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Immunodiagnostic Approaches in Schistosomiasis

Immunodiagnostic Approaches in Schistosomiasis

Proceedings of ICGEB/TDR Symposium, Shanghai, 1990

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The International Centre for Genetic Engineering and Biotechnology (ICGEB) was established to assist developing countries in realizing the promise of "new" biotechnology to improve human health and alleviate problems of food production.

ICGEB acts as a central resource for its Members by providing the necessary "critical mass" environment to pursue advanced research in biotechnology, and thus strengthen their R&D capabilities. The chief functions of the Centre are to:

- undertake research and development in molecular and cell biology for the benefit of developing countries and
- offer advanced training and access to state-of-the-art facilities and services

ICGEB has hitherto been managed as a project of the United Nations Industrial Development Organization (UNIDO), but will become an autonomous intergovernmental organization when 24 of its member countries have ratified its statutes. So far (August 1991), 23 of the 43 present member countries have taken this step.

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Two component laboratories at Trieste, Italy and New Delhi, India, constitute the permanent facilities of the Centre, which also consists of an expanding network of affiliated laboratories in member countries. The research staff at each component laboratory consists of senior scientists directing research groups composed of junior scientists, support staff and trainee fellows from the Member Countries.

The Centre presently (1991) employs some 150 staff at its interim facilities in Trieste (2000 m^2) and New Delhi (1800 m^2). It is planned to reach the full complement of research and administrative staff, at both components, by the end of 1993.

Construction of new facilities of 6000 m^2 and 10,000 m^2 for Trieste and New Delhi, respectively, is scheduled for completion by the end of 1992. New guesthouse facilities are already available in New Delhi and there are similar plans for Trieste.

Funding takes place under a rolling five year programme which is annually reviewed and extended. The current operational budget, for the 1989-1994 period, provides for a total expenditure of $58 million. Funds are provided by the Governments of India and Italy, the Research Area of Trieste — the authority that administers the science park where the Trieste component is located — as well as voluntary contributions from member countries.

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The research and development programme of ICGEB is dedicated to the application of genetic engineering and biotechnology to problems of relevance to the developing world. The programme currently consists of three main areas:

- human health, with emphasis on infectious disease control and vaccine production;
- agribiology, with emphasis on crop improvement; and
- biomass conversion, with emphasis on lignocellulose degradation.

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The Special Programme for Research and Training in Tropical Diseases, known as TDR (Tropical Diseases Research Programme), is co-sponsored by the United Nations Development Programme (UNDP), the World Bank, and the World Health Organization (WHO). TDR is administered by WHO in Geneva and supported by voluntary contributions from governments, international organizations, foundations, and other non-governmental agencies.

TDR was constituted in 1978 with the twofold objective of promoting the development of new ways to prevent, diagnose, and treat the major tropical diseases, and to strengthen the research capabilities of scientists in countries where the diseases are endemic.

These diseases are malaria, schistosomiasis, filariasis (both onchocerciasis or river blindness and lymphatic filariasis or elephantiasis), trypanosomiasis (African sleeping sickness and Chagas disease), the leishmaniases and leprosy. Collectively, these diseases directly or indirectly affected an estimated one billion people.

Over 4,500 scientists from 135 countries are part of TDR's worldwide network of experts. Their work, in a wide range of disciplines, is bearing fruit in the form of new disease control tools.

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Immunodiagnosis is as old as the discovery of antibodies and their key role in infection. Indeed, this indirect means of detection of infectious agents has proved to be an important adjunct to microbial cultivation techniques, greatly facilitating correct diagnosis.

Although the usefulness of immunodiagnosis in bacteriology and virology is undisputed, the more complicated relationship with the host of protozoal and helminth infections limits some of the advantages. These organisms, generally established in the host in the intermediate or long term, have evolved ways to successfully challenge the defence mechanisms of the body, thereby upsetting the normal kinetics of antibody formation. The protective immunity often seen after most bacterial or viral diseases is generally absent in parasite infections. In most of these infections, direct parasitological examination remains today the only unequivocal means of diagnosis.

In schistosomiasis, serological techniques have been available since early this century but have, for the reasons mentioned, not yet played a role in efforts to control the disease. Although there is no case to be made for serology in highly endemic areas, it has become increasingly evident that parasitological techniques frequently, even after repeated examinations, fail to reveal low-intensity infections. For this reason, in spite of the often difficult interpretation of the results, epidemiological survey teams have tentatively incorporated serology as a way of improving the diagnostic record in geographical areas characterized by a low level of transmission.

For national programmes of schistosomiasis control, the importance of useful and reliable immunodiagnosis is obvious. In addition, there is an imperative need for sensitive and specific methods of evaluation for the envisaged future trials of a schistosomiasis vaccine. The current developments in this area are being followed with great interest and the collaboration with the Institute of Parasitic Diseases of the Chinese Academy of Preventive Medicine in this endeavour is highly appreciated.

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Immunodiagnosis of Schistosomiasis

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Scope of the problem

Schistosomiasis is typically a life-long chronic disease. This is due partly to the irreversible morbidity caused by granulomatous reactions to schistosome eggs deposited in the tissues and partly to the longevity of the parasite, but it depends mainly on the slow and inefficient development of immunity to this parasite. After the primary infection early in life numerous reinfections, initially largely unopposed, occur over the first 10 to 15 years of age resulting in a relatively rapid build-up of parasites followed by a slow decrease. The introduction of safe and effective chemotherapeutic treatment has improved the control of schistosomiasis immensely but a proportion of the population, usually directly related to the initial prevalence in the area, requires retreatment. Although reinfection is common in these individuals, the generally lower worm burdens following the introduction of wide-spread chemotherapy has highlighted the need for improved diagnostic procedures.

Clinical examination and investigations by X-ray or ultrasonography all represent indirect means of diagnosis since the morbidity and not the underlying infection is targeted. The only unequivocal way of determining the presence or absence of living schistosomes in the body would be to actually dislodge and retrieve them from the blood but, in practice, the finding of eggs in stool or urine specimens or circulating parasite antigens in serum or other body fluids is usually sufficient for the diagnosis. Recombinant schistosome DNA probes would provide a direct diagnosis but, unfortunately, the advantages of this technique can only be utilized in the snail intermediate host. Unbound by these limitations, serology addresses direct or indirect diagnostic aspects depending on whether antibodies or antigens are used as the catching reagent. An overview of the different approaches to diagnosis is depicted in Figure 1.

Direct parasitological techniques are now well standardized and the inclusion of quality control has improved diagnosis in the field. Such methods have proved useful in numerous epidemiological surveys and in selecting patients for treatment in areas with moderate to high intensity of infection but the relative insensitivity of stool and urine examinations has become apparent in areas characterized by low prevalence and low intensity of infection[1]. This has resulted in an increased interest

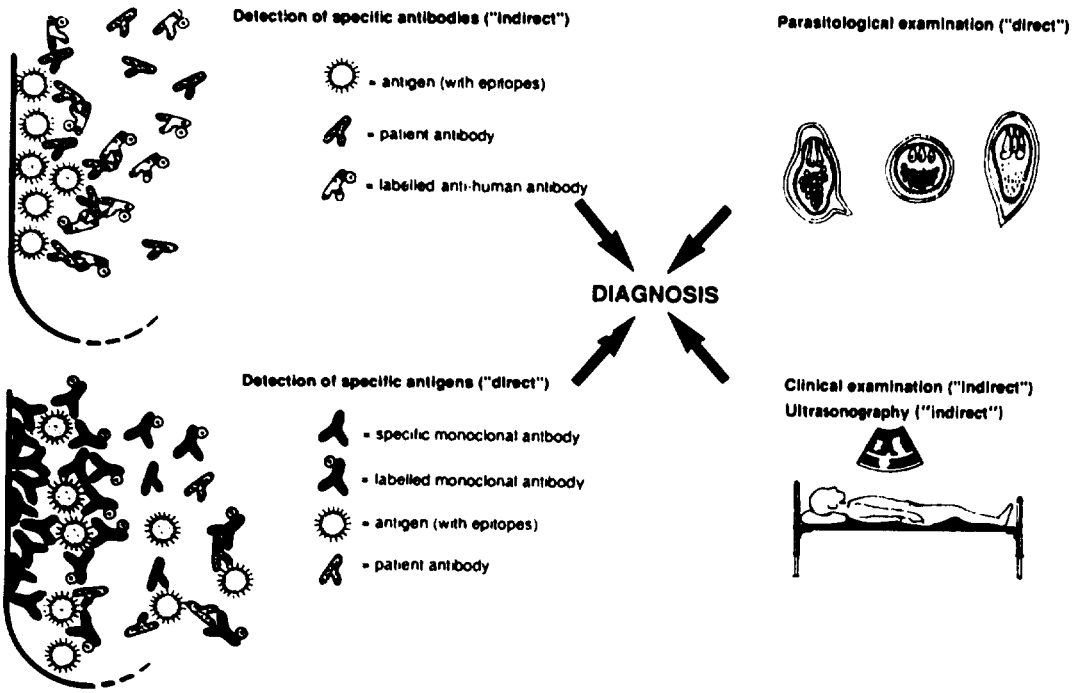

Figure 1 Schematic overview of direct and indirect approaches to diagnosis of schistosomiasis

in implementing serological methods but the urgent need to do so is hampered by the lack of suitable techniques for use under field conditions. Constraints include expensive equipment, costly and/or labile reagents and, commonly, the need for skilled personnel. In addition, mass-produced standardized assay kits are not yet available.

Antibody detection

The slow reduction of specific antibody levels after treatment diminishes the value of antibody detection in endemic areas, except in very special cases: for estimates of prevalence in the few populations which have not been previously investigated or treated, and in surveys of areas where schistosomiasis was eradicated a long time ago. Some preparations, however, such as for example the CEF6 antigen which seems to preferentially elicit antibodies of short-term duration[2], could conceivably be useful for post-treatment surveys in endemic countries. Since parasitological techniques are insufficiently sensitive when applied in controlled areas with very low intensity of infection and prevalence, antibody detection is often used for want of better techniques. That this approach is not without its merits is shown by the common use of serology in China as well as in the Philippines.

Antigen detection

Technical obstacles related to sensitivity and specificity have long precluded the assessment of prevalence of active infection but there are now signs that these difficulties are being overcome. Application of the hybridoma technique, permitting large-scale production of antibodies with exactly defined specificities, has resulted in the development of sensitive ultraspecific techniques for the detection of different schistosome constituents released into serum. In particular, the presence of a 70k Da circulating anodic antigen (CAA) in humans infected with schistosomiasis, as revealed by the enzyme-linked immunosorbent assay (ELISA), corresponds well with actual infection[3]. The results of these studies showed a sensitivity of around 90%, whilst the specificity was always 100%. In addition, some schistosome antigens, including CAA, are excreted in the urine, thus opening an approach to collection of specimens which will probably have a high rate of acceptance in the field. Recent promising studies indicate that urine specimens can be successfully used for diagnosis in patients with infection due to *Schistosoma haematobium* as well as to *S.mansoni* [4,5]. It is likely that this approach will also be valid in active schistosomiasis due to other species.

Immunodiagnostic techniques

Serology can basically be approached in two different ways: batch techniques which are useful for large-scale testing in well-equipped laboratories and sample techniques which can be used individually in the field. Laboratory-based testing generally requires expensive equipment and skilled personnel; yet costs are considerably reduced by automated procedures geared to bulk processing of sera. Although the standardized routine of execution also renders this approach reliable, the commonly experienced lack of infra structure in most endemic areas makes field diagnosis obligatory. Screening techniques utilizing equipment and reagents that can be incorporated into field kits would therefore be useful. Elements considered essential in the development of such kit assays are:

a) easily collectable test specimens (blood or preferably urine);
b) a simple and standardized procedure with clear-cut interpretations permitting rapidly available results (ideally while the patient waits);

c) the possibility to store and use reagents under hot and humid conditions;
d) inexpensive production and distribution of diagnostic kits.

Several serologic methods have been used for the diagnosis of schistosomiasis[6,7]. Those most commonly and widely used include various forms of ELISA, indirect immunofluorescence (IF), gel precipitation techniques, indirect haemagglutination (IHA) or latex agglutination tests (LAT), and the circumoval precipitin test (COPT). The majority of these tests can be carried out with less than five microlitres of plasma drawn from the top of a haematocrit tube after centrifugation.

ELISA requires a series of successive incubations with serum and labile reagents (conjugate and substrate) which must be performed under carefully standardized conditions demanding a high degree of technical skill. It is a typical laboratory-based technique but, if prefabricated standard kits are used, variations such as, for example, dot-ELISA[8] lend themselves to individual diagnosis in the field without the need for special equipment and experience.

IF can be performed with specimens of either frozen or lyophilized antigen sections. Although it is typically geared to individual diagnosis, the need for an expensive and complicated microscope, experienced personnel and delicate reagents precludes its use outside the laboratory.

Gel precipitation techniques do not exhibit high sensitivity and require at least six hours of diffusion in a fragile layer of agar and are thus not practical under field conditions. On the other hand, they do not require expensive equipment, reagents or skilled personnel.

IHA is normally performed in one hour and reading can be carried out with the naked eye. Reconstituted lyophilized antigen-coated erythrocytes can be used and, as in most ELISA applications, one microtitre plate is adequate for the screening of 94 specimens.

LAT utilizes a suspension of latex peptides with fixed antigens, the lyophilization of which improves stability. The test can be carried out on a commercially available plasticized card or other suitable surface by mixing a drop of whole blood or serum of the patient and a drop of reconstituted antigen. It can be read with the naked eye after two minutes of gentle shaking.

COPT is a simple and useful gel precipitation technique but the substantial number of washed schistosome eggs and amount of serum needed for large-scale applications could be a constraint. There is also a need for standardization of interpretation so that results from different geographical areas can be accurately compared.

