical laboratory have motivated the development of systems os non-isotopic labelling (cold probes). Many non-radioactive chemicals, mainly biotin, have been linked sucessfully to probes and showed to be valuable in various hibridization procedures. The use of probes conjugated directly to alkaline phosphatase represents another advance in this area, since they can be assayed easily in a conventional laboratory setting and the results obtained with them are adequate. The main disadvantage of these "cold probes" is their lower sensitivity, although some investigators have claimed to achieve results comparable to radioactive probes[6,57].

Hybridization

The diagnosis of infectious diseases in clinical samples can be done by *in situ* or filter hybridization (blot). For *in situ* hybridization (ISH) one uses histological sections from samples obtained in surgery (biopsy) or after death (autopsy), or even cellular smears such as blood cells, mucosal scrapings (cervical, bronquial etc.) or sediments of organic fluids (urine, ascitic fluid etc.). Iniatially, the preparations are treated with proteases in order to permit probe access, to expose the target sequence and to lower the non-specific adsorption of the probe. Subsequently, the samples are denatured by heat, hybridised and developed in microscope slides. The information gained is precious, since the technique detects infectious agents in their topographical site within the structure of the tissue. Moreover, ISH allows: 1. the analysis of small samples, with only a few cells; 2. the identification of a micro-organism even when only a minority of cells is infected; 3. to know if the infectious agent is in inside a lesion (e.g. a neoplasia) or if it is in normal adjacent tissues; 4. retrospective studies, since fixed material in paraffin blocks can be analysed with success; 5. since it is able to detect mRNA, it can inform about genetic expression of the micro-organism.

Filter hybridization always depends on previous solubilization of the nucleic acids present in the samples to be analysed. For dot blots, nucleic acids are adsorbed directly to filter. In Southern blots DNA is initially cleaved by restriction endonucleases and the resulting fragments are separeted according to their size by electrophoresis in agarose gels, transferred to the filter and hybridised with appropriated probes. For filter hybridization, solube DNA is more properly obtained from fresh samples, although there are tecniques that permit nucleic acid solubilization and isolation from histologial sections

(histoblots). The main advantages of filter hybridization are high sensitivity and specificity, besides the fact that of samples can be analysed simultaneosly.

Development

For radioactive probes the development consists on autoradiography of filters or microscopic slides. For non-radioactive probes, the hybridization result appears as a fluorescent or colored signals, visible at the microscope (ISH) or the naked eye (blots).

In hybridization with biotinylated probes the signal depends on the appearence of a chromogenic substance formed by an enzyme over a substrate. The enzyme (commonly peroxidase or alkaline phosphatase) is conjugated to streptavidin or avidin, which in turn has great afinity to biotin. Hybridization having occured, a complex biotin-avidin-enzyme-substrate is formed at sites of probe hybridization with the target and results in a colored precipitate.

Figure 1 shows the basic aspects of molecular hybridization with DNA probes.

POLYMERASE CHAIN REACTION

In the short history of molecular biology, only a few events have had so much impact than the discovery in 1985 of the technique of Polymerase Chain Reaction (PCR), a method that permits the amplification of specific segments of nucleic acids. PCR is based on repetitive cycles of *in vitro* synthesis of DNA segment, using a DNA polymerase in presence of nucleotides and co-factors. In the fisrt step the native double-stranded DNA is denatured by heat. In the second step two DNA primers are anneled to complementary sequences on opposite strands of the template DNA and thus flank the region of interest. Finally, DNA polymerase synthesises a complementary strand of DNA through the extension of each annealed primer. An essential feature in PCR is that the products of amplification act as templates for subsequent synthesis. In the next cycle, the two double-stranded DNA segments are again denatured to primers and copied (Figure 2). By repeating these cycles one obtains an exponential DNA synthesis, since in each cycle the number of segments of interest is doubled (hence the name chain reaction). Theoretically, after 20 cycles of amplification 2^{20} (1 million) copies are obtained. So, a single copy of a DNA sequence (e.g. one gene or one virus per cell) can be recognized by the extraordinary sensitivity of this technique.

With the advent of programmable thermal cyclers and the discovery of Taq Polymerase, a thermostable DNA polymerase isolated from a thermophilic bacteria, PCR tecnique is now a partially automated procedure that permits the analysis of many samples at the same time.

