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Molecular diagnosis of parasites

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Summary. New developments in molecular biology have generated exciting possibilities for improved diagnosis of parasitic diseases. Through gene cloning and expression and peptide synthesis, defined parasite antigens can be produced in vitro for use in serodiagnosis, while nuclear hybridization techniques offer a vastly improved approach to identification of parasites in the tissue specimens of infected hosts as a means of diagnosis. Furthermore, the advent of the polymerase chain reaction technique has made it possible to increase the sensitivity of nuclear hybridization techniques, through amplification of target DNA sequences of the parasites in test material, by in situ synthesis of these sequences prior to hybridization with the diagnostic probe. Finally, through the use of monoclonal antibody technology, it is possible to design highly specific and sensitive serological assays, as well as assays for parasite antigen detection in tissue fluids and in the excreta of infected hosts, as a means of diagnosis.
Key words. Parasites; DNA; oligonucleotides; probes; monoclonal antibodies; diagnosis.

Introduction

Diagnosis is an essential element in the management of disease, both at the level of individual patient care and at the level of disease-control in populations. The diagnostic tests used should be simple, rapid, specific and highly sensitive, and ideally they should differentiate between closely related parasites if the disease syndromes they cause require different management approaches. They should be suitable for field application, and the cost for the tests should be within the means of the communities affected by the disease. With most parasitic diseases, the tests currently in use do not meet these criteria.

Recent developments in molecular biology, however, have opened new avenues for a vast improvement in parasite detection. Firstly, recombinant DNA technology offers a means for cloning and expression of genes encoding specific parasite antigens needed for use in designing highly specific and sensitive diagnostic tests. Secondly, nuclear hybridization techniques have shown a high potential for use in parasite diagnosis with increased specificity and sensitivity. Thirdly, monoclonal antibody technology has also given rise to highly sensitive and specific assays for detection of parasite antigens in the tissues of the infected host.

The aim of this review is to highlight the potential for these new technologies in parasite diagnosis. The emphasis will be placed on disease diagnosis rather than parasite characterization per se, which will be reviewed in a separate article in this journal issue[9].

DNA diagnostics

Parasite DNA is made of nucleotide sequences which can be divided into two broad classes, namely, those sequences which exist in single copy and those which are in multiple copies within the parasite genome. The repeated DNA sequences, known as 'satellite' DNAs, have no known cellular function and typically they contain a simple concensus sequence that is repeated thousands and often millions of times. In *Trypanosoma brucei* and *T. cruzi*, for instance, these elements constitute 12% and 9% of the genome, respectively[3, 4, 11, 23].

Parasite species-specific differences can be found in either class of DNA sequences, but it is among the repetitive DNA sequences that the most convenient ones for diagnostic purposes are to be found. The repetitive sequences give good sensitivity when used as hybridization probes for detection of complementary sequences in small amounts of test DNA extracted from parasites or in whole organisms.

The principle of DNA hybridization is that a single-stranded DNA fragment containing the specific DNA sequences, such as parasite species-specific sequences, is identified and, preferably, purified. It is then labelled

with a tracer (often a radioisotope) and used to probe whole parasite DNA (purified) or whole organisms. Prior to application of the probe, the test parasite DNA is treated with denaturing agents and split into single strands. When the probe is applied to the specimen, the sequences in the probe will hybridize with complementary DNA sequences of the parasites. The bound label can then be revealed by autoradiography if the probe was labelled with a radiosotope.

With some probes, however, the number of sequence repeats may be so few that the sensitivity of the test will be low. The sensitivity can be remarkably increased by incorporation of an amplification step based on the DNA polymerase chain reaction [26]. This technique permits a rapid synthesis of desired sequences from genomic DNA, by DNA chain extension simultaneously from two opposing primers, catalyzed by DNA polymerase. A DNA sequence present in low copy numbers can thus be amplified, within acceptable limits of accuracy [11], several thousand-fold prior to exposure to the hybridization probe.

An example of a diagnostic probe based on repetitive DNA sequences is that for *Plasmodium falciparum* malaria [2, 5, 10]. The probe is a DNA fragment containing a sequence of 21 base pairs that is repeated several thousand times in the malaria parasite genome. Blood from infected patients is lysed and spotted directly onto nitrocellulose paper and the malaria parasites identified on the basis of hybridization of parasite DNA with complementary DNA sequences present in the labelled *P. falciparum*-specific probe. The technique is sensitive enough to detect 10 pg of purified *P. falciparum* DNA (equivalent to 100 parasites) and in field studies it has been observed that it can detect approximately 40 parasites per µl of blood. This approach has several advantages, namely, it detects current infections; it can be quantified; and it can be adapted to field use in that blood obtained from a finger prick can be applied directly to nitrocellulose paper so that there is no need for extraction of DNA.