Monoclonal reagents

Antigen detection relies increasingly on monoclonal antibodies (MAbs) as catching reagents and although the specificity of these reagents cannot be surpassed, their extremely narrow range of reactivity may render the results prey to error. Unless a battery of MAbs including specificities for different determinants is utilized, epitopes which are not expressed on all circulating antigens may be missed. In this way, sensitivity can be increased without sacrificing specificity. Once characterized and in production, MAbs provide reproducible reagents which can be prepared in large quantities at relatively low cost. MAbs reacting with genus-specific epitopes may prove useful in qualitative diagnostic tests, whereas MAbs to species specific antigens would provide important data for epidemiological and taxonomic investigations. Diagnostic assays require high affinity antibodies, whilst antibodies with a lower affinity facilitate antigen purification for antibody detection. Immunodiagnosis of schistosomiasis relies on particular MAbs with specificities to:

a) antigens common to all species infective to man, excluding animal-specific schistosomes;
b) antigens exclusive for certain populations or regions, e.g. species-specific antigens;
c) certain types of antigens, e.g. dominant surface structures and excretory secretory molecules, which commonly elicit humoral immune responses in infected humans;
d) antigens which pass through the glomerular membrane and are detectable in the urine.

In addition, MAbs reactive with different human antibody classes and isotypes are needed for analyzing the humoral response in immune and non-immune individuals.

Reagents based on recombinant DNA techniques

Ideally, antigen preparations should consist of pure native specific molecules which can actually be achieved with the aid of the recombinant DNA technique which permits mass-production of standardized protein. DNA sequences can be manipulated to attain expression of the corresponding antigens and different recombinant sequences can be further recombined to create new gene combinations. So far, however, only one attempt has been made to produce cloned diagnostic schistosome antigens[7]. Although the results of preliminary trials using fusion proteins have not been unequivocal this approach would be the solution to unwanted cross reactions.

In a direct approach, this technology has been used for the preparation of DNA probes which have proved to be a sensitive and highly specific means of demonstrating the presence or absence of an infectious agent in the host. Unusually stable and with no need for gene expression, such probes can define even minor differences. They have been used for the diagnosis of several microbiological infections including parasitoses but may not be suitable for diseases due to multicellular organisms. Although schistosome fragments may be found in the blood of the host, this is probably infrequent and mainly associated with treatment. Application of this approach for serological surveys might therefore produce a high proportion of falsely negative results.

When applied to the snail intermediate host, on the other hand, DNA probes may be utilized for determining strain differences. This technique has its main application in the field of taxonomy but it is also of value in general malacology and epidemiology. In addition, schistosome DNA sequences can be used for differentiation between schistosome strains[8] and as an indicator of the sex of cercariae[9]. These applications, however, fall somewhat outside the present discussion and, although DNA probes have several attractive features, they are unlikely to be of diagnostic relevance in the human situation.

Interpretation of results

When evaluating an assay, the relation of test results to the real situation falls into four different categories, as depicted in Figure 2. The theoretical reliability of diagnostic techniques is generally expressed in terms of sensitivity (percentage of infected subjects positive in the assay) and specificity (percentage of correctly diagnosed non-infected subjects in relation to all those negative in the test). Based on these measurements, an index was proposed by Youden[10] according to which the test performance can be expressed as the following quotient (using the symbols in Figure 2):

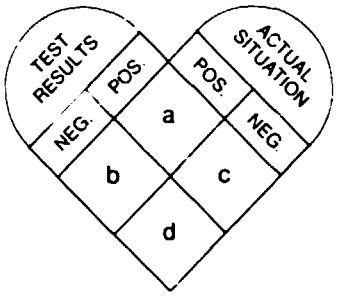

Figure 2 The relation between test results and the real situation. The letters signify the variable number of individuals in each category

$$\frac{(a \times d) - (b \times c)}{(a + b) \times (c + d)}$$

Theoretically, this index varies between -1 and +1 but since a test of any standing will result in a higher proportion of positive results in those subjects who are indeed infected as compared to controls, it is practically always above zero. However, the significance of an assay depends not only on sensitivity and specificity but also on the frequency of infected subjects in relation to examined individuals. By ignoring the effect of prevalence, the Youden index failed to account for how accurately an assay predicts presence or absence of infection in a given geographical area. This prompted the introduction of predictive values of positive (PV_{pos}) and of negative test results (PV_{neg}) which can be calculated according to Galen and Gambino[13] by another set of equations:

$$PV_{pos} = a/(a+c) \quad \text{and} \quad PV_{neg} = d/(b + d)$$

The test efficiency, defined as the proportion of an investigated population which is correctly classified by the assay as being either infected or non-infected, can be expressed as:

$$\frac{(a + d)}{(a + b + c + d)}$$

In immunodiagnosis of schistosomiasis, cross-reaction with antigens from other helminths whose endemicity coincides with that of schistosomiasis, is a serious source of error[14]. It needs to be emphasized that efforts to improve methodological

sensitivity are of little value until the problem of specificity has been fully mastered. Insufficient attention to this fact has resulted in a disproportionate growth in the number of serological techniques, leaving basic methodological parameters inadequately addressed. The practical usefulness of assays must therefore be carefully estimated before implementation in the field. In order to assess the situation in the area of schistosomiasis serology, two collaborative studies have been undertaken[15,16]. The results indicate that crude *S. japonicum* egg antigen, in spite of its unpurified nature, is relatively specific and correlates also to some degree with the intensity of infection. A third collaborative study, carried out in China with the aim of comparing different *S. japonicum* antigens and techniques in local use, emphasizes this conclusion while showing a generally good correspondence between results from different laboratories and regions in spite of the many different techniques used[17]. Although more work is still needed, these studies contribute to a beginning of standardization for large-scale use of schistosomiasis serology. It should, in this context, be mentioned that the utilization of molecular biology for production of pure candidate vaccine antigens is providing an increasing number of pure antigens which can, in some cases, also be diagnostically utilized.

Research priorities

The collaborative study on immunodiagnosis in China has emphasized the importance of investigating reagents and procedures in clinically well-characterized patients in a geographically defined region. This approach should now be extended to other geographically diverse endemic areas with the aim of testing the performance of assays in different settings and in relation to different species and strains of schistosomes in order to:

a) define various serodiagnostic test kits in order to ensure that reproducible standardized batches of reagents are produced;
b) investigate the usefulness of assays capable of antigen detection in large-scale human trials;
c) develop assays permitting quantitative determination of actual worm burdens for use in human vaccine trials;
d) investigate if sensitivity can be improved by combining sets of MAbs with different specificities;
e) investigate the role of immune complexes; in particular, to find out if masking of important epitopes by patient antibodies reduces sensitivity in antigen detecting assays;
f) characterize, immunologically and biochemically, useful diagnostic schistosome antigens found by screening with MAbs of unknown specificities.

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Community-Based Immunodiagnosis of Schistosomiasis

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Summary

During the past decade impressive advances have occurred in our understanding of the molecular biology of human schistosomiasis. Together with the rise of biotechnology this progress heralded the emergence of new diagnostic technologies which promised rapid, affordable and appropriate new methods for the diagnosis of schistosomiasis at community level where the vast majority of infected individuals reside. However, progress in the development of such field methods has not been as rapid as expected and the challenges that remain have been underestimated. This paper attempts to assess these challenges, to review the requirements of community/field level schistosomiasis immunodiagnostics from various user perspectives and to specify the performance characteristics required of the new assays. The lack of design criteria which reflect the demands of the field, and the inadequate validation of new methods have impeded progress in this area. More research effort is needed on the design and development of simplified detection systems for circulating or excreted schistosome antigens or metabolites to provide definitive, quantitative diagnoses relevant not just to infection and transmission parameters, but also to disease and morbidity patterns. Finally, it must be recognized that what is needed at peripheral levels of the health system is a core technology capable of addressing communities' priority health problems beyond a single disease.

Introduction

There have been impressive advances in our understanding of the molecular biology of human schistosomiasis during the last decade. In the early 1980's these advances coincided with the rise of biotechnology, and together, this progress heralded the emergence of new diagnostic technologies; technologies which promised rapid, affordable and appropriate new methods for the diagnosis of schistosomiasis at community level where the vast majority of infected individuals reside[1]. However,

progress in the development of such tools has not been as rapid as expected and the challenges that remain have been underestimated. This paper attempts to assess these challenges, to review the needs for community/field-level schistosomiasis immunodiagnostics from various user perspectives, to specify the performance characteristics required of the new assays, and to outline a general strategy for their development.

Schistosomiasis and the Field Perspective

The parasitological diagnosis of schistosomiasis at community level is now possible due to the availability of practical quantitative methods (urine filtration and faecal smear)[2]. Even though these parasitological methods have less than desired sensitivity, field workers are unlikely to replace them with the new generation of more sensitive "field" immunodiagnostic methods. The reason for this is rooted in the lack of an overall strategy for a coordinated approach to managing the required research and development of field applicable immunodiagnostic technologies and from the lack of a clear understanding of the context in which field or community based tests are ultimately applied.

What do we mean when we use the term "field" with respect to diagnostics? For some, the field is any service laboratory environment outside of the research laboratory. To others, it may be a laboratory (mobile or static) in a non-health service setting. More simply, the field may be any non-laboratory setting. The field may also be defined as those situations in which tests are conducted by non-technical personnel or it may mean peripheral or primary health services. Clearly there are many different "field" situations, some of which would demand different test design characteristics. All too often though, diagnostics developers stop at developing tests suitable for use at tertiary referral levels which test large numbers of specimens collected from the field for epidemiologic purposes. Such tests should not be confused with tests which are truly field applicable. *The predominantly rural nature of schistosomiasis demands tests which can be performed on-the-spot in rural communities in the absence of laboratory facilities and technical virtuosity.*

Whose view?

In developing field applicable methods, whose view of the field should prevail: the technology developer, or the technology user? Clearly it should be the latter, however, throughout the 1980's we have seen major diagnostic technology development projects, often funded by international development agencies, fail in their attempt to marry the promises of biotechnology to the needs of primary health care diagnostics. These failed, often because the technology users were not adequately involved in setting the design criteria for the methods. *The field cannot adapt to the technology; the technology must adapt to the field.*

What assumptions are common?

Since most immunodiagnostic tests are developed in laboratories in the industrial world and then adapted in various ways to the developing world, it is to be expected that certain assumptions will be made that may not always pertain. For schistosomiasis, one assumption is that in all countries where schistosomiasis field tests will be applied, laboratory services exist as an integrated part of the health system. However, in Africa especially, it is difficult to identify any office within Ministries of Health which is solely responsible for the coordination of health laboratory services from a national level. In such a country one expects to find laboratory capability at the central or national research centre (usually at least one

centre); at tertiary referral hospital(s) (one or a few); at regional or provincial hospitals (perhaps 20); at district and parastatal hospitals (100); and at division level health centres (400). Beyond this is the periphery of the health system where we approach the field settings in which laboratory facilities do not normally exist and these include ward, township or parastatal dispensaries (1800) and community health posts (8000). Immunodiagnostic capability presently reaches down only to district hospital level, however the laboratory referral and support hierarchy often operates sub-optimally even between national and district levels. The health system is not well geared to support laboratory diagnostic methods at community or field level. Hence such methods will need to be highly independent. An epidemiologic test designed for field application will normally have a more direct link to the national level and may not need to be as robust in this regard. *Corollary support for laboratory services as an integral component of the health system in developing countries must be contemplated if field level use of immunodiagnostics is to be sustainable.*

A second important assumption is that a good diagnostic test can be applied in all field situations. This would be ideal; however, field objectives are so varied that this becomes unrealistic. In schistosomiasis, field applications may include studies to establish baseline data prior to vaccine field trials; studies during vaccine field trials or population chemotherapy trials; community-based surveys associated with national control programs; primary health care diagnosis; surveillance in low prevalence situations following successful control; and sentinel site surveillance following eradication. Some of these are epidemiologic applications while others are diagnostic. This dichotomy of applications demands specific design features. *Tests designed for diagnosis should not (directly) be used for epidemiology.* The problems created by such applications will be illustrated later.

Problems and Challenges for Field- or Community-Based Immunodiagnosis for Schistosomiasis

Field realities

There are several constraints to effective use of laboratory tests at field level. All must be considered when designing new methods. Already mentioned above is the fact that there will be relatively little support from the health system itself. Furthermore, those performing the test will likely have minimal technical qualifications. There is less supervision at peripheral levels than normally required. Quality control and quality assurance will need to be built in. There will likely be no electricity or cold chain. Instrument maintenance will be difficult. Resources in general will be scarce and motivation difficult to maintain. Finally, and perhaps most important for schistosomiasis, is that competing health system costs demand that the recurrent costs per test be very low. The main problem that determines most of these realities is the fact that the field setting exists largely outside the health system.

Competing technologies

Another challenge to the development of effective immunodiagnostic tests for schistosomiasis is the fact that practical, field applicable, direct and indirect diagnostic methods already exist and are used. These include urine filtration, faecal thick smear, reagent stick tests[2], and even ultrasonography[3] and possibly questionnaires[4,5]. The main advantages of many of these approaches is that they allow rapid unit testing and continuous flow processing of subjects at community level rather than batch testing. In addition, very efficient field protocols exist for the

operational flow of registration, diagnosis, treatment and health education using such methods[2]. However, most immunodiagnostic tests popular today (eg. ELISA) are designed for batch testing at a central referral site (usually district level or higher) thus requiring a costly return visit to report results and provide treatment. There are no schistosomiasis immunodiagnostic tests rapid enough for on-the-spot testing with results available within 30 minutes that would work within existing protocols. That being said, deficiencies in the parasitologic methods are increasingly recognized[6,7]. The most important is the lack of sensitivity due to the variability of egg excretion in stool or urine, and poor correlation with intensity of infection and morbidity for the same reason. Recent studies by Lengeler *et al.*[8,9] have confirmed that a single urine filtration for *Schistosoma haematobium* detects only about 67% of infections and 44% of intense infections (>50 eggs per 10 ml). Furthermore, these studies indicated that a single haematuria reagent stick test was as predictive as five sequential daily urine filtrations. This sends two important messages to the developers of indirect diagnostic methods such as immunodiagnosis for schistosomiasis:

1) *the performance of immunodiagnostic tests cannot be validated using specimens from patients classified on the basis of a single parasitologic test; and*
2) *immunoassays to detect circulating or excreted antigens or morbidity markers should be superior to single parasitologic tests.*

Low prevalence applications

Tests designed and validated for diagnostic applications cannot be used directly for all epidemiologic applications. This is because in diagnostic applications there is a clinical pre-selection of patients such that prevalence in the testing population is usually moderate and clinically important infections may be intense. In some epidemiologic applications, especially following control programs, prevalence may be low and infection intensities may be light. Nevertheless it is common practice that tests designed for serodiagnosis are used indiscriminately in seroepidemiology. This often results in the unexpected poor performance of the method or erroneous conclusions. For example, assume a situation in which a successful control program or intervention reduces schistosomiasis prevalence from 40% to 1%. If an immunodiagnostic test with a sensitivity of 95% and a specificity of 90% would be used to measure this reduction, one would be lead to the erroneous conclusion that initial prevalence was 44% and the post-intervention prevalence was 11%, an apparent four-fold reduction, rather than the true 40-fold reduction that was achieved. Furthermore, if one had set a goal to reduce prevalence to less than 10%, one would falsely conclude that the intervention had failed. The reason for this is that the predictive value of a positive result before and after the intervention would be 86.4% and 8.8% respectively. As prevalence falls, specificity of the test used must increase in order to maintain the same predictive value for positive results[10].