The amplifyed DNA, intact or after digestion by restriction enzymes, can be identified and characterized by dot blots, by Southern blots or, simply, by electrophoretic migration in agarose or polyacrylamide gel.

Thus, PCR emerges as a simple and programmable procedure, having properties of great specificity (adjustable by selection of the primers and temperature of annealing) and enormous sensitivity (controled by the number of cycles, usually betwen 20 and 40). It is not surprising, therefore, that PCR is rapidly becoming the technique of choice for diagnosing infectious diseases by amplifying short genomic segments of viruses, bacteria, fungi or protozoa, even in presence of a large excess of host DNA. However, the use of PCR for medical diagnosis should be exercised with extreme care, since its best advantage is also its major drawback: due to its enormous sensitivity, contamination of the reaction with even a single molecule of the product under study can produce a false-positive result.

All these tools have revolutionised the diagnosis of infectious diseases. However, this technology is still largely a privilege of research laboratories and usually confined to the academic environment. Its widespread application for medical practive will require years or decades. Yet, new simplified approaches to some of these techniques are being developed and progressively applied to solve daily medical problems.

DIAGNOSIS OF INFECTIOUS DISEASES

For space limitations, a complete review of all infectious agents cannot be done in this article. Only those more important viruses, bacteria and parasites will be discussed.

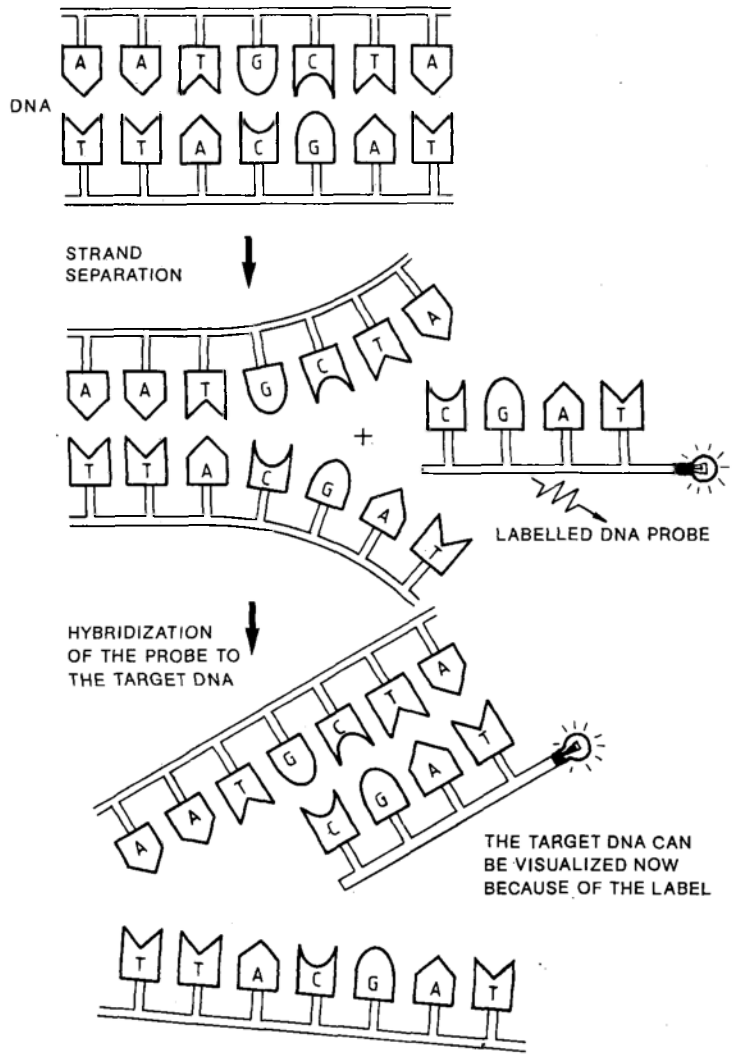

Figure 1 - Hybridization of a DNA probe to a target sequence. The target DNA is initially denatured and subsequently hybridised to a labelled complementary probe. After washings to remove unbound probe, the signal comes out by autorradiography or by chemical reactions that result in a visible compound.

VIRAL DISEASES

The techniques of molecular hybridization with DNA probes and PCR have become valuable methods of analysis and investigation in microbiology. Greater progress in this area has been observed in the study of viral diseases.

