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Perspectives on the diagnosis of parasitic diseases in the tropics

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Abstract

Recent progress in the diagnosis of tropical parasitic diseases is reviewed, with emphasis on the potential of new technologies. Unsolved diagnostic questions are highlighted. Sustainable breakthroughs in the development of diagnostic tools can only be achieved by an integrated approach involving health workers and managers, researchers and specialists from industry. Although the technical development of a tool is central, more emphasis should be laid on identifying diagnostic necessities, defining precise diagnostic objectives and guaranteeing dependable validation, which must include the validation of the test in the setting in which it will be ultimately used.

Introduction

The major cause of morbidity and mortality in the developing countries of the tropics is the high prevalence of infectious diseases. Besides the plethora of
microbes causing gastrointestinal and respiratory infections, there are major diseases of parasitic origin, caused by pathogenic protozoa and helminths. Table 1 summarizes the major plagues of developing countries in tropical and subtropical Africa, Asia and Latin America. Two major programmes of the World Health Organization (WHO) are focussed on the control of acute respiratory infections and of diarrhoeal diseases with the central objective of reducing severity and mortality. The main objective of these programmes is the improvement of acute case management; diagnosis does not have high priority. In the developing world, specific diagnostic exploration is often impossible. A febrile patient will automatically be treated for malaria, before other pathogens are taken into consideration. In many health centers and small hospitals in rural areas laboratory facilities are rather limited owing to lack of trained personnel, adequate equipment or sufficient operating funds. In contrast, in major cities the diagnostic laboratory potential is very often no different from that in the industrialised countries.

The field of diagnostics has made enormous progress in the last decade, primarily as a result of advances in biotechnology. Recombinant DNA and hybridoma technologies as well as peptide chemistry allow the production of highly specific reagents for the diagnosis of infectious diseases. The Polymerase-Chain-Reaction (PCR) with its fantastic power of amplifying very small amounts of specific DNAs, boosts sensitivity to levels beyond imagination. New "generations" of test kits for viral hepatitis or HIV infections etc. testify to the rapid development of diagnostics. If they can be produced in connection with an appropriate robust and simple test design, and at a low cost, such techniques could make their way to the rural health centers or hospitals. An example is the
new "dipstick" test for antibodies to HIV developed by the Programme for Appropriate Technology in Health (PATH) in Seattle.

Before highlighting recent advances and open questions on the diagnosis of some tropical diseases, I should like to express my views on some basic prerequisites for the development of diagnostic tools. Reviewing the literature I often get the impression that many tests have been developed with no very precise ideas of what purpose they will serve, and in what kind of environment they will be used. Validation is often hampered by the fact that ill-defined specimens ("accidently" found in a freezer) have been analysed. It should be self-evident that diagnostic goal(s) have to be clearly defined and an appropriate technique carefully chosen before a costly test development is started. Only a precise definition of the diagnostic goal(s) allows a relevant validation.

For most acute viral or bacterial diseases, the major diagnostic aims are either to detect present infection or to analyse the immune status. For many parasitic diseases diagnostic issues are more complex. This stems from two factors. Firstly, the patient often has a lifelong history of contact with the disease. Secondly, each disease has a wide clinical spectrum ranging from states of latent infection or asymptomatic carriers to acute or chronic pathology, including life-threatening conditions. Relevant diagnostic questions associated with individual cases might be related identifying the stage of infection, assessing morbidity, identifying subjects who are at risk of developing severe morbidity, assessing the parasite load in helminth infections, and so on. For public health purposes, diagnostic issues are different and ask for other diagnostic tools. An epidemiologist has to quantitate transmission or to assess the impact of control
measures on incidence, prevalence, overall morbidity, or transmission.