Very low prevalence applications

Immunodiagnostic tests are often called upon to replace parasitologic tests to monitor areas where schistosomiasis control has been highly successful and prevalence is very low. This is an important role. However, in such situations a diagnostic strategy relying on a single immunodiagnostic test will not work because of the low predictive values of positive results mentioned above. A screening and confirmation strategy using more than one test will be required and even this will need adjustments for different levels of prevalence.

To illustrate how this might operate, let us imagine a hypothetical situation in which immunodiagnostic specimens from 10,000 subjects are tested in the field in a low prevalence schistosomiasis setting (<1%)(Table I). The diagnostic strategy is to screen all specimens using a sensitive, practical, and inexpensive immunodiagnostic field test. All positive subjects will be then asked to provide faecal specimens for laboratory based testing before diagnosis is confirmed and treatment offered. The hypothetical immunodiagnostic test has a sensitivity of 98%. We can vary the specificity to estimate the effect on the number of parasitological tests required. By definition, the parasitologic test has a specificity of virtually 100%. Let us say that the sensitivity of a single parasitologic examination is 67% and that three sequential examinations would be done to bring the accumulated sensitivity up to the range of the serologic test. By varying the specificity hypothetically, one can calculate using predictive values and false positive rates, how many stool examinations would be required at each level of immunodiagnostic specificity between 80% and 99.9%. From there it is a simple matter to calculate the cost per case detected. In this example, the predictive value of an immunodiagnostic positive ranges from 4.7% to 90.8% when specificity ranges from 80% to 99.9%. At an immunodiagnostic specificity of 80%, over 6000 parasitologic confirmations would be required. The situation does not improve much even at 90% specificity. However, between 90% and 99% specificity, the number of parasitologic confirmations declines rapidly to a manageable number of around 600. Improving immunodiagnostic specificity higher

Table I Effect of immunodiagnostic specificity on case detection costs in a population with a low prevalence of schistosomiasis when a low cost immunodiagnostic field test is used to identify individuals for more costly parasitologic confirmation.

Field Immunodiagnostic Screen* Unit Cost = 1			Parasitologic Confirmation† Unit Cost = 10		
Specificity	PV_{pos}‡	False +ve Rate	No of Specimens Tested (a)	No of Confirmatory Tests (b)	Cost Case Detected§ (1a+10b÷100)
80	4.7	95.3	10,000	6,234	723
90	9.0	91.3	10,000	3,264	426
99	49.7	50.3	10,000	591	159
99.9	90.8	9.2	10,000	324	132
99.99	99.0	1.0	10,000	297	130

* The Field Immunodiagnostic Screen has a specificity shown in the table and a sensitivity of 98%.

† The Parasitologic Confirmation has a specificity of 100%, and cumulative sensitivity of 67%, 89%, and 98% on the first, second and third specimens. Three specimens are tested for each immunodiagnostic positive.

‡ PV_{pos} = predictive value of positive results.

§ Prevalence in the population is 1%, hence there are 100 true cases in the sample of 10,000.

than 99% does not result in dramatically fewer parasitological test requirements. In this example, it is clear that optimal specificity of the immunodiagnostic test deployed in such a strategy would need to be somewhere between 90 and 99%.

The task now would be to select the optimally cost-effective screening and confirmation strategy which suits the intended application and then select or develop an immunodiagnostic test with the required level of specificity. Different strategies would be required for different orders of magnitude in prevalence. Relative (Receiver) Operating Characteristic Curves or predictive values[10] can be calculated in advance to determine what level of sensitivity and specificity would be required of the immunodiagnostic test. Again, *field test design criteria must be decided in advance and the technology developed to meet the criteria.*

Inadequate validation of field immunodiagnostics

The vast majority of diagnostic tests ever developed never leave the laboratory in which they were developed. Those that perform well under such optimum conditions are sometimes adopted for field use and often perform surprisingly poorly. The reason for this high attrition rate is that tests are developed frequently on an *ad hoc* basis, usually exploiting a new technical approach to the production of the primary ligand. This is then placed into the most sensitive assay system available and optimized against a small panel of well defined clinical specimens from subjects with a limited spectrum of infections arising following transient visits to endemic areas. Such specimens do not reflect the nature of specimens typical of subjects indigenous to endemic areas. The new assay is then used in a variety of field trials. It is only at this time that the true performance characteristics of the test are revealed. However, the most important performance characteristics can often be determined well in advance of progressing to costly field trials. Moreover, targets for such characteristics should be set before tests are developed and the development process should be driven by these criteria.

Lack of minimum validation criteria

Validation criteria should be set early in the assay development process specifying the minimum target values acceptable for the following test parameters: sensitivity; specificity; positive and negative predictive values (assumes knowledge of the intended application); cross-reactivity (assumes knowledge of the important differential diagnoses possible in the intended application); and reproducibility[1]. In reviewing papers on schistosomiasis diagnostic technology, it is very difficult to find reports in which all of the above parameters have been measured and reported.

Design Needs for Community- and Field-Applicable Immunodiagnosis for Schistosomiasis

The growth of the market for physician's office testing in developed countries (in some ways, analogous to field testing in primary health care settings) has lead to a number of very rapid and practical, albeit costly, immunodiagnostic test formats. By establishing partnerships with industry, some of these formats could be adapted to

Low cost (< 0.10 US$)
Rapid (minutes)
Unit (vs. batch) test format
Robust (no instrumentation or cold chain required)
Built-in positive and negative controls
No specimen or reagent volume measurement ("dip-stick" format)
No specimen pre-treatment
Uses non- or less invasive clinical specimen, eg. urine, saliva, stool, or capillary blood
Appropriate: Sensitivity
Specificity
Predictive Values
Cross-reactivity
Reproducibility
Provides an index of morbidity or prognosis
Manufacturable in developing countries
Serves as a core-technology for additional diseases

This is not an impossible list. In fact HIV-1 antibody-detection systems are emerging which are approaching all of the above-mentioned criteria.

How can this be achieved for schistosomiasis? **A "Development by Design"** approach is required. This invokes a strategic plan which involves a goal oriented, user directed, product development approach. Such a plan requires **cooperative research and development** with close links between the bench and the field at all stages; between basic and applied research; and between academia and industry. **Basic research** is needed to focus on the pathophysiology of infection and disease to identify the most important target markers and to develop molecular biological approaches to reagent production. **Applied research** is needed to establish proof of concept, develop prototype technology, validate the assay and conduct field trials. **Industry** partnership is required to refine the prototype to an appropriate technology, scale-up production, establish quality control, conduct kit development, and initiate technology transfer. Such cooperative "R&D" needs to be effectively funded and managed. For diseases such as schistosomiasis, this will probably depend on national and international public funding agencies such as WHO using direct project management executed by an international, non-governmental organization oriented to appropriate technology.

Conclusion

In conclusion, immunodiagnostic tests for schistosomiasis are not yet widely used at field/community levels, although certain developments, particularly regarding the detection of circulating and excreted antigens show promise[11,12]. Despite the increasing availability of superior and adequate reagents, in order to accelerate progress towards technologies which are truly field applicable, we must have a better understanding of the needs of field-based testing if we expect the methods to be appropriately developed and used sustainably. The lack of design criteria which reflect the demands of the field, and the inadequate validation of new methods continue to impede progress in this area. A case is made for concentrating efforts on the development of simplified detection systems for circulating or excreted schistosome antigens or metabolites, designed to provide definitive, quantitative diagnoses

relevant not just to infection and transmission parameters, but also to disease and morbidity patterns. The days have passed when a single laboratory or research group can carry a promising diagnostics development project all the way from concept to a finished product useable in the field. A call is made for more strategic research and development management process which continuously recognizes the participation and the needs of the field while coordinating the necessary cooperation between basic research, applied research and the skills of the diagnostics industry. Sustainable use of immunodiagnostics at field level will require a strengthened and more effective hierarchy of laboratory services throughout the health systems of countries where schistosomiasis is endemic. Finally, it must be recognized that what is needed at peripheral levels of the health system is a core technology which will eventually be capable of addressing communities' priority health problems beyond a single disease.

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Serological Techniques for Diagnosing Schistosomiasis in the Individual

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Summary

Serological techniques to demonstrate circulating antibodies are powerful tools to detect cases of human schistosomiasis not only in acute disease ("Katayama syndrome") but also in later stages of infection since the sensitivity of parasitological methods is rather poor.

We analysed 50 of our outpatients with positive serological tests and no prior treatment for schistosomiasis retrospectively in terms of their past and present history, exposure, clinical and laboratory findings (stool and urine examinations, eosinophilic blood counts). In only 21 cases (42%) was infection confirmed by parasitological findings (18 *Schistosoma mansoni*, two *S. haematobium*, one mixed infection). All patients had lived in areas known to be endemic for schistosomiasis (45 for at least one year, 47 in Africa) and most of them gave a clear history of exposure. In 28 (56%) cases eosinophilic blood counts were > 400/mm^3. Extremely high values (> 2000 eosinophils/mm^3) were found in two patients during acute disease.

For screening purposes we currently test patient sera against two different antigens of *S. mansoni* (an adult NP40 extract and a crude soluble egg antigen [SEA]) by an enzyme-linked immuno-sorbent assay (ELISA). Those sera which react with at least one of the two antigens are further tested by an indirect immuno-fluorescent antibody test (IFAT) using frozen sections of male worms. More than 5000 sera were screened during the last three years. All 30 sera from patients with parasitologically proven, active *S. mansoni* infections were positive in one of the two ELISA screening-tests (28 with NP40, 27 with SEA) of which 27 were confirmed by the IFAT (reciprocal titre > 80). This screening method seems to be less sensitive for *S. haematobium* (only five cases; four detected by ELISA, two by IFAT).

In contrast to our earlier findings in permanent residents in endemic areas, specific IgE-antibodies (measured by a radio-allergosorbent test, RAST) were of

limited diagnostic value in our outpatients. Eleven out of 16 cases (nine with total serum IgE < 200 kU/l) were RAST-negative.

Recent developments in the field of antibody-detection methods (use of recombinant antigens, analysis of subclasses of IgG antibodies) are reviewed. However, the main limitation of all serological methods, i.e. the long persistence of serum antibodies after treatment over long periods of time, remains.

Introduction

Definitive diagnosis of schistosomiasis requires the demonstration of viable eggs in faeces, urine or histological samples. It is, however, well known that the sensitivity of parasitological methods is rather poor. This applies even to concentration methods, such as the formol-ether sedimentation technique (after Ritchie) or the standard 10 ml-filtration method. The mean sensitivity of the former was found to be 47.6% for *Schistosoma japonicum* when multiple samples from individuals living in an endemic area in Japan were analyzed[1]. The standard filtration method for *S. haematobium* reached a mean sensitivity for a single examination of only 40% when the overall prevalence was set at 100 % for five samples[2]. This study was carried out on children in the United Republic of Tanzania, who were all heavily infected (>50 eggs/10 ml in at least one urine sample). It proved that daily fluctuations of egg-output are high. Parasitological methods are even less helpful for diagnosing schistosomiasis with a low parasite burden after limited exposure, or in chronic infections with a low egg-output. In addition, parasitological methods are not suitable for detecting cases of acute disease ("Katayama" syndrome) or when worms have not yet started to produce eggs (prepatent period).

Therefore other criteria for diagnosing schistosomiasis have to be considered. Gelfand suggested a clear history of exposure as a second major criterion for diagnosis besides the parasitological evidence[3]. The risk of exposure, however, is often difficult to assess because epidemiological patterns are highly focal. Positive serology, blood eosinophilia and clinical symptoms of acute disease (fever, dry cough, headache, abdominal pain, anorexia, haematuria [in urinary schistosomiasis]) are considered by Gelfand minor diagnostic criteria.

To evaluate our serological methods for diagnosing schistosomiasis in the individual, we analyzed the parasitological and serological findings of 420 outpatients whom we saw between 1985 and 1990. Additionally, we evaluated our serological techniques with sera of parasitologically proven cases sent to our laboratory and studied the files of 50 outpatients with positive serology in terms of the application of the diagnostic criteria mentioned above.

Methods

For screening purposes we currently use two different antigen preparations in a micro-ELISA system: membrane antigens of adult *S. mansoni* (detergent (Nonidet P40; NP40) extract of a pellet after PBS extraction) and crude soluble egg antigens (SEA) prepared from *S. mansoni* eggs from infected laboratory animals. Before 1987 a PBS-extract of adult *S. mansoni* was used. These antigens are part of a multi-antigen screening test for helminths (*Schistosoma, Fasciola, Echinococcus, Acanthocheilonema, Toxocara* and *Trichinella*) which was introduced in our laboratory in 1982[4]. Sera which react with at least one of the two *S. mansoni*

Table 1 Reactivities of parasitologically proven sera in ELISA and IFAT

Parasitological findings	N	ELISA (OD) NP40 N(>0.3)	NP40 mean (SD)	SEA N(>0.6)	SEA mean (SD)	IFAT reciprocal titre N(>80)	IFAT reciprocal titre GMRT*
S. mansoni	30	28	0.96 (0.58)	27	1.32 (0.67)	27	372
S. haematobium	5	3	0.62 (0.65)	4	1.30 (1.05)	2	145
S. mekongi	1	1	(0.37)	1	(1.40)	0†	(80)

* GMRT = geometric mean of reciprocal titres

† IFAT became positive (reciprocal titre 320) one month after treatment

Abbreviations: NP40: adult *S. mansoni* membrane extract, SEA: soluble *S. mansoni* egg extract

antigens are further tested by an indirect immunofluorescence antibody test (IFAT) using frozen sections of adult *S. mansoni*.[5] This test was established in our laboratory in 1968.