HEPATITIS

Hepatitis B

The diagnosis of hepatitis B is routinely made by the detection in serum of specific antigens or antibodies. However, these serologic markers are inconstant and their absence does not rule out

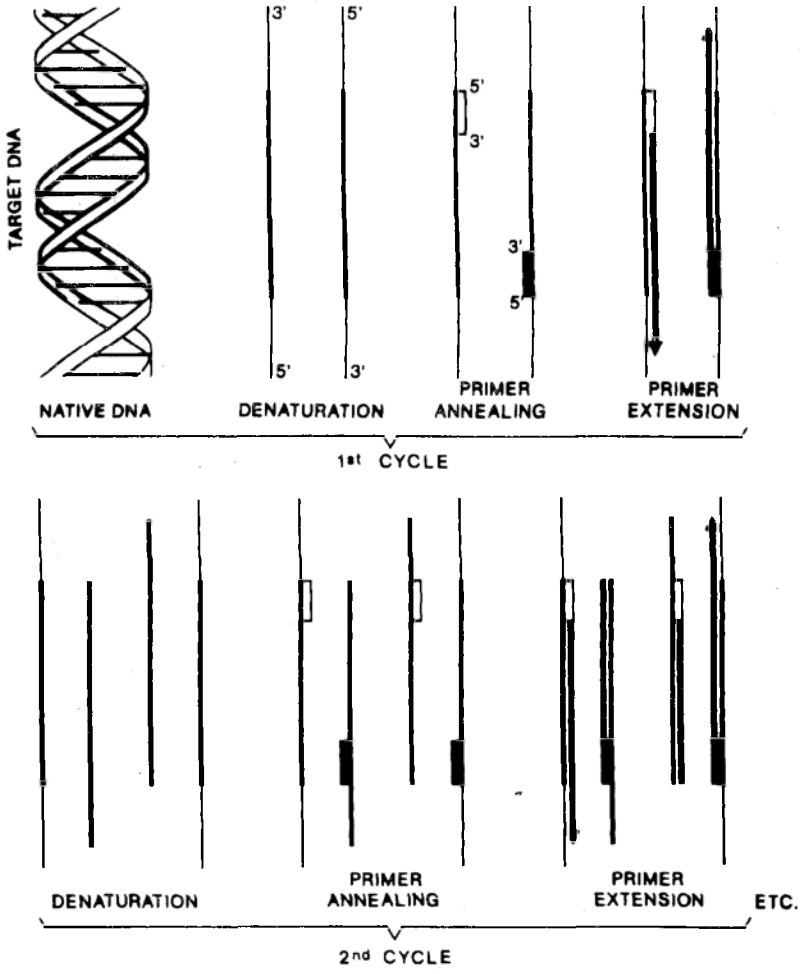

Figure 2 - Polymerase chain reaction. In the first cycle, the target DNA is denatured by heat, annealed to primers and copied by the action of a polymerase. The synthesised products act as templates for the second cycle, in which the same steps are repeated. For each cycle the number of segments of interest is doubled. At the end of n cycles (usually 20 to 40), one theoretically obtains 2^n copies of that sequence.

infection by the virus (HBV) nor indicates absence of infectivity. Direct virus detection is difficult by lack of a simple and practical system of cell culture.

In the past few years there has been a growing interest in viral DNA detection in serum or in liver using Southern or dot blot, which can detect HBV DNA even when their serologic markers are not found[5]. By dot blot with radioctive probes, one can detect low quantities of virus in blood, of the order of 10^4 particles per sample. However, this procedure is not able to detect smaller amounts of virus still sufficient for transmitting the disease. In chimpanzees, for instance, 10^2 or more viral particles can transmit the infection.

More recently, PCR technology has revolutionised this area. Using different primer sets and oligonucleotide probes, it is now possilble to detect HBV when present in levels of 10^1-10^2

particles/ml[37 64]. Therefore, PCR is the most sensitive method not only for diagnosis but also for the screening of samples in blood transfusion centers.

Hepatitis C

The great majority of post-transfusion hepatitis worldwide is of non-A, non-B type (NANBH), which is caused by the hepatitis C virus (HCV), an RNA virus only recently characterised by recombinant DNA technology[13].