It is obvious that one single diagnostic test cannot answer all those questions. The prime test parameters, sensitivity and specificity, must be carefully adapted to optimize the predictive value for either a positive or for a negative test result. One should not forget that predictive values are dependent on prevalence. This means that the threshold value of a quantitative test must be chosen according to the level of prevalence but also to the purpose that the test is to serve. For example, for HIV diagnosis maximal specificity (excluding false-positive results) is a high priority for the diagnosis of individual cases. On the other hand, one would aim at a high predictive value for a negative results, accepting false-positives, for screening blood products for transfusion. For some diagnostic issues the diagnostic a strategy combining a sensitive screening test to be followed by a more specific confirmatory test might be c. acceptable solution.

Highlights on advances and problems in the diagnosis of some tropical diseases

1. Malaria

Specific diagnosis of malaria is usually achieved by microscopic examination of a blood specimen. However, the detection of a malaria case might be a time consuming and costly exercise, especially in areas with a low level of transmission. For an experienced microscopist 10 to 20 parasites per µl blood are considered to be the detection limit when analyzing a thick blood film. For
public health purposes - e.g. to assess the impact of control measures - a more efficient method would be of great advantage. During the last decade, alternative diagnostic approaches have been developed using three techniques, namely DNA probes, antigen detection and quantitative buffy coat (QBC) analysis.

Most DNA probes for *Plasmodium falciparum* (either genetic probes or synthetic oligonucleotides) detect a family of 21 base-pair tandem repeats which comprise about 10% of the genome (for review see 1). With the most sensitive procedures, using isotope-labelling and long exposure times, the sensitivity, claimed for parasites obtained from in vitro cultures, was 20 to 50 parasites per µl. However, using blood samples from infected individuals sensitivity was considerably lower (approx. 200 to 1'000 parasites/µl). A comparative study of four hybridization assays with different probes and procedures (part of a vaccination trial) confirmed that all of them were less sensitive than microscopic examination of thick blood films or in vitro cultivation (2). In fact, the DNA probes had a disappointing sensitivity of only 5 to 28% compared to culture and 13 to 40% compared to thick films. The application of DNA hybridization as an alternative diagnostic method for malaria is thus still in its initial phase. Further improvements, especially of sample preparation and of the detection system may increase sensitivity.

A second alternative approach to the diagnosis of malaria is the immunological detection of red blood cell-associated antigens or soluble antigens in the serum. Two test principles have been used so far. In the first, a competitive radio-immunoassay, malaria antigens (in blood lysates) were used to bind polyclonal (3) or monoclonal antibodies (4) and thus inhibit the subsequent binding of these
antibodies to antigens bound to a solid phase. The results of preliminary attempts to use this method were far from satisfying. The sensitivity was much too low (several thousand parasites/µl). A much better sensitivity was reported by a different technique, an immunoradiometric assay (IRMA) (5). An excellent correlation was reported between IRMA binding activity and parasitaemia for in vitro cultured parasites. However, this correlation was less satisfactory when blood samples from patients were analysed. A major problem is the interpretation of antigen-positive results from microscopically-negative individuals. One explanation is the persistence of antigen from an infection that has been treated. Antigens could be detected by IRMA as long as 2 weeks after the disappearance of parasitaemia. This could cause confusion and lead for false diagnostic of other febrile illnesses. In addition, considering the common practice of self-medication for malaria in endemic areas, parasite antigen detection in human blood might not be a satisfactory alternative to conventional microscopy in prevalence surveys.

Antigen detection assays have proven their merits for detecting infective mosquitoes (6). Two-site immunoenzymatic methods using monoclonal antibodies against the repetitive epitope of the P. falciparum circumsporozoite antigen are now reliable and applicable tools in the field measuring impact of control on transmission or giving valid answers to entomological questions (7).

