

RECENT ADVANCES IN THE LABORATORY DIAGNOSIS OF MALARIA

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Keywords: Malaria, diagnosis, serology, monoclonal antibodies, DNA probes.

INTRODUCTION

Although serological methods are well established for the immunodiagnosis of bacterial and viral infections, their application to parasitic infections is relatively recent.¹⁻⁷ Even now, only a few laboratories use such methods routinely. The diagnosis of most parasitic infections rests primarily on the demonstration of the causative organism, and malaria is no exception.

MICROSCOPICAL DIAGNOSIS

The discovery of malaria parasites is credited to the examination of stained blood films with light microscopy.⁸ At present, most laboratories still depend heavily on this method for the diagnosis of malaria. It is adequate for the routine diagnosis of individual cases since it allows not only the identification of the different species and stages, but also quantitation of parasitaemia. In principle, the sensitivity of this method allows the detection of a single parasite in 250,000 erythrocytes, which is equivalent to 0.0004%.⁹ The effectiveness of malaria diagnosis by blood smears can be improved by direct observation of centrifuged blood. In a recent study using a commercially available modified haematological apparatus, the QBC tube, the sensitivity of diagnosis by direct centrifugation was at least 8 times better than the conventional approach.¹⁰ However, if large numbers of blood films have to be examined, the limitations of microscopic diagnosis become apparent. Extended periods of examination are needed to detect sparse parasitaemias usually found in infected individuals from endemic areas. The method is therefore time-consuming, labour-intensive, and requires very experienced personnel. More efficient and sensitive diagnostic approaches such as immunological techniques may therefore be valuable.

SEROLOGICAL DIAGNOSIS

Conventional serological methods have been tried previously for the diagnosis of malaria.¹⁻¹³ Depending on the requirements,

serological assays may be tailored for the detection and quantitation of malarial antibodies, or they may be designed for the demonstration and measurement of malarial antigens. From the immunodiagnostic point of view, antibodies which cross-react between the different strains of a plasmodial species but not with other species of malaria parasites are most useful. However, the mere detection of malarial antibodies has limitations since the presence of serum antibodies does not necessarily indicate an active disease. Although the antibody levels may reflect the immune response, they are by no means reliable for measuring malarial protective immunity. The prime purpose of an immunological procedure for the detection of malarial antigens in serum or blood is to identify those people with active or ongoing infections.

Early work on malarial antibody detection was based on gel precipitation¹¹ and indirect haemagglutination.¹² Crude antigen preparations were usually made from extracts of placentas infected with *Plasmodium falciparum*, parasitized blood from patients and from *in vitro* cultures. The methods were plagued with poor specificity, low sensitivity and reproducibility problems.⁴⁻¹⁷ These limitations subsequently precluded their use as immunodiagnostic tools.

Recently a new generation of sensitive immunoassays has been developed for immunodiagnosis of malaria. Indirect immunofluorescence (IFA),¹⁸ radioimmunoassay (RIA),¹⁹ and enzyme-linked immunosorbent assay (ELISA)²⁰ are techniques which have found wider acceptance.

Immunofluorescence

IFA has been the reference method for malarial antibody determination for the last decade.^{18,21,22} This method has the advantage that "intact" or, at least, morphologically identifiable parasites are used as the antigen and permits specific antibody-antigen reactions to be recognized on defined anatomical

structures of the parasites. However, on the debit side, it must be recognized that immunofluorescence microscopy is labourious and reading of the results may be subjective.

Radioimmunoassays and enzyme-linked immunosorbent assay

RIA and ELISA for the detection of *P. falciparum* antigens have been developed with the sensitivity of detecting 1–10 parasites per 1,000,000 red blood cells.²³⁻²⁵ There are, however, some inherent limitations related to standardization of the tests. The main limitation has been the lack of standardized reagents, especially antigens. The principal source of malarial antigens has been blood from infected individuals or experimental animals and, more recently, *P. falciparum* asexual stages from *in vitro* cultures.^{9,26,27} Extracts prepared from parasitized erythrocytes contain a wide range of antigens, including normal red blood cell components which pose a problem since sera from endemic areas frequently contain high levels of anti-normal red blood cell antibodies.^{7,28} Although this problem can be overcome by use of purified antigens, their production and purification are not standardized. Endogenous enzyme activity in red blood cells also compromises the use of certain enzyme markers in ELISA.²⁹ Although the sensitivity of IFA is comparable to that of ELISA in sera with high antibody titres, discordance between the two assays has been observed with serum samples of low titres.³⁰ The discordance suggests that ELISA is less sensitive in detecting low titre serum activity.³¹