Current diagnosis of hepatitis C and screening of blood donors is based on the detection of a serum antibody directed to a recombinant protein coded by the virus (C100-3)[43]. Although serelogic tests with anti-C100-3 have represented a substantial advance in HCV detection, there are some problems. Fisrt, not every blood sample positive for anti-C100-3 has a real risk of transmitting the disease[23 67]. Second, the test can give false-negative results, as anti-C100-3 is directed against the regulatory and not to structural proteins of the viral envelope. It is also negative when assayed before seroconversion, which occurs around 22 weeks after infection or 15 weeks after the onset of clinical signs of hepatitis; moreover, in about 40 % of acute NANBH patients no antibody ever develops[1].

The sequencing of the viral genome has made possible the design of PCR systems to analyse clinical samples for diagnosis in patients with liver disease and for screening of blood donors[23]. Due to its great sensitivity, PCR is able to identify very small amounts of the virus, even in the absence of its serum markers. Currently, many research laboratories worldwide are investing much in PCR technology for detection of HCV, emphasizing out its effectiveness for diagnosis, epidemiological studies and selection of blood donors[20 23].

Other hepatitis

DNA probes or PCR methods are also available for diagnosis of hepatitis A[35], D[16 71] and E[52].

Rotavirus

Human rotavirus is the most prevalent cause of acute diarrhea in children up to two years old. In practive, the diagnosis of rotavirus infection is confirmed by detection of viral antigens by ELISA tests. It also can be made by visualization of viral particles by electron microscopy (EM) or by RNA analysis by polyacrylamide gel electrophoresis (PAGE). However, EM and PAGE are available only in research centers.

In last decade, molecular hybridization technology started to be used and proved useful for detection of rotaviruses A and B[17 19]. Using Oligonucleotide probes complementary to conserved regions of the viral genome, different viral types could be detected[2]. When cloned probes derived from specific segments were employed, many distinct serotypes could de identified[70].

Human papillomavirus

Human papillomavirus (HPV) has notorius tropism for the squamous epithelia of skin and mucous membranes, where it is associated with proliferative lesions of varying malignant potential. The most frequent and important lesions are located in genital system. There, among more than 60 HPV types now known, some (mainly types 6 and 11) have been implicated in genesis of benign or low-grade lesions, whereas others (notably types 16 and 18) have been associated preferentially with high-grade lesions or malignant neoplasms[41].

Accurate detection and precise typing of HPV is really relevant for better understanding of the natural history, epidemiology and oncogenic potential of HPV. Conventional methods of HPV detection usually have low sensitivity and none can discriminate viral types. For these reasons, among all viral diseases, HPV infection is the one that has derived the to date largest benefit from molecular biology techniques. Besides detecting the virus with great sensitivity, molecular hybridization techniques are unique in identifying HPV types safely. As stated above, this aspect is crucially relevant in HPV infection.

All molecular biology procedures described previously have been used, largely with success, for the diagnosis of HPV infection in genital lesions. The samples can be obtained by biopsy or by cervico-vaginal scrapes. Dot blotting is the simplest and most practical method, since it is easy to perform and multiple samples can be tested at each time[68]. Its sensitivity, however, is lower than that of other procedures. Southern blotting is very sensitive and considered the "gold standard" for diagnosis of HPV infection. Other advantage of this method is the possibility of studying HPV physical

state in cells[40], since in the majority of malignant lesions the viral DNA is integrated in the host genome. Yet, due to its laborious handling, Southern blotting is performed only in research centers. With the simplification of the methodology due to the availability of non-radioactive probes, HIS in cervical tissues can be now applied in many laboratories. In our experience[25], HIS has shown to be useful for detecting and typing HPV in samples routinely received for histopathological analysis. Its sensitivity is lower than that of Southern blotting, but it has some advantages over the last method: 1. as individual cells are analysed, it can recognise the virus when only a few cells are infected; 2. permits information if the virus is within a lesion or if it is found in normal adjacent tissues.

The PCR technique is now being used with increasing frequency for HPV detection. When performed with the essential and desired care, PCR has shown to be very effective in HPV identification[42 62]. Using pairs of primers homologous to a conserved region in many HPV types, it was possible to amplify sequence of different types of HPV[57 62]. In positive cases, virus typing can be achieved by subsequent hybridization with specific probes. This technological improvement has great importance, since it reduces time and reagents necessary to testing several samples.

Although fresh specimens are preferred, molecular hybridization tests can be done in old samples fixed for months or years and stored in paraffin blocks. This is very valuable to retrospective studies, which are of enormous importance for the better understanding of HPV infection.