An effective method for the rapid detection of acute infections - where the lengthy procedure of DNA hybridization and present antigen detection assays are inappropriate - was recently developed by Becton-Dickinson (Franklin Lakes, N.J.). The clever principle of this QBC technique, staining parasitic DNA with
acridine-orange and concentrating infected red blood cells by centrifugation, is based on a modified microhaematocrit tube. Infected red blood cells can be easily detected in a 1 - 2 mm broad band using fluorescence microscopy. High sensitivity is to be expected since a larger blood sample can be rapidly examined (55 µl vs about 0.4 µl equivalent to 200 fields of a thick-film preparation). In a first field evaluation, the QBC method appeared to be at least 8 times as sensitive as conventional microscopy, detecting an additional 10 % of infections not diagnosed by conventional microscopy (8). In our experience with imported malaria cases QBC has proved to be as sensitive as classical microscopy (9), and in a holoendemic area we confirmed a slightly higher sensitivity of QBC. The great advantage is the remarkable time gain for microscopic reading; the test needs about 1 compared with 10 - 15 minutes for thick films. This is especially important if there are many negative samples to be screened. For dependable species diagnosis, however, blood has to be reexamined by conventionally stained blood-films.

2. Amoebiasis

It is estimated that approximately 10 % of the world’s population are infected by Entamoeba histolytica and that in some developing countries invasive amoebiasis is among the 10 leading causes of death (10). Imperfect diagnostic tests limit our perception of the magnitude and severity of this disease. The two major problems concerning diagnosis are to find more efficient methods to detect an intestinal infection, and simpler ways to distinguish pathogenic and non-pathogenic forms (isolates). Even using concentration methods for
microscopical detection, a reliable diagnosis needs repeated stool examinations. From our own data using the "SAF"-method (11), which involves fixation and concentration of the parasites, we calculated - from analyses on multiple specimens - that at least 4 stool specimens are needed to guarantee a predictive value for a negative result of 0.99 (9). If only one false-negative out of 1'000 stool specimens is acceptable, one would have to analyse 10 stool samples from each patient.

Since it is evident that dependable stool examinations are strenuous, time-consuming, expensive and rely on a high level of microscopic skills, alternative diagnostic approaches were attempted. The first development of an ELISA test to detect stool antigens was described in 1978 (12). Using a commercial immunozyyme test kit (Millipore Corp. Bedford, Mass.) conflicting results were reported with regard to sensitivity and specificity. This test depended on polyclonal antibodies, but more recent attempts utilized monoclonal antibodies for antigen capture (13). Unfortunately the excretion of antigens, like that of trophozoites or cysts, is irregular and, therefore it is still necessary to analyse of multiple stool specimens to reach an acceptable level of sensitivity (own results).

Recently, first results using a DNA hybridization technique as an alternative to microscopy were reported (14). The diagnostic clones, detecting highly-repeated parasite DNA sequences, reacted specifically with as few as 800 amoebae, but did not distinguish between pathogenic and non-pathogenic zymodemes of E. histolytica. Further investigations with multiple sampling of individuals are needed to determine reliable predictive values.
When it comes to the analysis of (potential) virulence of an isolate, the current "gold standard" is the zymodeme analysis introduced by Sargeaunt and coworkers (for review see 15). The isoenzyme profiles obtained after electrophoresis allow the distinction of virulent and avirulent E. histolytica isolates. This technique is, however, too time-consuming for most diagnostic laboratories. New ways of distinguishing E. histolytica possessing pathogenic and nonpathogenic zymodemes were opened by using either genomic DNA (16) or cDNA probes (17). Preliminary results indicate that pathogenic isolates of E. histolytica are genetically distinct from nonpathogenic isolates. In addition to their diagnostic use, these probes could serve as tools to investigate the molecular basis of pathogenicity.

Serology is an important tool to aid in the diagnosis of suspected extraintestinal involvement or cases of bloody diarrhoea or chronic colitis. A wide range of methods, ranging from the very simple (e.g. Latex agglutination) to the rather sophisticated (time-resolved fluoro-immunoassay), were evaluated (for review see 18). For all of them, the major dilemma is to interpret a positive serological finding in an endemic area. This is related to the fact that antibodies due to past infections persist and that antibody titers can be low in the early stages of liver abscess formation. A solution to this might be the choice of (an) appropriate diagnostic antigen(s) - produced by recombinant DNA technique or as synthetic peptide(s) - in combination with class- or subclass-specific antibody detection of an early immune response after invasion. In view of the high mortality due to invasive amoebiasis, the development of a robust and reliable immune assay (e.g. dipstick or dot blot test) seems to me to be an urgent priority.
3. Intestinal Nematodiasis