Sensitivity

The sensitivities of the methods are summarized in Table 1. All 30 cases with parasitologically proven *S. mansoni* infections reacted positively with at least one of the two antigens. Figure 1 gives the correlation of ELISA- with IFAT-results. The results of the ELISA using the adult NP40 antigen correlate well with those of the IFAT. In contrast, this does not apply to SEA-ELISA and IFAT. Twenty-seven of the 30 parasitologically-proven sera were considered positive by IFAT (reciprocal titre >80). Out of the three sera with an IFAT titre of 80 (borderline result), all three reacted weakly with NP40, two (one strongly) with SEA (Figure 1).

Similarities between antigens of different species enabled *S. haematobium* and *S. mekongi* infections to be detected using *S. mansoni* antigens. Sensitivity for *S. haematobium*, however, seems to be considerably lower than for *S. mansoni*. Four out of five sera were positive in the ELISA and only two in the IFAT (Table 1).

The reactivity of 67 IFAT-positive sera with NP40 and SEA is demonstrated in Figure 2. Four sera reacted only with SEA, 10 sera solely with NP40. Only one IFAT-positive serum was negative for both ELISA antigens.

Specificity

The specificity of the two ELISA antigens was evaluated with sera from 108 patients with parasitologically proven tissue helminth infections, clinical signs highly suggestive for trichinellosis or positive serological results for toxocariasis (Figure 3). Three out of 108 sera gave false-positive results with NP40. The specificity of

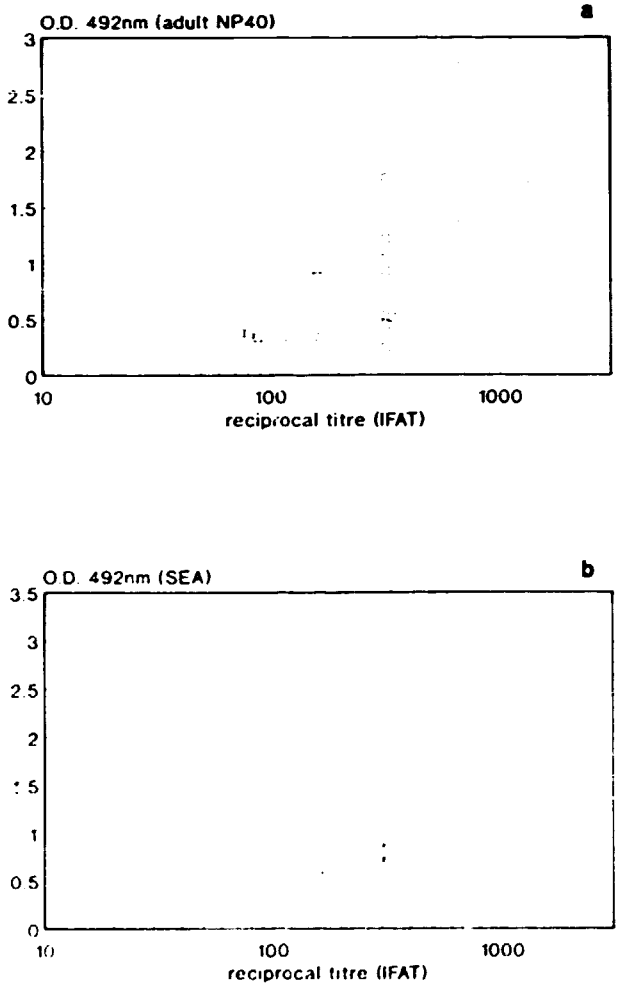

Figure 1 Serological results for 30 parasitologically proven *S. mansoni* sera in the indirect immunfluorescent antibody test (IFAT) and in ELISA using adult NP40 (a) or SEA (b) as antigens (dotted lines: threshold values for a positive result).

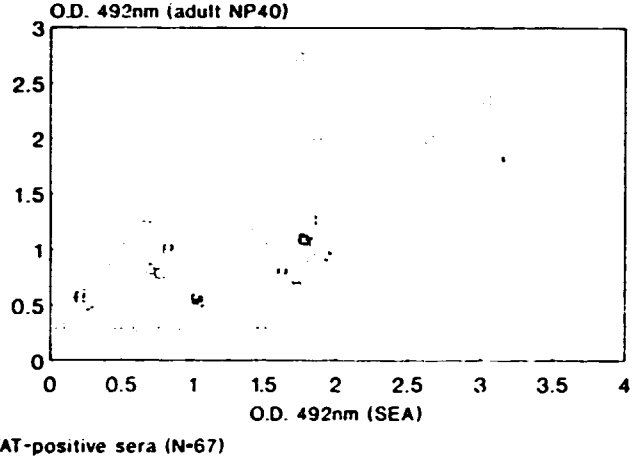

Figure 2 Reactivity of 67 sera with a positive IFAT (reciprocal titre > 80) against adult NP40 and SEA antigens in ELISA (dotted lines: threshold values for a positive result)

crude SEA was lower. The main problem is that of crossreacting anti-Trichinella antibodies (sera kindly provided by Dr. K. Cuperlowic, Beograd). Crossreactivity is due to a variety of glycoproteins as demonstrated by radio-immunoprecipitation and 2D-gel-electrophoresis[6]. Since in our screening procedure all sera are simultaneously tested against an excretory/secretory antigen preparation of *Trichinella spiralis* muscle larvae (kindly provided by Dr. Gamble, Beltsville) a serological diagnosis can still be made since the reaction patterns are different. Sera of patients with trichinellosis are more reactive against the homologous antigen (mean O.D. (± S.D.): 2.09 ± 0.85) than against SEA (mean O.D. (±S.D.): 0.73 ± 0.50). A single false-positive serological result (SEA-ELISA O.D.= 0.73, IFAT reciprocal titre = 640) was due to a *Capillaria hepatica* infection in a Swiss child with no exposure in the tropics.

Results and Discussion

Parasitological and serological findings in Swiss expatriates

Experts of the Swiss Development Agency and their family members who had lived overseas for more than two years (mean: four years) and who attended our outpatients clinic between 1985 and 1990 were studied[7]. Schistosomiasis mansoni was detected in 12 out of 420 individuals (2.9%), eight of whom were serologically positive, one was borderline, three were not tested (children). A positive serological result was found in 20 individuals (5.2% of all individuals tested; no serology in small children). Three out of 12 cases with positive serological results and negative parasitological findings had been treated for schistosomiasis in the past. Four of the

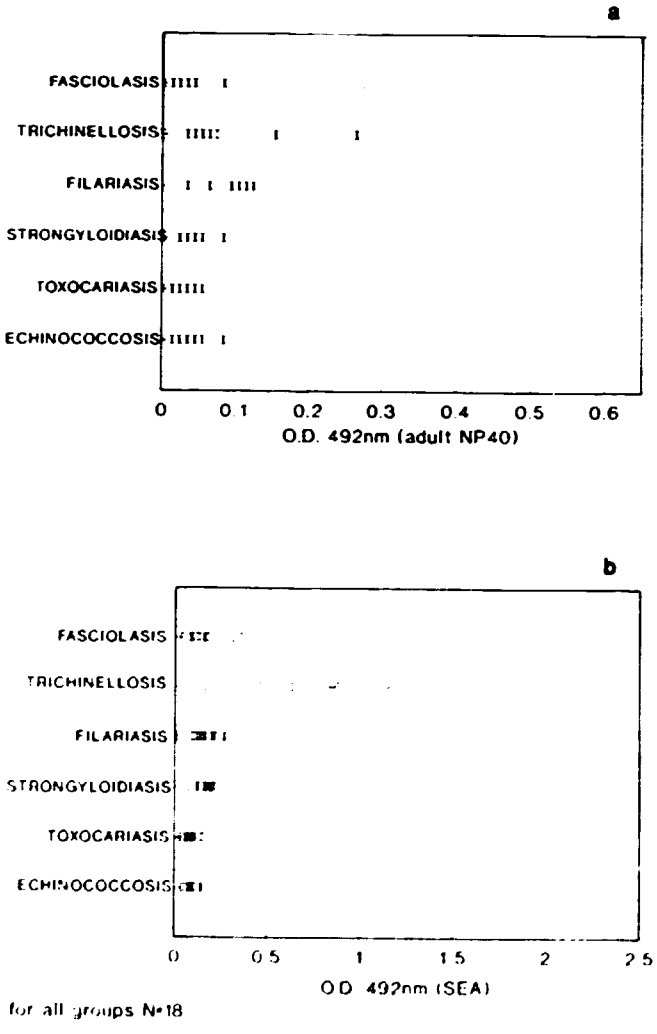

Figure 3 Reactivity of sera from individuals with parasitologically proven helminthic infections, clinical signs of trichinellosis or positive serology for toxocariasis in ELISA using adult NP40 (a) or SEA (b) antigens (dotted lines: threshold values for a positive result)

remaining nine cases had elevated eosinophilic blood counts (> 400/mm³). Positive serological results were clustered (12 cases in five families of which five cases in three families were parasitologically positive). Twelve out of 20 seropositive cases had returned from Rwanda (exposure in Lake Kivu!), four from Chad. We conclude from these data that amongst the criteria which contribute to establishing a diagnosis of schistosomiasis in expatriates who have lived some years in endemic areas, serological methods are of great importance.

Evaluation of positive serological results

History of exposure, clinical findings and laboratory results were studied in 50 outpatients with positive serological results but no prior treatment for schistosomiasis. Parasitological confirmation of infection was achieved in 21(42%) cases (18 *S. mansoni*, two *S. haematobium*, one mixed *S. mansoni*/*S. haematobium* infection). In some cases only one egg could be demonstrated in one stool sample out of two to three. All seropositive cases had been to areas endemic for schistosomiasis either as tourists or as temporary residents. Only two (4%) stated that they had had no risk of exposure. Clinically, only three were suspected to be infected (two with symptoms of acute disease, one case with haematuria).

Table 2 correlates parasitological, haematological and serological findings with the duration of travel or residence in areas endemic for schistosomiasis.

Table 2 Data of outpatients with positive serological results grouped according to the duration of risk of exposure and to parasitological findings

Travel/Residence in Endemic Area	N total	Eosinophils per mm³		ELISA Mean OD (SD)		IFAT GMRT*
		N (>400)	Mean (SD)	NP40	SEA	
< 6 months	5†	4	1949 (900)	0.47 (0.23)	1.13 (0.72)	160
0.5 - 5 years	20	12	404 (275)	0.79 (0.33)	1.03 (0.54)	437
parasit. pos	6	5	458 (174)	1.03 (0.27)	1.40 (0.39)	513
parasit. neg	14	7	381 (305)	0.70 (0.31)	0.89 (0.53)	407
> 5 years	25	12	456 (327)	0.92 (0.62)	1.23 (0.70)	263
parasit. pos	14	7	476 (352)	0.98 (0.71)	1.35 (0.70)	240
parasit. neg	11	5	429 (288)	0.86 (0.49)	1.09 (0.68)	302

* GMRT = geometric mean of reciprocal titres

† one parasit. positive, two acute cases

Table 3 Data from expatriates with parasitologically proven infections grouped according to IgE levels

Group (IgE levels) (kU/l)	Age (y)	Sex	Stay in endemic area (y)	Eosinophilia %	Total IgE (PRIST*) (kU/l)	RAST	IFAT recipr. titre	Parasite
A. >1000	62	M	23	14	3050	POS	320	*S. mansoni*
	36	M	5	12	4750	POS	<80	*S. haematob.*
	27	M	0.3	22	1870	POS	320	*S. mansoni*
	31	M	0.1	26.5	1400	neg	160	*S. haematob. /S. intercal.*
B. 200-1000	32	M	4	7.5	980	POS	160	*S. mansoni*
	42	M	1.3	22.5	480	POS	320	*S. mansoni*
	22	M	1	20.5	400	neg	160	*S. mansoni*
C. <200	54	M	25	6.5	185	neg	<80	*S. haematob.*
	38	M	14	2.5	52	neg	160	*S. mansoni*
	19	M	4	6.5	170	neg	<80*	*S. mansoni*
	26	F	2.5	11	90	neg	160	*S. mansoni*
	34	M	1	45	24	neg	320	*S. mansoni*
	31	F	1	0	20	neg	1280	*S. mansoni*
	23	M	1	17.5	175	neg	320	*S. mansoni*
	31	M	0.8	2.5	63	neg	320	*S. mansoni*
	24	F	0.3	18	130	neg	<80†	*S. mansoni*

* PRIST = Paper RadioImmunoSorbent Test

† One month post-treatment: IFAT reciprocal titre 160

Individuals were grouped into those with short (< six months), intermediate (up to five years) and long (> five years) periods of residence. As expected, the proportion of cases with positive parasitological findings increased over time with risk of infection/reinfection. Schistosomiasis is only rarely seen in tourists travelling for a few weeks in endemic areas. The risk is highest for temporary residents (see results for expatriates). Very high eosinophilic blood counts were significantly more frequent in early stages of infection, which confirms published results[8] In later stages of infection, eosinophilic blood counts rarely exceed 1000/mm^3; in 21 out of 45 cases (47%) eosinophilic blood counts were even below 400/mm^3. Serological results showed great inter-individual variation, but were generally not related to duration of risk of infection. Only in IFAT and in NP40-ELISA were differences between early and late infections observed (Table 2). In contrast, mean reactivity against SEA was similar in all three groups but was consistently higher in parasitologically proven cases.

Specific IgE antibodies as diagnostic criteria

High levels of circulating IgE are generally associated with infections due to tissue-dwelling helminths. Our earlier studies in Africans in endemic areas revealed high serum IgE levels (mean: >4000 kU/l) and a high prevalence (60-80%) of specific IgE anti-schistosome antibodies in a radio-allergosorbent test[9] Later we found that the sensitivity of this RAST assay was considerably lower in expatriates: IgE antibodies against adult *S. mansoni* antigens were only found in 14 out of 29 (48%) proven cases[10].