Another example of a parasitic disease where the DNA hybridization technique has been found to be useful as a diagnostic procedure is the human disease leishmaniasis, caused by several species of *Leishmania* parasites. The initial lesion in this disease is located in the skin at the infection site, in which the parasites multiply within the phagolysosomal vacuoles of the host macrophages. A rapid, accurate identification of the parasites in the skin lesion could contribute significantly to proper management of the patient since the mode of treatment and follow-up will differ depending on the infecting *Leishmania* species.

Leishmania belong to the order Kinetoplastida. The distinguishing feature of this group of protozoa is the kinetoplast organelle, a unique mitochondrial structure containing a concatenated DNA. This kinetoplast DNA (kDNA) consists of two components: the maxicircle and the minicircle. The maxicircle, 20,000–40,000 base pairs in size, encodes the mitochondrial genes, and the minicircle, which is approximately 2,500 base pairs, has no known coding function. By restriction enzyme digestion of minicircle kDNA, followed by resolution of restriction fragments on agarose gels, species-specific sequences were demonstrated in *Leishmania*. Therefore it was possible to use labelled minicircle kDNA as a diagnostic probe for detection and identification of *Leishmania* parasites in tissue touch blots by nucleic acid hybridization [1, 27]. The minimum number of parasites detected by this technique was 10^2–10^3. There was, however, a slight cross-hybridization between *L. tropica* and *L. mexicana*. This problem was easily eliminated through addition of unlabelled kDNA of the cross-reacting *Leishmania* species to the probe prior to hybridization. Genomic DNA probes with species-discriminatory characteristics also exist, but most are probes for single-copy sequences, which are not suitable targets for diagnostic probes [1].

A repetitive genomic DNA sequence for *T. cruzi*, the causative agent of Chagas disease, has also been used as a diagnostic probe [3, 4, 6, 7, 12]. This probe consists of a 195-base pair repeating unit that constitutes 9% of the parasite genome. Cloned DNA fragments containing this repetitive element have been shown to be useful as probes for identification and counting of *T. cruzi*-infected cells by dot-blot hybridization. The sensitivity of this assay permits detection of phenol-extracted DNA of 30 parasite genome equivalents. The probes can be used for direct detection of parasites in mammalian blood and tissue touch blots.

RNA diagnostics

Another approach to molecular diagnosis of parasites involves the use of synthetic oligonucleotides for detection, by hybridization, of species-specific sequences in ribosomal RNA of the parasites. This approach involves the identification of ribosomal RNA sequences which are parasite species-specific. Oligonucleotides complementary to the species-specific sequences of ribosomal RNA are then synthesized, labelled and used to detect homologous sequences in the ribosomal RNA of the parasites by hybridization.

Oligonucleotides complementary to species-specific sequences of ribosomal RNAs of four human malaria parasites, *P. falciparum, P. vivax, P. malariae* and *P. ovale*, have been synthesized and applied to the diagnosis of human malaria [14, 25]. The sensitivity of these assays is of the order of less than 10 parasites and the target ribosomal RNA sequences are stable even in dehydrated cells.

Constraints of DNA diagnostics

DNA probes are now available for the detection and identification of a variety of parasites [1, 6, 7, 10, 20, 21, 24]. The major logistical problems for field application of

DNA probes are the preparation of specimens, hybridization of the probe and, most importantly, the requirement for radiolabel and subsequent detection of the hybridization signal by autoradiography. A number of alternative labelling techniques, such as direct enzyme linking to the DNA probe, fluorescent probes, and the use of antibodies to detect DNA hybrids, will circumvent this constraint. This approach, coupled with the use of synthetic oligonucleotide probes, which generally permit short hybridization times, and greatly simplified protocols should provide the frame-work for field application of this exciting technology.

Immunodiagnosis

The essential element in immunodiagnosis is an adequate supply of species-specific test antigens and/or highly specific antibodies. Because parasites contain thousands of potentially antigenic polypeptides, glycoproteins and glycolipids, many of which are shared with unrelated species or phyla and even with bacteria, there has been great difficulty in developing sufficiently specific immunodiagnostic tests.

Diagnostic serology

Recombinant DNA techniques permit the cloning and expression of genes encoding synthesis of antigens of diagnostic interest. This development allows the production, in abundance, of pure antigen for immunodiagnosis, and will ensure a degree of specificity that hitherto has not been possible. Care should be taken, though, to ensure that the antigen selected is recognized during natural infection by the host immune system and that the recombinant product retains this antigenicity. Diagnostic serology can also be improved in terms of specificity by the use of monoclonal antibody in competitive assays. Despite the refinements in technology, however, interpretation of diagnostic serology has many pitfalls. For example, false negative results may be encountered in the early stages of infection before the host has had time to respond immunologically, or more importantly, in the young, non-responsive, or malnourished host incapable of mounting an effective antibody response despite heavy parasitization. Similarly, modulation of host immunity by the parasites themselves can give negative results. Finally, selection of test antigen is often made on the basis of availability. An antigen that simply happens to be available may not necessarily be the most appropriate antigen for diagnostic use.