The detection of intestinal infections due to nematodes is still the field of classical microscopical techniques. Since morbidity is related to worm burden, the quantitative assessment of egg counts is relevant. Recent studies have clearly demonstrated the impact of nematode infections on health, growth and physical fitness. Programmes for the control of nematodiasis are now being actively reassessed by WHO, the World Bank, UNESCO and UNICEF. However, there is no consensus on whether diagnostic screening before treatment is a required component of such programmes. The issue of mass treatment versus diagnostic screening has recently been discussed (19). The central question still remains unanswered is it acceptable to treat individuals without knowing their infection status? Besides the economic issue - a screening component increases programme cost by a factor of 2 to 6 - diagnostic screening is laborious and requires trained technicians and laboratory equipment. Diagnostic screening would therefore only be possible if simpler diagnostic tools were available. For severe hookworm infections, haematocrit values have been shown to be a possible indicator.

A negative consequence of any diagnostic screening, which often seems to be ignored, is that a significant proportion of infected individuals remain untreated, since the level of compliance with stool sampling is reported to be of the order of 50 to 70% (19). If one opts for diagnostic screening (to be in concordance with good medical practice) one ought to add an educational component to the control programme in order to enhance compliance. This problem illustrates the fact that the diagnosis of infectious diseases includes more than technical
laboratory aspects. A diagnostic procedure has to find its place in a given health system, and has to be accepted by the health personnel and by the population concerned.

4. Filariasis and onchocerciasis

There are major limitations to the parasitological diagnosis of tissue-dwelling nematodes. Infections remain parasitologically unidentified during the long prepatent period (until adult worm produce microfilariae) and in light infections, as well as in individuals with an acquired immunity to circulating microfilariae. In addition, the periodicity of certain blood microfilariae necessitates the sampling of blood at night, which presents a significant obstacle for epidemiological studies. The replacement of night-blood by an alternative method is one of the defined diagnostic goals of the Tropical Diseases Programme (TDR) of WHO.

For one possible approach, the detection of circulating antigens, a variety of immunological methods have been utilized, with polyclonal or monoclonal antibodies as reagents. To take the example of a monoclonal antibody-based immunoradiometric assay (IRMA), evaluations have clearly shown an association of patent infection with detection of the target epitope, and a good correlation between levels of serum antigen and blood microfilarial counts (20). However, circulating antigens could also be detected in amicrofilaraemic subjects with acute symptoms of lymphatic filariasis, as well as in about half of the asymptomatic amicrofilaraemic individuals. Similar findings were also reported using other test systems.
The interpretation of a positive antigen test for an amicrofilaraemic individual is rather difficult. Does it mean that we are detecting a latent infection, or is antigen detection in "endemic controls" a sign for active immunity? From what has been said and in view of the possible interference with host antibodies, detection of circulating antigens seems not to be the appropriate approach to replace microfilarial blood counts, but it could be used to follow the effect of filaricidal drugs administration, as was shown in a study in Papua New Guinea (21).

Specific DNA probes were produced to diagnose Brugia malayi. A method to detect microfilariae in blood specimens has been developed (22). There are ongoing efforts to adapt this technique for field application.

For onchocerciasis, major advances can be reported on two diagnostic issues which are especially relevant for the Onchocerciasis Control Programme (OCP) in West Africa. The first is related to the differentiation of the savannah and the forest forms of Onchocerca volvulus using specific DNA probes (23). These forms differ in the symptoms produced: The savannah form more often leads to blindness. This differentiation is therefore relevant with regard to identifying the potential pathogenicity of parasites carried by vectors invading the control area. The second issue is investigating the recrudescence of transmission in the control area, where an early indicator for reinfection is an urgent need. It has been shown that in early infections specific antibody detection is more sensitive than parasitological examination of skin snips (24). The old problem of the poor specificity of serological tests can be overcome by using recombinant antigens selected for high specificity (25,26,27,28). Owing to individual variations in the immune response, a mixture of several recombinant antigens will increase
sensitivity. For that purpose a collaborative study, including candidate antigens from several laboratories, has been organized within the TDR programme with the aim of developing a reliable field test.