In just about 50% of all schistosomiasis patients we observe normal IgE levels (<200 kU/l) and negative RAST results. Detailed results for 16 outpatients are given in Table 3. In conclusion, IgE antibodies are rarely present in early stages of infection which confirms published data[11,12] So far we do not know why only half of our schistosomiasis patients mount an IgE response in later stages of infection. The pattern of exposure ("trickle" infections over a long time) or concomitant other helminthic infections might give a clue as to why most Africans develop high IgE antibody levels.

Follow-up criteria after chemotherapy

Some outpatients were closely followed for periods up to one to two years after treatment (data not shown). As shown by others[12], antibody titres only exceptionally decrease significantly within one year after treatment. We even observed that positive serological results persisted for more than five years after successful treatment in situations where there were no risk of reinfection. Eosinophilic blood

Table 4 Recent developments in antibody-detection methods using purified or recombinant antigens

Antigens	Origin	Method	References
CEF6	eggs *S. mansoni*	ELISA	Doenhoff *et al.* (1985)[14]
MSA-1	eggs *S. mansoni*	RIA	Stek *et al.* (1985)[15]
gp-2	eggs *S. japonicum*	ELISA	Tracy *et al.* (1985)[16]
Sm31/32*	adult *S. mansoni*	Immunoblot	Ruppel *et al.* (1987)[17] Idris and Ruppel (1988)[18]
Cysteine proteinases (CP1 and CP2)	adult *S. mansoni*	ELISA	Chappell *et al.* (1990)[19]
Sm31/32*	recombinant antigen	ELISA	Klinkert *et al.* (1988)[21]
Haemoglobinase	recombinant antigen	Dot-blot	Chappell *et al.* (1989)[22]
70 kDa peptide†	recombinant antigen		Newport *et al.* (1988)[20]

* Sm31 (CP2) has homology to Cathepsin B, Sm32 (CP1) is identical to haemoglobinase
† Homology with heat shock protein 70 (hsp-70)

eosinophils should not be expected promptly after treatment in all cases. The assessment of the success of treatment in seropositive patients is difficult as long as eosinophilic blood counts remain above normal values.

Recent developments in antibody-detection tests

Since the multi-center study organized by WHO in 1982 in which different methods were compared[3], the main advances have been in antigen purification, especially of SEA[14-16], and in the production of recombinant antigens (Table 4). Analysis of sera of schistosomiasis patients by immunoblot techniques demonstrated the heterogeneity of individual immune responses. This approach also allowed the identification of major diagnostic antigens: e.g. cysteine proteinases of adult worms[17-19] or a 70 kDa peptide (heat shock protein)[20]. Three of these antigens were produced by recombinant DNA technology[20-22]. Their diagnostic value, however, has not been fully assessed so far.

The availability of defined antigens is a prerequisite for the standardization of serological techniques. Further research on sera of well-documented patients is needed to design serological tests which will make it possible to discriminate between active and past infections and to quantify the intensity of infection. Antigen-detection assays could perhaps contribute more to the solution of some of these problems.

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Collaborative Study on Antigens and Methods for Immunodiagnosis of Schistosomiasis in China

Background and Study Design

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Background

Since the report of the last human case of *Schistosoma japonicum* infection in Japan in 1976, widespread schistosomiasis in the Far East is currently found only in China and the Philippines. Both these countries have operational national control programmes in the well-defined endemic areas. In the 1950s, when coordinated control efforts started in China, 378 counties were endemic for the infection. Since then, it has been eliminated in 150 counties and cities and brought under effective control in 110, but it is still prevalent in 118, the majority related to the river Yangtse [1,2].

Traditionally, schistosomiasis is diagnosed by direct parasitological techniques which are useful in areas with moderate to high intensity of infection. However, the relative insensitivity of stool and urine examinations makes them less suitable for well-controlled areas where both the prevalence and the intensity of infection are generally low. Various antibody detection methods have been implemented as adjuncts to faecal examination, especially in China. Since antibody titres diminish in the absence of reinfection, and eventually disappear or reach very

low levels, this approach could be a useful tool in the maintenance phase of control programmes.

Immunological diagnosis of schistosomiasis has been available for the better part of this century and was first applied to *S. japonicum* infection[3]. However, it was not until the advent of modern biotechnology that these assays were improved sufficiently enough to offer an alternative to parasitological diagnosis. Two collaborative studies have been carried out with the aim of identifying guidelines for the implementation of serology in this discipline[4,5]. In both of these studies, crude *S. japonicum* soluble egg antigen (SEA) preparations showed high sensitivity and adequate specificity compared to standard parasitological techniques. In addition, the quantitative sero-reactivity of the characterized *S. japonicum* egg antigens correlated directly with the intensity of infection in all age groups. However, differentiation between past and current infection will continue to pose a problem until improved immunological or new biochemical tests are widely available. In addition, the occurrence of cross-reactions with other infectious agents, helminths in particular, constitutes another obstacle yet to be overcome. In spite of these constraints, there has been a considerable growth in the number of immunodiagnostic techniques, thus prompting a renewed assessment of the situation.

Objectives of the study

The main aim of this third collaborative study is to evaluate the sensitivity and specificity of a number of different serological assays for the diagnosis of schistosomiasis due to *S. japonicum*, including different antigens in general use in different localities. The ultimate goal is to identify antigens and test systems which could be proposed for use in national control programmes.

The data will be analyzed to determine whether 1) infection can be assessed both qualitatively and quantitatively by serology; 2) clinical morbidity can be correlated to these results; and 3) past and present infection can be differentiated using antibody detection.

Participating laboratories

In China, the national control programme for schistosomiasis relies on reference laboratories which belong to Institutes of Parasitic Diseases situated in the different provinces. Seven of these laboratories, all currently performing serological analysis for schistosomiasis on an independent basis, were selected because of their experience in immunodiagnosis and their access to sera from individuals infected with schistosomiasis. Four of the laboratories are situated in regions still considered endemic for this infection:

Institute of Parasitic Diseases, Hubei Academy of Medical Sciences, Wuhan;
Hunan Provincial Institute of Parasitic Diseases, Yueyang;
Department of Parasitology, Nanjing Medical College, Nanjing; and
Institute of Parasitic Diseases, Sichuan Academy of Medical Sciences, Chengdu.

Three laboratories are situated in areas where schistosomiasis was highly endemic before 1965 but where the disease has been eradicated for more than 20 years:

Institute of Parasitic Diseases, Chinese Academy of Preventative Medicine, Shanghai;
Department of Parasitology, Shanghai Second Medical University, Shanghai; and
Department of Parasitology, Suzhou Medical College, Suzhou.

Table 1 Number of subjects who provided sera by age and sex

Age	Sex		Total
	Male	Female	
1-4	2	0	2
5-9	9	3	12
10-14	52	27	79
15-24	96	84	180
25-44	135	90	225
45+	47	25	72
Total	341	229	570

In addition, the Division of Parasitic Diseases, Centers for Disease Control (CDC), Atlanta, United States also participated. This laboratory specializes in the development and application of serological techniques and has a close collaboration with Chinese laboratories.

The serum bank

All participating laboratories, except CDC, took part in the collection of sera for the study. The persons providing the sera were all examined clinically and subjected to a range of laboratory tests relevant to schistosomiasis and other clinical states, as depicted in the form shown in Figure 1. Stool tests were carried out using the modified Kato cellophane faecal thick smear technique[6] examining two slides from each sample. In addition, the miracidial hatching test[7], which is widely used in China, was performed on more than half (353) of all stool samples.

Characterized sera were obtained from 579 individuals but due to incomplete knowledge, either missing parasitological data or inadequate information regarding the subjects providing the samples, nine entries had to be eliminated. For this reason, the study will refer only to 570 sera (Table I). Although no particular sector of the population was favoured, sera from young adult males turned out to be in the majority (Figure 2). However, the serum bank does not represent any special area or ethnic group within the regions selected for the study.

Of these 570 sera, 418 come from subjects living in the provinces of Hunan, Hubei, Sichuan and Yunnan which are all endemic for schistosomiasis. The liver was palpable more than two cm below the costal margin in more than 53% of these subjects and 3% had undergone splenectomy. As many as 47% had been treated more than once while, on the other hand, 32% had never received any kind of chemotherapy for schistosomiasis. At the time of examination, 15 had ascites, 40 had a cough, 135 had diarrhoea, 26 had fever and 46 had some type of neurological disorder. Two hundred and fifty seven of them had *Ascaris*, 34 *Trichuris*, 28 hookworm and six *Fasciolopsis* infection. This group consists of four categories:

UNDP World Bank WHO
Special Programme for Research and Training in Tropical Diseases (TDR)

Collaborative Study of Schistosomiasis Immunodiagnosis in China

Form 1: Initial Examination

Identification

Laboratory | Village | Patient no. | Date of examination (day / month / year) | Name:

Sex [M] [F] Age Occupation

1 = Student 5 = Factory worker
2 = Teacher 6 = Housewife
3 = Farmer 7 = none
4 = Fisherman 8 = other (specify)

1-15

Treatment History

Number of treatments

Year of last treatment 19

Drug used in last treatment

1 = Praziquantel, 2 = Amoscanate,
3 = Antimonials, 4 = Furapromidum-Dipterex

16-20

Stool Examination

Hatching test (No. of miracidia) 38-39

Number of eggs counted: Slide 1 40-42; Slide 2 43-45

Occult blood in faeces [Y] [N] [ ] not done 46

Ascaris [Y] [N] Fasciolopsis [Y] [N]

Hookworm [Y] [N] Clonorchis [Y] [N]

Trichuris [Y] [N] other (specify) [Y] [N]

47-49 50-52

Symptoms / Clinical Signs

	in the past	at present
Liver size (mid-sternum)	cm (21-22)	
Liver size (mid-clavicle)	cm (23-24)	
Spleen size	cm (25-26) [R] removed (27)	
Ascites	[Y] [N] [?]	[Y] [N]
Coughing	[Y] [N] [?]	[Y] [N]
Diarrhoea	[Y] [N] [?]	[Y] [N]
Fever	[Y] [N] [?]	[Y] [N]
Neurological	[Y] [N] [?]	[Y] [N]

28-37

Blood Examination

Sedimentation rate mm/h

Haemoglobin g/100 ml

Eosinophilia %

53-60

Remarks: [Y] [N]

61

Figure 1

Table 2 Classification of subjects providing sera

Subjects from areas endemic for schistosomiasis:	
Acute infection	15
Chronic infection	308
Advanced infection	19
Previously infected	76
Subtotal	418
Subjects from areas non-endemic for schistosomiasis but endemic for other parasitic infections:	
Ascariasis	26
Clonorchiasis	37
Paragonimiasis	10
Parasitologically negative controls	9
Subtotal	82
Subjects from areas non-endemic for schistosomiasis, clonorchiasis and paragonimiasis:	
Parasitologically negative controls	70
Total	570

1. **Acute infections**: patients with severe clinical manifestations and recent exposure to snail-inhabited water whose symptoms include liver enlargement, palpable spleen, fever, urticaria, cough, nausea, vomiting and diarrhoea. Although these individuals are not always egg-positive they invariably show positive intradermal tests, positive serology and leucocytosis, mainly in the form of eosinophilia.
2. **Chronic infections**: subjects with hepatomegaly but otherwise mild symptoms such as dyspepsia and abdominal pain, sometimes associated with mucus or bloody discharge. These patients are always egg-positive and they show positive intradermal tests and positive serology.
3. **Advanced infections**: patients with clinical signs of liver cirrhosis such as hepatosplenomegaly, ascites, oesophageal varices and other collateral circulation. These patients do not all excrete eggs but they generally show positive serology and often have an inverse albumin to globulin ratio. Dwarfism also classifies patients to this category.
4. **Previously infected**: successfully treated subjects.

The remaining 152 sera constitute different sets of controls: 26 come from *Ascaris* infected individuals; 47 from subjects with other trematode infections such as paragonimiasis (from a region non-endemic for schistosomiasis in Hubei Province) and clonorchiasis (from regions non-endemic for schistosomiasis in the provinces

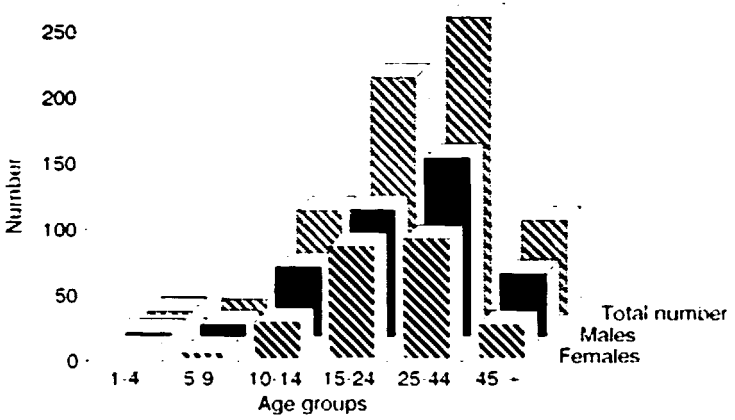

Figure 2 The collaborative study serum bank: distribution of sera by age and sex

of Sichuan and Anhui); and 79 parasitologically negative controls: nine from Hubei Province and 70 from individuals living in the Shandong and Shanghai provinces which are non-endemic for schistosomiasis (Table 2). Only subjects less than 20 years of age were chosen from the latter province since the disease was not eradicated there until the 1960s.

The hatching test was performed on about 75% (316) of the stool samples from the 418 individuals in the endemic areas (Table 3). Of the 149 samples in which no eggs were found, 56% had one or more miracidia, while eggs were found without any miracidia being detected in only two cases. Subjects were classified for the serological analysis as parasitologically negative (no *S. japonicum* eggs in the faeces and no miracidia according to the hatching test) or positive (at least one egg of *S. japonicum* in the faeces sample and/or at least one miracidium in the hatching test). Based on these criteria, only 327 of the 418 in the endemic group were positive including all persons in the "acute infection" or "chronic infection" subgroups (Table 4). As can also be seen in this table, all those assigned to the subgroup of "previously infected" were parasitologically negative, while the cases with "advanced infection" were equivocal in this respect.

In coding the sera for the study, 53 sera were given duplicate numbers in order to permit the reproducibility to be tested within each laboratory. Finally, aliquots of 0.5 ml of all sera were prepared and stored at -20 °C until dispatched as one package in dry ice (crushed ordinary ice was used for short transports). Each participating laboratory received a set of the sera, including the extra duplicate sera, which was kept at -20 °C or colder, until investigated.