Cytomegalovirus

Cytomegalovirus (CMV) is a major cause of congenital infection in humans. it has been estimated that 60-80% of individuals in developed countries and virtually 100% of those living in developing countries will be infected with it during their lifetime[26]. In most people the infection is asymptomatic, but when the immunologial defense drops for any reason (immunosuppressive drugs in allograft recipients, pacients with cancer AIDS etc.) it may become serious and can even be lethal. CMV can cause pneumonitis, hepatitis, colitis, infeccious mononucleosis like picture retinitis, anemia and other pathology. With the advent of chemotherapy for CMV[35], the precise identification of the virus is of crucial importance, mainly in a compromised host or after transplantation. For a review of conventional diagnostic methods for CMV infection, see CHOU (1990) [14]. Through molecular biology techniques, CMV detection can be attained by ISH in histological sections[24] or in cell smears[30], by dot blot of urine[15] and by PCR from blood, urine or fresh or fixed tissues[58]. However, a positive CMV PCR result should be cautiously interpreted, since CMV infections are usually of latent form and the majority of adults in the normal population are infected.

Retrovirus

Retroviruses are single-stranded RNA viruses. Following cell entry and by the action of a reverse transcriptase, the RNA genome is converted to double-stranded DNA (provirus), which integrates into the host cellular genome and remains there for the life of the cell.

The human retrovirus so far characterized are HIV-1 and HIV-2, causative agents of Acquired Immunodeficiency Syndrome (AIDS), and HTLV-1 and HTLV-II, associated with T-cell leukemias. Retrovirus infections are usually diagnosed by serologic tests, but some factors limit the efficiency of these methods[36 55]: 1. the transcription dormancy of the proviral genome; 2. the small number of infected cells in peripheral blood; 3. the small number of proviral copies per infected cell; 4. in newborns, the presence of passively acquired maternal antibody and the lack of reliable tests for HIV-specific IgM. Because of this and due to its great sensitivity, PCR has become the best procedure for detection of these viruses[36]. Because of the small number of infected circulating cells, other hybridization procedures such as ISH and Southern blot can give false negative results.

PCR has proven to be very useful for HIV identification in the following conditions: 1. confirming infection in seropositive individuals negative by virus culture[48]; 2. infected individuals prior to seroconversion[33]. Clinical and epidemiological evidence shows that HIV can establish a latent infection with low viral expression and absence of specific antibody response. In this situation, the infected person is a potential source of

spread of the virus; 3. resolving the infection status when results of the Western blot assay are ambiguous[36]; 4. newborn or seronegative children born to seropositive mothers[39 55]; 5. seronegative individual at high-risk of infection[33 50]. PCR is also effective in determining the type of the virus[51].

Other viruses

In a lesser frequency, molecular hybridization techniques are also being applied to detection of adenoviruses, enteroviruses, *Herpes simplex* virus[72], Epstein Barr virus[63] and rubella virus[32].

BACTERIAL DISEASES

Molecular hybridization technology for the diagnosis of bacterial disease is still less than for viral infection. However, the potential usefulness of this methodelogy is clearly demonstrated in certain conditions. The bacteria most studied with this approach are listed below.

Helicobacter

Many successful molecular hybridization procedures are now available for detecting *Helicobacter pylori*. The most used are ISH or dot blot in gastric biopsies[66]. PCR performed in fresh tissues or in gastric aspirate specimens can detect very low numbers of bacteria (100 or more per sample)[65]. With such sensitivity, PCR is also well-suited for verifying eradication followinf therapy. The next step seems to the use of PCR in more accessible samples, such as saliva, bool or feces.

Campylobacter

Many *Campylobacter* species have emerged as important causes of gastrointestinal disease their clinical picture being indistinguishable from illnesses associated other enteropathogens. Conventional identification of distinct species of these bacteria is troublesome and more practical and accurate methods are highly desired. Hybridization techniques with DNA probes, including non-radioactive ones[12], can provide results comparable to culture and represent a viable alternative for routine application[61].

Escherichia coli

For each *E. coli* strain of medical interest there are specific DNA probes[25 44 46], which belong to two categories: cloned genes linked to one phenotype (e.g. one toxin) or synthetic oligonucleotides homologous to specific sequences.