5. Schistosomiasis

Morbidity in chronic schistosomal infections mainly related to the magnitude of egg production, which is a function of the number of adult worms. I like to restrict discussions on that aspect taking the case of urinary schistosomiasis. The identification of heavily infected individuals is therefore an important diagnostic issue, in order to prevent morbidity by timely drug treatment. The standard diagnostic procedure is the urine filtration technique which produces quantitative egg counts. However, multiple daily samplings of infected individuals revealed extreme fluctuations of the egg output over time (29), and even in heavy infections egg-negative results were not infrequently observed. Analysis on a single day detected only 44% of heavily infected children. A single urine examination is therefore not a reliable indicator for measuring the actual worm load. Repeated urine examinations require an excessive amount of work even for a single patient, and are certainly not realistic for public health purposes.

When community diagnosis (identifying villages for which urinary schistosomiasis is a major health problem) is the diagnostic aim, diagnostic tools simpler than urine filtration have to be available. Reagent strips to test urine for blood or protein have been evaluated (30). Both indicators have a high sensitivity for detecting egg-positive urine specimens but specificity was not optimal, especially for proteinuria. Results from two different endemic areas showed
significant variations which shows that locally defined criteria for the interpretation of test results are necessary. In a recent study in Tanzania, testing for microhaematuria was found to be a reliable indicator (93 % sensitivity and 92 % specificity) for heavy infections (29).

An interesting approach is the immunological detection of parasite antigens in the urine. Especially the groups of Drs. Deelder & de Jonge (University of Leiden) have put much effort in the development of antigen detecting assays over the last ten years. The detection of the "circulating anodic antigen" (CAA) in the urine by a monoclonal antibody-based two-site immunoenzyme assay had a sensitivity of 97% (31). If further validation attests adequate specificity and a simpler test format can be designed, such a test could become an interesting candidate for public health purposes. Preliminary studies point to an additional possible use of this antigen-detection assay, to monitor the efficacy of chemotherapy (32).

A completely different approach to measure morbidity in schistosomiasis is the use of ultrasound (33). Various studies have been shown that this is an efficient technique which is applicable and acceptable in the field at community level.

A completely different approach, which may prove valuable for public health purposes, is the use of questionnaires based on the disease-perception of members of the community. In a study of urinary schistosomiasis a questionnaire administered to teachers and schoolchildren was tested as a diagnostic tool (34). In comparison to urine filtration, this cost-effective way of screening revealed a sensitivity of 100 % and a astonishingly high specificity of 87 % for
schools with a high infection rate. Using this approach of rapid assessment with key informants combined with selective reagent stick testing (performed by instructed teachers in schools identified by the questionnaire) the distribution of \textit{S. haematobium} in a rural district with over 300'000 people could be mapped within a period of 4 months at a total cost of less than 1 US cent per inhabitant (34). Currently this diagnostic approach is validated for schistosomiasis in different African countries.

\textbf{Perspectives on future developments}

The enormous potential of new technologies is being very efficiently exploited for the diagnosis of diseases which are of concern for the industrialized world. Although recent advances in the diagnosis of tropical diseases are discernable, they are only a few real breakthroughs which have resulted in diagnostic tools that are appropriate for widespread routine use. The development of a diagnostic test is a rather complex and costly process (major stages are listed in table 2). For each step, the definition of precise objectives is needed; this requires the multidisciplinary collaboration of various specialists. Too often, researchers are not fully aware of the problems of developing countries with respect to urgent diagnostic needs and appropriate techniques. On the other hand, field workers have insufficient knowledge about new diagnostic possibilities. Field research networks were recently incorporated into the TDR programme to recruit more scientists for field related research. Operational programmes, known as FIELDLINCS, have been initiated to improve interactions between researchers, managers and health workers from health ministries and national disease control
programmes.