Table 3 Number of eggs (as determined by two Kato examinations) by number of miracidia (as determined by the hatching test) in all cases investigated with both techniques

No. of Eggs	Number of Miracidia				Total
	0	1-5	6-29	30+	
0	83	51	15	0	149
1-9	2	60	67	16	145
10-49	0	1	6	13	20
50+	0	0	0	2	2
Total	85	112	88	31	316

Materials and methods

The participating laboratories have each tested the sera with their own routinely used techniques. The antigens for the tests were produced locally. Each laboratory normally utilizes two or three assays from the following list of test systems which are all included in the study:

Circumoval precipitin test (COPT) with lyophilized eggs;
COPT with heated and sonicated ova;
Indirect immunofluorescence with adult worm sections;
Indirect immunofluorescence with eggs in liver sections;
Indirect haemagglutination with soluble egg antigen (SEA);
Latex agglutination with SEA;
Enzyme-linked antigen counter-immunoelectrophoresis with SEA;
Enzyme-linked immunosorbent assay (ELISA) with SEA;
ELISA with gut-associated antigen (GAA);
ELISA with the major serological antigen (MSA);
Rapid ELISA on PVC films with urea soluble egg antigen (USA);
Avidin-biotin-ELISA with SEA; and
Dot-ELISA with SEA.

A detailed description of each test, including the characteristics of the antigen used, will be given in the final publication of the results of this study planned for later this year.

In order to compare crude and purified egg antigen preparations, all laboratories have carried out the Falcon Assay Screening Test (FAST) ELISA with crude SEA and with the *S. japonicum* microsomal antigen (JAMA)[8]. Due to the relative insensitivity of the stool examination it is planned to also test the sera for circulating antigens so as to include a comparison with one more direct test for schistosome infection. It is also planned to perform Western blots[9] with the objective of providing an improved assessment of antibody specificity for the evaluation of cross-reactions.

UNDP World Bank WHO
Special Programme for Research and Training in Tropical Diseases (TDR)

Collaborative Study of Schistosomiasis Immunodiagnosis in China

Form 2: Serological Results

Identification

Institute Sample no

Remarks:

This form was designed to accomodate all agreed upon combinations of techniques and antigens. Each laboratory should only perform and report those combinations which are routinely used at that site, plus JAMA FAST-ELISA (C4).

Serological Results

Antigens	Antigen	Technique	Pos	Neg	Numerical Result
A AWS					
B GAA					
C JAMA					
D LEA					
E LSE					
F MSA					
G SEA					
H SSE					
USA					
	E	1			
	E	1			
	E	1			
	C	4			
		5			

Techniques

1 ELISA
2 ABC
3 DELISA
4 FELISA
5 COPT
6 ECIE
7 IFA
8 IHA
9 LAT

Abbreviations Used

AWS		ELISA	
GAA		ABC	
JAMA		DELISA	
LEA		FELISA	FAST ELISA
LSE		COPT	
MSA		ECIE	
SEA		IFA	
SSE		IHA	
USA		LAT	

Figure 3

Table 4 Results of stool examination in the four study categories of subjects living in the endemic areas

Category	Negative	Positive	Total
Acute infection	0	15	15
Chronic infection	0	308	308
Advanced infection	15	4	19
Previously infected	76	0	76
Total	91	327	418

Statistical analysis

The participating laboratories have reported their serology test results to WHO Headquarters, Geneva, on the form represented in Figure 3. These data will be processed in conjunction with the clinical information and with other laboratory results provided earlier on Form 1 (Figure 1), using an IBM 370 computer and SPSS software. The specificity and sensitivity will be evaluated based on the results of the stool examinations and the qualitative assessment of the sera determined by criteria established by each laboratory for each antigen/test system. The serological results will also be compared with those of the test for circulating antigens which, in addition, will be directly correlated to the egg counts. The results of the duplicate sera will be analyzed qualitatively and quantitatively in order to assess the level of reproducibility within each laboratory.

All data will be analyzed according to the methodology presented in earlier collaborative studies[4,5]. Multiple regression will be carried out to determine the contribution of egg counts, morbidity, age and sex to the quantitative serological results. A full account of this analysis will be presented in the final publication which will also include a detailed discussion of the results.

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Field Trials for Immunodiagnosis with Reference to *Schistosoma mansoni*

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Summary

In our epidemiological studies of schistosomiasis mansoni performed in the Sao Paulo State over a period of 20 years, the higher prevalence rates found by immunological, in comparison with parasitological techniques (2.5 to 4.0 times higher), were due to lower intensity of infection in relation to other Brazilian areas. Recently, an immunoepidemiological survey was carried out in Pedro de Toledo (Sao Paulo State) where *Biomphalaria tenagophila* is the sole vector, transmitting *Schistosoma mansoni* infection at a low intensity (geometric mean = 58.5 eggs/g faeces), and the majority of parasite carriers are asymptomatic. Thus, immunofluorescence (IFT), intradermal (IDT) and Kato-Katz (KKT) techniques were evaluated in the population of that area for further determination of the true prevalence of schistosomiasis by a probabilistic model. The sensitivities of IFT, IDT and KKT were 98.8%, 92.8% and 32.4%, respectively, and their specificities were 78.9%, 81.8% and 100%. The overall prevalences given by these techniques were 55.5%, 51.8% and 14.3% respectively, but the corresponding true prevalence was 44.3%. Therefore, the immunological data indicate the population displaying a low parasite egg excretion rate does not reflect a necessarily low prevalence for *S.mansoni* infection. Also in untreated populations with a low intensity of infection, immunological techniques are useful and allow the estimation of the true prevalence based on their sensitivity and specificity. The parasitological techniques, however, are still needed to afford information about the intensity of infection.

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Introduction

Schistosoma mansoni is the only causative species of schistosomiasis in Brazil, and presently, about 30 million inhabitants are believed to be exposed to this infection. According to estimates made by our group, at least five million individuals are infected by *S.mansoni* in this country.

The National Control Programme, begun in 1976, has effectively reduced the morbidity; however, the potential risk of transmission still exists throughout the endemic areas, particularly near the numerous man-made bodies of water[1,2].

Biomphalaria glabrata is the major intermediate host among several snail species, but *B. tenagophila* is also found in a few areas.

We have been working since 1980 in the Municipality of Pedro de Toledo (Sao Paulo State), where *B. tenagophila* was a sole snail species; *S. mansoni* transmission level was low; and the majority of the parasitized individuals showed no clinical symptoms[3].

Our experiences over a period of 20 years, in the State of Sao Paulo, where the intensity of infection is generally low to moderate in comparison with that from other Brazilian endemic areas, have demonstrated that the prevalence rates provided by immunological techniques are always higher than those from parasitological methods[3,4,5].

Therefore, an epidemiological survey was conducted in untreated residents from rural and urban areas of Pedro de Toledo, using immunological and parasitological techniques, in an attempt to determine true prevalences with the use of a probabilistic model[6].

Materials and Methods

Population study

A total of 4,158 residents from rural and urban areas of Pedro de Toledo, with no previous schistosomiasis treatment, were studied.

Immunological and parasitological techniques

Blood samples were collected on filter paper and immunofluorescence technique (IFT) was carried out with adult worm sections[7], for the detection of IgG antibodies. The intradermal technique (IDT) was performed on subjects of 14 ars and older, as recommended[8]. Simultaneously, stool examinations were done by Kato-Katz quantitative technique (KKT)[3], preparing three slides per sample.

Statistical analyses

The sensitivity, specificity, positive and negative predictive values[9], and Youden (J) index[10] were calculated for each of the techniques here utilized. The probabilistic model[6] was used to determine the true prevalence. Also, Spearman's correlation coefficients[11] were calculated for the studies of epidemiological findings.

Results

A total of 4,158 residents from Pedro de Toledo were investigated for epidemiological purposes, but only 1,453 from the age group equal or higher than 14 years could be assessed concomitantly by three techniques (KKT, IDT and IFT), and 1,044 from age group under 14 years, by KKT and IFT.

The low parasite egg excretion rate (58.5 eggs/g faeces) was verified, and Table 1 illustrates the variability seen in five further parasite egg detections in subjects presenting positive results for immunological techniques and negative in KKT, upon first examination.

Table 1 Variability of parasitological technique (KKT)* in six residents from Pedro de Toledo (Sao Paulo State, Brazil) with low *S.mansoni* infection and positive for immunological techniques (IFT and IDT)†

	Stool Sample Collection (St) and Slide (s)																	
Case Number	St_1			St_2			St_3			St_4			St_5			St_6		
	s 1	s 2	s 3	s 1	s 2	s 3	s 1	s 2	s 3	s 1	s 2	s 3	s 1	s 2	s 3	s 1	s 2	s 3
1	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
3	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Kato-Katz technique

† Immunofluorescence and intradermal techniques

The agreement of positive results provided by IFT and KKT is higher than that by IDT and KKT, but apparently IFT as well as IDT gave high rates of false positives in relation to KKT (Table 2). However, those apparent false positive results were interpreted as " infected" (> or = 14 years) in case the positivity was observed for both immunological techniques, because we considered that KKT had low sensitivity (Table 3). Whereas those 352 children with KKT negatives but IF positives were dissociated as positive or negative in terms of probability through the following equation:

$$\text{True prevalence } (P_t) = \frac{\text{Presumed prevalence } (P_p) + \text{Specificity} - 1}{\text{Sensitivity} + \text{Specificity} - 1} \quad \text{(Ref. 6)}$$

in which KKT P_p = 14% (146/1,044); specificity = 100% and sensitivity = 32.4% (212/655). The latter value was taken from the data provided by the adult group, i.e. 14 years and older (Table 4), by assuming that the sensitivity for KKT was the same for the group of children under 14 years.

Based on these two interpretation criteria, a study of diagnostic performance of each technique could be carried out (Table 4). The IFT afforded high sensitivity in both age groups, but its specificity was satisfactory only for children.

Table 2 Agreement between immunofluorescence (IFT), intradermal (IDT) and Kato-Katz (KKT) techniques for the diagnosis of Schistosomiasis mansoni, in the study of 2,497 untreated residents of Pedro de Toledo (Sao Paulo State, Brazil)

Age group	Techniques	Agreement	
		Positive %	Negative %
<14 years	IFT/KKT	95.9 (140/146)	60.8 (546/898)
≥14 years	IFT/KKT	96.7 (205/212)	44.5 (552/1,241)
	IDT/KKT	77.8 (165/212)	52.6 (653/1,241)
	IDT/IFT	67.6 (604/894)	73.3 (410/559)
All ages	IFT/KKT	96.4 (345/358)	51.3 (1,098/2,139)

The true prevalences obtained theoretically by applying the above equation gave similar values to the data furnished by any of the three techniques (Table 5).

Accordingly, the 43.2% (40.2-46.2%) prevalence of schistosomiasis seen in children was close to that of 45.1% (42.5-47.7%) found for the adults. However, the rural population was considerably more infected than the urban population, with a prevalence of 50.7% (47.1-53.7%), compared to 39.1% (35.5-42.7%), respectively. Also, rural females were more infected than the urban females, with prevalences of 40.9% (35.7-46.1%) and 29.1% (24.6-33.6%), respectively.

Positive and high Spearman's correlation coefficients (Rs) were observed between the prevalences provided by KKT and IFT in the population divided either by age group (Rs = 0.670) or by settlements (Rs = 0.950).

Discussion

In our epidemiological studies of schistosomiasis mansoni performed in different areas o. Sao Paulo State, Brazil, over a period of 20 years, the high prevalence rates revealed by immunological techniques (2.5 to 4.0 times higher) in comparison with parasitological techniques were due to the low sensitivity of the latter, which in turn were associated with low or moderate intensity of infection[3,4,5]. Therefore in a population such as Pedro de Toledo, where the intensity is low, the evaluation of the Schistosomiasis Control Programme is critical if based exclusively on one stool examination.

The sensitivity of KKT determined here was 32.4% and this value seems consistent with that found by some authors (Mota et al. XXVII Congr. Soc. Bras. Med. Trop., 1981, Caldas Novas, Brazil, pp. 103-104). The evaluation of KKT sensitivity

Table 3 Combination of immunofluorescence (IFT), intradermal (IDT) and Kato-Katz (KKT) techniques for the diagnosis of Schistosomiasis mansoni, in the study of 2,497 untreated residents of Pedro de Toledo (Sao Paulo State, Brazil), and criteria of interpretation.

Age Group	Technique			Number of cases		Frequency %	Interpretation criterion
	KKT	IFT	IDT				
≥ 14 years	-	-	-	407			
	-	-	+	145		54.9 (798/1,453)	Negative
	-	+	-	246			
	-	+	+	443			
	+	+	+	161			
	+	-	-	44		45.1 (655/1,453)	Positive
	+	-	+	4			
	+	-	-	3			
Total				1,453			
< 14 years	-	-		546			
	-	+		352	47*	56.8 (593/1,044)	Negative
					305†		
	+	+		140		43.2 (451/1,044)	Positive
	+	-		6			
Total				1,044			

* - Negatives, † - positives, calculated by the probabilistic model[6]

is more difficult than for immunological techniques because of great daily variation in individual egg counts (Table 1), as well as of considerable influence of stool consistency.

The low sensitivity of KKT is one of the significant causes of the discrepancy between the parasitological and immunological techniques (Table 2). Neverthless, other factors such as some cross-reactivities with non-related parasitoses or with non-human cercaria allergies may cause false positives in immunological techniques.

The interpretation criteria adopted for the immunological techniques (Table 3) allowed us to verify the diagnostic features of each of three techniques. IDT and IFT yielded high values of sensitivity, 92.8% and 98.8%, respectively (Table 4), and they are close to those reported[12,13]. However, the specificities obtained by the same techniques were in general lower than those found by different investigators[7,12,13]. These low specificities can be ascribed mainly to the type of population selected for the studies, because the specificity of IFT, we found, is comparable to that verified in the study of similarly sampled populations to ours[14].