Application of monoclonal antibodies

The advent of cloning technology has provided monoclonal antibodies for improved diagnostic tests.³² This has offered several advantages. Firstly, once a specific product has been cloned, an inexhaustible and renewable supply is established. Secondly, since cloning separates molecular elements in a mixed pool, their isolation from similar elements in the native background is theoretically absolute. Therefore, cloning of antibodies eliminates unwanted reactions that are invariably present in the conventional type of antiserum. Thirdly, the sensitivity and specificity of a cloned reagent is controlled by the user, rather than the purity of the crude reagent itself. The development of diagnostic tests depends on the isolation of monoclonal antibody that recognizes an epitope represented on all, or almost all, serological variants of the pathogen of

interest, but not on other organisms or host tissues.³³

Monoclonal antibodies with defined specificity have been widely used for serotyping *P. falciparum* by the indirect method of immunofluorescence.³⁴⁻³⁷ With appropriately defined monoclonal antibodies, immunofluorescence provides relatively high specificity and is, at least, as sensitive as existing methods of identification.

Although monoclonal reagents may have a higher proportion of total immunoglobulin directed at a specific antigen, this advantage is frequently offset by their usually lower binding affinity. Therefore, in any existing assay that is sufficiently specific when polyvalent reagents are used, the substitution of monoclonal antibodies may not necessarily enhance the sensitivity of antigen detection.³³

USE OF DNA PROBES

Recent advancement in gene cloning technology has made it possible to separate and grow a particular sequence of DNA independently from the genome of *P. falciparum*.³⁸ Like monoclonal antibodies, cloned DNA fragments are beginning to find a place in diagnostic work. DNA probes specific for human malaria, particularly that of *P. falciparum*, are being used in preliminary trials for the detection of clinical isolates in the blood of infected patients.³⁹ The probes specific for *P. falciparum* are dispersed, highly repeated DNA sequences of 21 base pair tandem repeats.⁴⁰⁻⁴² To identify the complementary target DNA sequences in *P. falciparum* clinical isolates, infected blood extract is spotted directly onto nitrocellulose filter paper. The spots are hybridized to radiolabelled DNA probe, and are subsequently identified by autoradiography. The stringent requirement for complementarity as a precondition for strand reassociation is the basis for the great specificity of the DNA-hybridisation probe test.⁴³⁻⁴⁵

Besides the obvious advantage of bulk sample-handling, an advantage of DNA probes for the diagnosis of *P. falciparum* is the high specificity which is attributed to the unique 21 base pair tandem repeats of the probes specific for *P. falciparum*.^{46,47} In malaria with moderate parasite densities, the method also compares favourably in sensitivity with routine microscopy. However, its sensitivity for detecting low parasite densities common in malaria endemic populations may be limited.⁴⁸ Furthermore, correlation of hybridization densities with parasite densities is constrained by the potential for variation in

the number of repeated target sequences in different *P. falciparum* isolates.⁴⁹⁻⁵¹ The sensitivity required of a *P. falciparum* probe will vary with its purpose and the population for which it is to be applied.^{39,52}

The actual applicability and usefulness of DNA probes as a clinical diagnostic tool has yet to be evaluated. The time within which radionuclide-labelled DNA probes can detect target DNA is quite unfavourable for clinical diagnosis. For visual detection of reactivity, overnight hybridization and/or autoradiography are usually required. DNA probes in combination with short autoradiographic exposure times are, at best, as sensitive as the classical microscopical technique of evaluating thick smears. Only if longer exposure times are used are the hybridization assays more sensitive than conventional light microscopy.⁵³ Besides, radionuclide-labelled probes are inconvenient for clinical use, both because they require specialized facilities and because radioactivity has short half-lives. An alternative approach makes use of the high affinity between biotin and avidin. Probes are synthesized with biotinylated nucleotides, and their presence is detected using avidin-conjugated enzyme and colour-producing substrates, as in an enzyme-linked immunoassay.⁴⁸

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