Training of scientists from developing countries, and the development of research capacities there, are important components of programmes of WHO, United Nations Industrial Development Organization (UNIDO) and others. In the field of biotechnology, the International Centre for Genetic Engineering and Biotechnology, a major UNIDO project, fulfills this role. Goal-oriented research in diagnostics has to include test developers and test users from the beginning in order to ban costly mistakes and further due to ignorance of diagnostic needs, imprecise diagnostic objectives, inappropriate techniques or irrelevant validation. A dependable validation is a prerequisite for the production of a test kit. Such a validation has to include evaluating the test with defined specimens (e.g. from serum banks) to validate technical specifications. However this is not enough; it is vital also to prove the appropriateness of the method under "real-life" conditions outside the developer's laboratory. For its inclusion in a health system or control programme the applicability of the method and its acceptability has to be tested.

The final step is the production of a diagnostic test kit. For this, a partner from the industry has to be found. However, the decline in the interest of industry in the industrialized world in parasitic diseases is a major obstacle. Making profits from diagnostic tools is apparently the driving force for test development. The aim of the newly established Product Development Unit within the TDR programme is to accelerate the development of high priority products, and to stimulate collaboration with partners from the industry. In the future, partners may be more easily found in the developing world, as a result of the
programmes mentioned above.

I am confident that a goal-oriented, multidisciplinary approach, initiated and steered by international collaboration, will produce more and more appropriate diagnostic methods for large scale use. When such methods are available, they will be able to make a major contribution to achieving the ultimate goal of improving health in the tropics.

Acknowledgements

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References


Table 1. Estimates of prevalence and mortality of major infections in Africa, Asia and Latin America (from published data by WHO and World Bank)

<table>
<thead>
<tr>
<th></th>
<th>Infections</th>
<th>Mortality</th>
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<tr>
<td>Diarrhoeas</td>
<td>5'000'000'000</td>
<td>10'000'000</td>
</tr>
<tr>
<td>Respiratory infections</td>
<td>unknown</td>
<td>5'000'000</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>1'000'000'000</td>
<td>400'000</td>
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<tr>
<td><strong>Parasitic infections:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria</td>
<td>800'000'000</td>
<td>1'500'000</td>
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<tr>
<td>Amoebiasis</td>
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<td>75'000</td>
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<tr>
<td>Schistosomiasis</td>
<td>200'000'000</td>
<td>750'000</td>
</tr>
<tr>
<td>A.</td>
<td><strong>Identify diagnostic necessities</strong>&lt;br&gt;a) for communities&lt;br&gt;to assess prevalence, morbidity etc.&lt;br&gt;(to implement or to monitor control measures)&lt;br&gt;b) for individuals&lt;br&gt;to diagnose acute cases, identify individuals at risk to develop disease, assess immune status etc.&lt;br&gt;(to treat or to prevent disease)</td>
<td>Remarks</td>
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<td>B.</td>
<td><strong>Define precise diagnostic objective(s)</strong>&lt;br&gt;set specifications related to&lt;br&gt;a) predictive values&lt;br&gt;b) technical level&lt;br&gt;c) standards of performance (e.g. accuracy, precision, reproducibility)</td>
<td>Remarks</td>
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<td><strong>DECISION about step C &amp; D</strong></td>
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<td></td>
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<tr>
<td>C.</td>
<td><strong>Develop diagnostic method</strong>&lt;br&gt;a) produce reagents&lt;br&gt;b) establish appropriate technique</td>
<td>Remarks</td>
</tr>
<tr>
<td>D.</td>
<td><strong>Validate diagnostic test</strong>&lt;br&gt;a) related to fixed specifications&lt;br&gt;b) appropriateness in authentic environment (applicability, acceptance, efficiency)</td>
<td>Remarks</td>
</tr>
<tr>
<td><strong>DECISION about step E</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.</td>
<td><strong>Produce diagnostic test kit</strong>&lt;br&gt;(including quality control)</td>
<td>Remarks</td>
</tr>
<tr>
<td>F.</td>
<td><strong>Apply test</strong></td>
<td>Remarks</td>
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