Table 4 Diagnostic performance of Kato-Katz (KKT), immunofluorescence (IFT) and intradermal (IDT) techniques for schistosomiasis mansoni, in the study of 2,497 untreated residents of Pedro de Toledo (Sao Paulo State, Brazil)

Age Group	Technique	Sensitivity %	Specificity %	PV_{pos}* %	PV_{neg}† %	J Index‡
< 14 years	KKT	32.4 (146/451)	100 (593/593)	100 (146/146)	66.0 (593/898)	0.324
	IFT	98.7 (445/451)	92.1 (546/593)	90.4 (445/492)	98.9 (546/552)	0.908
≥ 14 years	KKT	32.4 (212/655)	100 (798/798)	100 (212/212)	64.3 (798/1,241)	0.324
	IFT	98.9 (648/655)	69.2 (552/798)	72.5 (648/894)	98.7 (552/559)	0.681
	IDT	92.8 (608/655)	81.8 (653/798)	80.7 (608/753)	93.3 (653/700)	0.746
All ages	KKT	32.4 (358/1,106)	100 (1,391/1,391)	100 (358/358)	65.0 (1,391/2,139)	0.324
	IFT	98.8 (1,093/1,106)	78.9 (1,098/1,391)	78.9 (1,093/1,386)	98.8 (1,098/1,111)	0.777

* Positive predictive value
† Negative predictive value
‡ J index from 0 to 1

Undoubtedly, IFT presented better diagnostic performance when applied to the early age group, and this is emphasized by positive and negative predictive values, as well as by the J index (Table 4). These findings recommend IFT for the diagnosis of schistosomiasis in children, who are mostly exposed to the infection in endemic areas for schistosomiasis[15]. Moreover, the sensitivity of IFT, as with any other immunological techniques, is less influenced by the intensity of *S. mansoni* infection than the parasitological technique[12].

The data obtained here indicate that IDT might be preferable to IFT in some special circumstances in which the emphasis is on adults, owing to its practical and economic aspects.

The probabilistic model described for the comparison of the diagnostic tests utilizes an equation which permitted us to correct the presumed prevalence given by the three techniques, according to their respective sensitivities and specificities (Table 5). There is a significant difference between the presumed prevalence provided by KKT (14.3%) and the true prevalence (44.3%), although this true prevalence is closer to those demontrated by immunological techniques. Thus, the immunological data indicate that a population displaying low parasite egg output does not necessarily have a low prevalence of schistosomiasis.

Table 5 True prevalences for schistosomiasis mansoni obtained based on parasitological (KKT)* and on immunological (IFT and IDT)† techniques, in the study of 2,497 untreated residents of Pedro de Toledo (Sao Paulo State, Brazil).

Age Group	Technique	Presumed Prevalence %	True Prevalence %
< 14 years	KKT	14.0 (146/1,044)	43.2
	IFT	47.1 (492/1,044)	43.2
≥ 14 years	KKT	14.6 (212/1,453)	45.1
	IFT	61.5 (894/1,453)	45.1
	IDT	51.8 (753/1,453)	45.0
All ages	KKT	14.3 (358/2,497)	44.3
	IFT	55.5 (1,386/1,497)	44.3

* Kato-Katz technique

† Immunofluorescence and intradermal techniques

Table 6 True prevalence of *S. mansoni* infection, in 1,453 residents of 14 years or older from Pedro de Toledo (Sao Paulo State, Brazil), according to sex and zone.

Parameter		Frequency %	(95% confidence interval)
Zone			
	Urban	39.1	(35.5 - 42.7)
	Rural	50.7	(47.1 - 53.7)
Sex			
	Male	56.0	(52.4 - 59.6)
	Female	34.7	(31.3 - 38.1)
Sex and Zone			
	Urban male	51.8	(46.2 - 57.4)
	Rural male	59.0	(54.2 - 63.8)
	Urban female	29.1	(24.6 - 33.6)
	Rural female	40.9	(35.7 - 46.1)

The corrected prevalences demonstrated: the rural population was more infected than the urban population; rural females were more infected than urban females; and the prevalence of schistosomiasis among children was close to that for adults, as expected, confirming our previous data[3] obtained from same area using only a parasitological method.

The Spearman's correlation coefficients indicate the prevalences obtained by KKT and IFT follow similar trends, even if the population is divided into different age groups or localities.

Therefore at the present stage of our work, IFT seems to be useful for the prevalence determination of untreated populations, and KKT necessary to verify the intensity of infection.

The data here presented may be helpful to establish a better framework for new investigations to be carried out in areas for schistosomiasis mansoni presenting epidemiological features resembling those from Pedro de Toledo.

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Seroepidemiology of Schistosomiasis Mansoni in Kenya Using Crude and Purified *Schistosoma mansoni* Egg Antigens: Results of a Field Trial

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Summary

In 1980/81 WHO conducted an interlaboratory trial of serological tests for schistosomiasis, and from the results it was concluded that no one antigen preparation or immunoassay system performed outstandingly better than the others with respect to sensitivity and specificity. Nevertheless CEF6, a purified fraction of *Schistosoma mansoni* eggs containing two cationic antigens, yielded relatively high levels of sensitivity and specificity in the diagnosis of schistosomiasis mansoni. This fraction was in addition found to have other desirable characteristics in terms of correlation of antibody levels with infection intensity and a high rate of conversion towards seronegativity following chemotherapy.

The performance of antibody detection methods has been further evaluated in a collaborative study in Kenya in which CEF6 was compared directly with crude soluble *S. mansoni* egg antigens (SEA). High sensitivity values (relative to determination of disease status by parasitological diagnosis) and a positive correlation between antibody activity and excreted egg numbers were obtained. Analysis of the results by age and sex indicated that data which are of use in an epidemiological context can be

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obtained by adoption of immunoassay test parameters appropriate for the endemic area in question. A significant problem was, however, encountered in respect of poor specificity of the reaction of the two egg antigens with blood samples from the schistosome endemic area (but not from a non-endemic area). The possibility that this is in fact due to poor sensitivity of parasitological examination of a single stool specimen is considered.

Introduction

In 1980/81 WHO organized an interlaboratory trial of serodiagnostic antigens for schistosomiasis. Eight participating laboratories in Europe and the United States were sent nearly 400 human infection and negative control sera by WHO, and the reactivity of the sera was tested against 17 different antigen preparations in nine different immunoassays. Following collation and evaluation of the results it was concluded that although none of the antigens or immunoassays involved in the trial had significantly outperformed the others, schistosome egg antigens had yielded marginally better results than adult worm antigens in terms of sensitivity and specificity[1]

One of the egg antigen preparations used in that trial was CEF6, a fraction purified from *Schistosoma mansoni* egg homogenate. Fraction CEF6 contains two cationic antigens z_1 and a_1[2], and during previous 'in house' testing in enzyme-linked immunosorbent assay (ELISA), CEF6 had shown greater sensitivity and specificity than either unfractionated egg extract or any other of the six fractions of egg homogenate yielded by cation exchange chromatography[3] In the WHO trial CEF6 also gave the highest combined values for specificity and sensitivity when tested against sera from a cohort of untreated individuals. Furthermore, CEF6 was the antigen preparation which gave: (i) the highest positive correlation between immunoassay result and intensity of infection as measured by the number of excreted eggs, and (ii) the greatest rate of conversion from sero-positivity to sero-negativity after chemotherapy[1]

In late 1984 and early 1985 further collaborative work was done in Kenya to compare directly the performances of CEF6 and crude unfractionated *S. mansoni* egg antigens in the immunodiagnosis of schistosomiasis mansoni by means of ELISA. The immunological tests were run in parallel with conventional diagnosis by parasitological detection of excreted eggs, and the results were assessed at the level of both the individual (i.e., serodiagnosis) and the study population as a whole (seroepidemiology). This report describes preliminary results from the project which indicate the potential usefulness of antibody detection as a seroepidemiological tool.

Materials and Methods

Human subjects

The blood and stool samples for the results presented here were from inhabitants of two areas of Kenya: (i) Kericho, where there is no transmission of schistosomiasis, and which therefore served as a negative control area; and (ii) The Kamanzi area of Machakos district, Central Province, Kenya, where *S. mansoni* (but not *S.haematobium*) is endemic.

The subjects in Kericho were 258 school children aged between six and 15 years. Nearly 1000 individuals from all age groups were examined in Kamanzi. Inhabitants of the latter study area were in a 'buffer' zone surrounding a population in Ilefune village which has been described in detail by Butterworth *et al.*[4] Demographic details of the Kamanzi population, including the subject's name, age and

sex had been recorded under a distinct identification number during the early part of 1984. There was no record of anti-schistosome chemotherapy having been given to any subjects before this study. The project was initiated only after the proposed experimental protocols had been scrutinized and passed as acceptable by the Kenya national ethical committee. After blood collection and examination of faeces in October November 1984, treatment (praziquantel, 40 mg/kg) was offered in April 1985 to all individuals with schistosome infection detectable by stool examination.

Stool samples were examined quantitatively for *S. mansoni* eggs by the Kato-Katz method[5] modified by Sturrock *et al.*[6] Two samples, each of 50 mg, from the same stool were examined, and the result converted to the number of eggs per gram stool (epg). Estimates of excreted egg numbers were performed once only on the Kericho subjects during the same fortnight that a blood sample was taken. In Kamanzi egg counts were performed on two occasions, the first (Survey 1) during July and August 1984, and the second (Survey 2) in November 1984, subjects in this population having donated their blood samples towards the end of October. Except when stated otherwise, egg excretion results from Survey 2 are used here for illustrative purposes, the stool samples in this survey having been obtained at a time nearest to that of blood collection.

Blood collection and enzyme-immunoassay

Subjects were finger-pricked for blood withdrawal and duplicate blood spots were collected on Whatman 3MM filter paper strips[7]. Each spot contained approximately 30 µl of blood, and after drying at ambient temperature the paper strips were packed in plastic bags together with anydrous copper sulphate as dessicant and transported by air freight to UK, where they were stored with dessicant at -70 °C until used. When required for testing in ELISA a circle estimated to contain 10µl blood was punched out from each dried blood spot, and the blood contained thereon was eluted into 1.5 ml of incubation buffer (i.e. 150 times dilution in phosphate buffered saline(PBS) Tween, which consisted of 0.5 ml Tween 20 in one litre PBS, pH 7.2).

ELISA was performed as previously described[1,8]. The wells of flat-based, 96-well microtitration plates (Flow Laboratories) were coated overnight with 'coating buffer' (0.05 M sodium carbonate bicarbonate solution, pH 9.6) containing the appropriate concentration of antigen (prepared as described below). The concentrations of antigens and the dilutions of blood samples and anti human IgG peroxidase conjugate used during the course of this project were predetermined by 'chequer-board' titration[1]. The coated plates were washed three times with isotonic saline solution containing 0.5 ml Tween 20 (washing solution), and 150 µl of eluted and diluted blood was added to each of the wells. The plates were incubated for two hours at room temperature, and then washed three times in washing solution. To each well were added 150 µl of a 1:2000 dilution of peroxidase-conjugated goat anti human IgG, the plates were incubated again for three hours at room temperature and the washing procedure repeated three times. To each well was added 150 µl of peroxidase substrate (1mg orthophenylene diamine in 0.1M phosphate citrate buffer, pH 5.5, and 0.05 ml of 6% H_2O_2). Colour development was stopped by addition of 0.025 ml of 8 N H_2SO_4 to each well. The time of termination of all the reactions in a plate was determined by the time at which reactivity in wells containing replicate samples of a reference positive serum (made up of a pool of serum from Kenyans known to be excreting *S. mansoni* eggs) gave an OD_{492} reading of approximately 0.75. Optical densities were read semi automatically using a Microelisa Minireader (Dynatech). Differences in reaction end points between plates were eliminated by normalizing reactivities of reference positive serum samples on all plates to an OD_{492} value of 0.75 exactly.

Antigens

The reactivity of blood from each patient was tested against four different *S. mansoni* egg antigen preparations, *viz.* unfractionated homogenates from eggs of a Kenyan and a Puerto Rican isolate of parasite, and batches of CEF6 prepared from each of these two geographical isolates. The origins of the parasites, their laboratory maintenance, and the methods of retrieving eggs from the tissues of heavily infected mice have been described elsewhere[10,11] Eggs were homogenized and centrifuged at 20,000 g for three hours, and the supernatant was used in ELISA as crude soluble egg antigen (SEA). Purified fraction CEF6 was isolated from SEA by cation exchange chromatograpy as originally described by Dunne *et al.*[2] with modification to a one-step method as described by Dunne *et al.*[11]

SEA and CEF6 were stored at -70 °C in PBS, pH 7.2, at concentrations of 5 mg/ml and 0.5 mg/ml, respectively. When required for ELISA they were diluted to concentrations of 10 µg/ml and 1 µg/ml respectively in coating buffer, and 150 µl of these antigen solutions were used to coat microtitration plates.

Results

No schistosome eggs were detected in any stool samples from the school children in the negative control area. In the *S. mansoni* endemic area 642 of 973 subjects (66%) were excreting eggs in Survey 1, and 618 of 887 (70%) in Survey 2.

Comparison of the ELISA results for Puerto Rican SEA with those for Kenyan SEA gave a correlation coefficient of 0.95, and similar comparison of results for CEF6 from the two parasite isolates gave a correlation coefficient of 0.98. The reactivity of Kenyan blood samples in ELISA was therefore not appreciably affected by the geographical origin of the parasite used to prepare the target egg antigens. The results presented here will therefore be only those that were obtained with antigens prepared from the Kenyan isolate.

Calculations of the mean ELISA reactivities of bloods from the two areas of Kenya (Table 1) indicate that samples from the endemic area were more reactive with egg antigens than those from the non-endemic area, and that in the endemic area reactivity against CEF6 was greater than against SEA. The distribution of ELISA reactivities of bloods from the two study areas against SEA and CEF6 are given in histogram form in Figure 1. In agreement with the results in Table 1, bloods from the endemic area were generally more reactive in ELISA with CEF6 than with SEA, as indicated by the relative displacement of the histogram bars to the right in Figure 1d when compared with 1b. In the non-endemic area very few blood samples gave ELISA OD_{492} values above 0.20 after reaction with either antigen preparation.