The most commom use of DNA probes for the diagnosis of these bacteria is colony hybridization, in which bacterial colonies are directly transferred to filters and subsequently hybridised. The specificity and sensitivity of the method are great and its performance is simpler than conventional procedures, which, as a rule, are expensive, difficult do perform and usually limited to reference laboratories. For all these reasons, molecular hybridization for detecting different strains of *E. coli* has represented a real advance in clinical diagnosis and for epidemiological studies.

E. coli detection can also be made by PCR, which recognises one single bacterium per reaction[47]. Besides their use in clinical samples, there is also great interest in the application of all these tests in detection of such bacteria in water[5] or potentially contaminated food[18].

Shigella

Enterocyte invasion by *Shigella* and by enteroinvasive *E. coli* (EIEC) is essential for the appearence of clinical symptoms of the infection. DNA probes correspondent to one segment of the plasmid responsible for invasion recognise efficiently virulent *Shigella* and EIEC strains[25 67]. On the other hand, the identification of a particular sequence within this plasmid made possible the development of a PCR amplification system substantially more sensitive than other tests[22]. Its major disadvantage is that it is unable to identify precisely the bacterial type, since it recognises indistinctively EIEC and various *Shigella* species.

Mycobacteria

Different types of mycobacteria are import cause of both morbidity and mortality. *Mycobacterium tuberculosis* and other mycobacteria species cause pulmonary diseases which are clinically very similar. Therapeutic agents have to be distinct for each type of bacterium, so the knowledge of the etiologic agent is crucial for treatment. Accurate identification of the many types of mycobacteria by conventional methods is time-consuming and difficult.

DNA probes for detecting mycobacteria of medical importance by dot blotting are already available and work well[49 54]. A more promissing strategy is PCR amplification of sequences conserved in all mucobacteria and hybridization of the amplified product with specific probes for *M. tuberculosis*, *M. avium-intracellulare* and *M. fortuitum*[7]. With this technique ten or more bacilli per sample can be detected.

A system for detecting *M. leprae* by PCR has been devised, by which few bacteria can be recognized[27]. This seems to be very important not only for identifying *M. leprae* in paucibacillary leprosy but also for distinguishing this agent from other mycobacteria in skin.

PARASITIC DISEASES

The use of techniques of molecular biology for diagnosing parasitic diseases is still in its initial phase but for some parasites significant progress has already been achieved.

Entamoeba histolytica

Of the 500 million people infected by *E. histolytica* worldwide, only about 10% develop clinically overt disease. Differences in virulence among *E. histolytica* isolates seem to be important for inducing lesions and symptoms, since there are pathogenic and non-pathogenic strains.

The diagnosis of *E. histolytica* in clinical samples can de done fast and accurately by dot blot[56]. Recently, DNA specific probes for pathogenic and non-pathogenic strains were obtained from tandemly repeated sequences present in extrachromosomal DNA, which can detect the parasite with good sensitivity[7 10].

By virtue of differences in restriciton pattern of certain genes in pathogenic and non-pathogenic strains, the amplification of these sequences by PCR and the subsequent electrophoresis analysis of the fragments resulted from digestion by restriction endonucleases allow the distinction of the strains with even greater efficiency[60]. By this method, 10 or more parasites can be recognized in a sample.

Other parasites

Through the use of total genomic DNA as probe, some authors have used dot blotting for detecting *Trypanosoma cruzi* in blood[3]. The amplification of a *T. cruzi* highly repetitive DNA sequence by PCR permits the identification of a single parasite in a sample[45]. With DNA probes derived from mitochondrial kinetoplast DNA (kDNA), species and subespecies of *Leishamania* can be identifyed by blots or by ISH[4]. Amplification of kDNA sequences by PCR improves considerably the detection of *Leishmania*. Many detection systems of *Plasmodium*, especially *P. falciparum*, can be performed for the diagnostic of malaria [4]. *P. falciparum* can still be identifyed by PCR[34]. The use of PCR for the diagnosis of giardiasis seems to be promising, since small numbers of the parasite (around 10^3 *Giardia lamblia* per sample) can be recognized[11]. So, compared to other diagnostic methods, PCR represents a real advance in the diagnostic of giardiasis. The infection by *Toxoplasma gondii* can be accurately diagnosed by PCR[27], constituting a progress over other identification tests for this parasite. For metazoa, specific probes are available for *Taenia solium*[53] and *Taenia saginata*[21] and are useful for diagnosing these parasites through Southern or dot blotting.

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