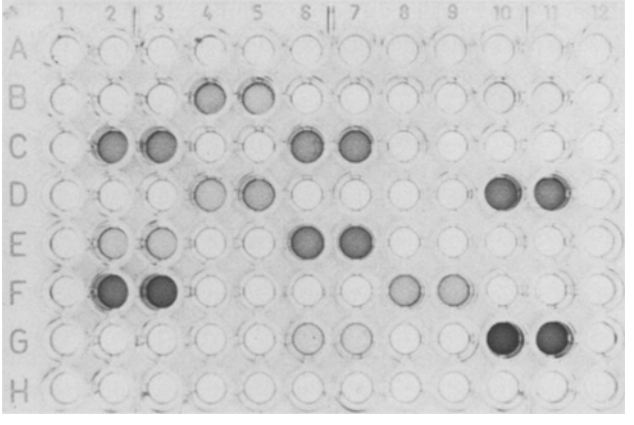
Antigen-detection

Immunodiagnosis by detection of parasite antigens in faeces or tissue biopsies, or in blood and other body fluids, is also feasible provided the antibodies employed in the assays are sufficiently specific.

'Antigenaemia' has been demonstrated in a variety of parasitic infections such as toxoplasmosis, malaria, hydatidosis and African trypanosomiasis by the ELISA method and by radioimmunoassay [13, 15–17, 19, 22]. Antigen-detection is an important approach to immunodiagnosis because the presence of antigens in appropriate host tissues provides ample evidence for an on-going infection as opposed to an infection that has already been cured.

The principle of the ELISA method for antigen-detection is as follows. A micro-ELISA plate or a polystyrene tube is coated with an antibody against the parasite in question (capture antibody). Excess antibody is drained off and test serum is added and incubated for a short time period. The excess serum is drained off. If the antigen were present in the serum it would be trapped by the capture antibody. To reveal this, an enzyme-labelled antibody against the same parasite antigen (i.e. the same antibody as was used for capture, or a different antibody but one that reacts with one other epitope on the same antigen molecule) is added. The plate or tube is then washed to get rid of excess reactants and substrate and chromogen is added to give a colour reaction (see fig.).

In the initial studies, the antibodies used for antigen-detection were either recovered from infected individuals [13], or were obtained by immunization of laboratory animals with crude parasite extracts [22]. Antisera thus produced are less specific and less standardizable and so the assays gave a high proportion of false positive results. With the advent of monoclonal antibody technology, there has been a tremendous improvement in the performance of antigen-detection assays, as exemplified by the progress made in the diagnosis of African trypanosomiasis.



Micro-ELISA plate assay showing a typical set-up of the antigen-trapping assay and the results obtained. Samples were not applied in the peripheral rows of wells. Negative (wells B2 and B3) and positive control sera (wells G10 and G11) were applied in duplicate. The rest of the wells received test sera in duplicate as for the controls. Weak reactions (wells G6 and G7), intermediate reactions (e.g. wells E2 and E3) and strong reactions (e.g. wells F2 and F3) were observed, reflecting differences in antigen titre.

In the case of African trypanosomiasis, species-specific monoclonal antibodies have been made against invariant antigens of the 3 major pathogenic species, *T. congolense*, *T. vivax* and *T. brucei*. These antibodies showed no cross-reaction with common blood parasites such as *Babesia*, *Anaplasma*, *Theileria* or *Plasmodia*[18].

In initial studies it was shown by sandwich ELISA that the antigens recognized by the monoclonal antibodies were present in extracts of forms of the parasites found in the infected mammalian host[18]. This finding led to investigations into the possibility of detection of the species-specific antigens in the blood of experimentally-infected hosts as a means of diagnosis.

Circulating antigens were detected in the blood of experimentally infected animals about 2 weeks following infection[16,19]. The antigen levels increased, though they showed some fluctuations, and antigens were persistently present in the circulation even on occasions when parasitaemia was not detectable by microscopic examination of the blood[16,19]. This type of assay is far more sensitive than the present parasitological techniques in the diagnosis of chronic trypanosomiasis, and in addition is capable of identifying the infecting trypanosome species (unpublished observations).

T. brucei is a complex of several trypanosome species comprising members other than *T. brucei brucei*, notably, *T.b. rhodesiense* and *T.b. gambiense*, which cause human sleeping sickness, and *T.b. evansi*, which causes the disease commonly known as 'surra' which afflicts a variety of livestock species, notably camels, water buffaloes and pigs. Hence, the antigen-trapping ELISA for *T. brucei* can also be used for diagnosis of human sleeping sickness[15] as well as 'surra'[17,19]. The beauty of these assays is that only one antibody is used in each test system for diagnosis of the disease in several animal species. Host species-specific anti-immunoglobulin conjugates are not required.

It has been demonstrated too that circulating antigens decline following the institution of effective chemotherapy[16]. Thus, parasite antigen-detection may also be a useful tool for monitoring the efficacy of chemotherapy.

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