Table 1 Mean ELISA OD_{492} results of blood samples from a non endemic area and an endemic area

	No. of samples	SEA	CEF6
Kericho (non-endemic)	254	0.06 ± 0.02	0.12 ± 0.04
Kamanzi (endemic)	995	0.38 ± 0.20	0.50 ± 0.24

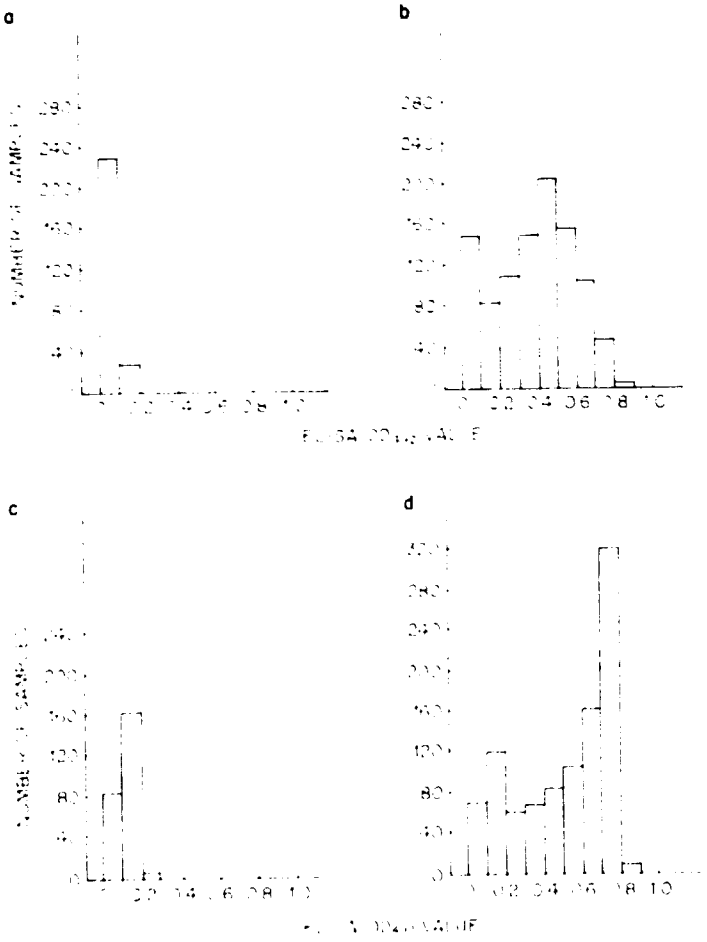

Figure 1 The distribution of ELISA OD_{492} readings given by blood samples from subjects living in a non-endemic area (a and c) and an endemic area (b and d) of Kenya. Reactivity of the bloods was tested against SEA (a and b) and CEF6 (c and d).

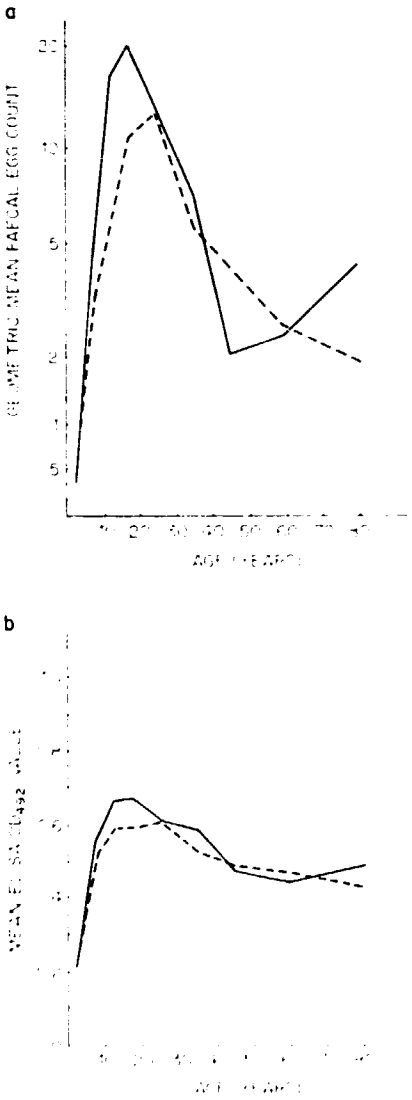

Figure 2 (a) The geometric mean stool egg counts in subjects of different ages in the *S. mansoni*-endemic Kamanzi area of Kenya. (b) The mean ELISA OD_{492} readings of bloods from the same subjects as in Figure 2a, using CEF6 as antigen.
(—) male subjects; (···) female subjects.

Table 2 Correlation of ELISA OD_{492} readings with parasitological infection intensity [$\log_{10}$ + 1) eggs/g]

	Correlation coefficients			
	All subjects	Age <10	Age 10-19	Age ≥20
SEA	0.58	0.61	0.46	0.49
CEF6	0.65	0.70	0.50	0.50

Figure 2a gives the geometric mean stool egg counts by age and sex for the population in the *S. mansoni* endemic area, and Figure 2b shows the mean ELISA OD_{492} readings for this population, using CEF6 as antigen. The two figures are similar in so far as both egg counts and antibody reactivity rise to a maximum in 10-19 year-old subjects and then decline with age such that 50+ year-olds had somewhat lower ELISA reactivity than the 5-9 year olds.

Table 2 gives the correlation coefficients between ELISA OD_{492} reactivity against Kenyan SEA and CEF6 and the ($\log_{10}$ + 1) number of eggs excreted by subjects in the endemic area. Children aged under ten had higher correlation coefficients for both antigen preparations than older subjects, and CEF6 gave a higher correlation coefficient than SEA with respect to the youngest age group, as well as for the population as a whole.

In Figure 3a, is plotted the prevalence of egg excretion according to age. Figure 3b gives the percentage of subjects of each age group with an ELISA OD_{492} reading > 0.20. The similarity in the shapes of the two respective curves for egg excretion and ELISA results is evident.

Table 3 indicates the sensitivity and specificity of the ELISA readings on SEA and CEF6 at OD_{492} cut-off points of 0.20 and 0.40. Both antigen preparations had sensitivities of greater than 90% at the lower cut-off value. Specificity of the serology was poor, however, indicating a relatively high proportion of subjects in the endemic area were antibody-positive, but parasitologically-negative.

Table 3 Sensitivity and specificity of ELISA with SEA and CEF6 in a *S. mansoni* endemic area of Kenya

	SEA		CEF6	
ELISA OD_{492} cutoff	0.20	0.40	0.20	0.40
Sensitivity %	94	65	97	87
Specificity %	64	83	59	76

Discussion

There are at present no immunodiagnostic tests for schistosomiasis mansoni in routine commercial production or use, and diagnosis of this infection is still for the most part achieved by parasitological examination of stool. The relatively poor

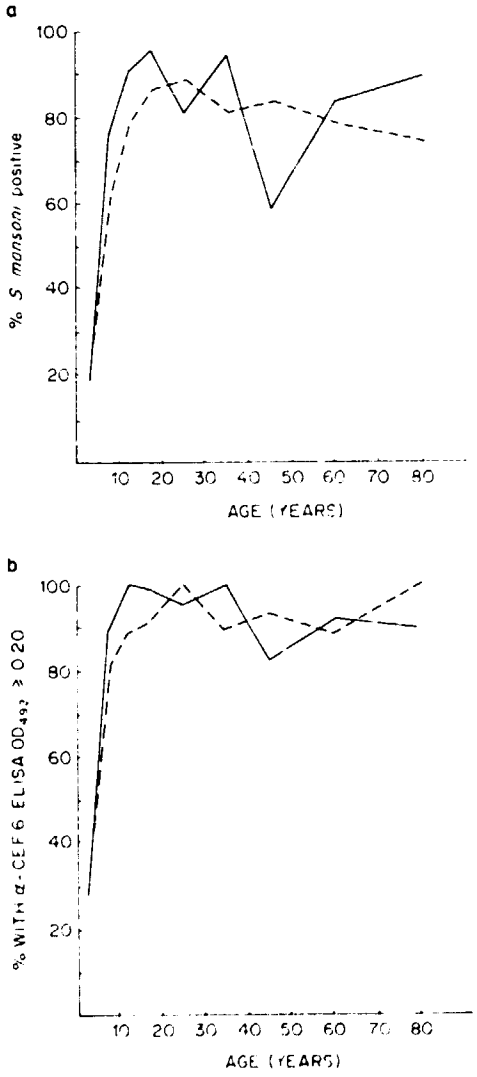

Figure 3 (a) The proportion of subjects of different ages in the Kamanzi study area that had one or more *S. mansoni* eggs in 100mg stool examined by the Kato-Katz method, expressed as a percentage of the total number of subjects in the respective age group. (b) The percentage of blood samples from the same population as in Figure 3a with anti-CEF6 ELISA OD_{492} readings > 0.20.
(—) male subjects; (---) female subjects.

financial return anticipated for products for control of tropical diseases is undoubtedly one factor which contributes to the slow rate of progress in this area of research. In addition, however, immunodiagnostic tests, particularly those based on the detection of anti-parasite antibodies, are often assumed to have inherent deficiencies which render them potentially less useful than parasitological techniques.

One common assumption is that antibody levels will generally fail to reflect the intensity of infection. Previous results with CEF6 alone[1], and the results presented here with respect to both SEA and CEF6 (Table 1) nevertheless indicate a significant degree of correlation between antibody titre and excreted egg count, particularly in the youngest age groups. There is, as a consequence of this relationship, some similarity of shape between the respective graphs for excreted egg counts and antibody activity (Figures 2a and 2b).

Another assumption is that antibody titres will remain high even in the absence of infection (e.g., after self-cure or elimination of the parasite by chemotherapy). Immunological tests based on the detection of circulating antigen levels naturally obviate this particular problem, and it has indeed been observed that the blood concentrations of schistosome circulating anodic antigen (CAA) decrease rapidly after treatment of schistosomiasis mansoni with praziquantel[12] However, both 'in-house' testing[13,14] and the WHO-sponsored interlaboratory trial[1] demonstrated that antibodies against CEF6 also declined within a period of 6-12 months after chemotherapy. The time taken for anti-CEF6 antibody titres to return, after treatment, to preinfection levels remains to be determined more exactly on a larger number of subjects.

In view of the high degree of sensitivity of antibody detection (Table 3), and the good correlation between antibody levels and infection intensity in the youngest age groups (Table 2), an alternative strategy that could be used to monitor the effectiveness of control programmes, and which by-passes the problem of antibody persistence after treatment, would be to determine the rate of conversion to sero-positivity in younger age groups that had not previously been treated.

The poor specificity of the immunoassay recorded in Table 3 reflects a relatively high percentage of subjects in the endemic area who were antibody-positive but in whose stools no eggs were detected. One explanation for this could again be in terms of the persistence of antibody in the absence of infection (in this case after self-cure, as there was no record of chemotherapy having been given to subjects in this study). The better correlation between egg counts and antibody levels in the younger age groups would be consistent with this notion. Alternatively it may be due in some part to an inherent insensitivity of a parasitological method in which only 100 mg of stool is examined. By this technique most of those people who are excreting 10 or fewer schistosome eggs/g will be recorded as having no infection.

The insensitivity of single examinations of unconcentrated stool has been noted previously[15,16] as has the variability in numbers of eggs found in successive stool samples from the same patient[17,18] In the present study it can be seen that specificity of the egg antigens was satisfactory with respect to blood samples from the non-endemic area (Figures 1a and 1c). In the endemic area the value for specificity increased to 85% when the pooled parasitological results from both Surveys 1 and 2 were used to estimate this parameter with respect to CEF6 at an ELISA OD_{492} cut-off of 0.40, compared with specificity values of 75-76% when results from only one parasitological survey were used in the calculation. The low specificity of the serological testing reported here may therefore be in part attributed to the low sensitivity of parasitology, but further work is needed to determine to what extent.

In conclusion, the results demonstrate the potential of antibody detection as an epidemiological tool. It will of course be necessary to determine whether similar

results can be obtained in other endemic areas, and the parameters of the immunoassay, such as the appropriateness of the cut-off point of OD_{492} 0.20 used in Figure 2b, may in any case to have to be periodically revalidated. Further work is thus required to establish whether antibody detection can indeed provide a cost-effective alternative to parasitological methodology.

Acknowledgements

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Evaluation of Recombinant *Schistosoma mansoni* Antigens Sm31 and Sm32 for Immunodiagnosis

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Summary

Two immunogenic *Schistosoma mansoni* antigens, Sm31 and Sm32, were evaluated as immunodiagnostic reagents. We compared the seroreactivities in ELISAs of partial and full-length proteins expressed in *Escherichia coli* as polypeptides fused to MS2 polymerase. The partial and full-length recombinant products of Sm31 yielded sensitivities of 56.9 and 69.6%, respectively. The same degree of sensitivity of 68.3% was calculated for partial and full-length fusions of Sm32. The overall sensitivity of both Sm31 or Sm32 presented as partial fusions was 82.2%. In comparison, the full-length fusion antigens resulted in a higher sensitivity of 88.6%. As expected, after praziquantel treatment of the subjects, egg counts in stool or urine samples were found to be markedly reduced. A decrease in seroreactivity to partial fusions was also measured, in contrast to full-length fusion products. The reason for this is unclear. Furthermore, the reactivities of Sm31 and Sm32 expressed as native non-fused recombinant antigens in rabbit reticulocyte lysates and in insect cells (using the baculovirus expression vector system) were assessed. The sensitivities of the non-fused antigens both translated *in vitro* or synthesised in the eukaryotic system were considerably higher (97.5%) than those obtained with the fusion proteins. This finding supports the view that adoption of native tertiary structures can improve the performance of recombinant antigens in diagnosis.

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Introduction

The potential use of two adult *Schistosoma mansoni* antigens, Sm31 and Sm32, as probes for the development of an antibody detection assay in the diagnosis of schistosomiasis had been shown by Ruppel *et al.*[1,2] In Western blotting of total *S.mansoni* proteins, sera from patients with schistosomiasis infections were found to recognise the two antigens consistently, whereas sera from uninfected persons or from patients with other parasitic diseases showed no reactivity[3,4]

We explored the applicability of Sm31 and Sm32 for diagnosis in the form of recombinant antigens. Molecular cloning techniques were used to isolate the two genes[5] and to produce purified recombinant antigens in sufficient quantities for immunodiagnostic assays. An *Escherichia coli* expression plasmid pEx34b was used to synthesise various parts of the *S. mansoni* antigens as fusions with the N-terminus of the RNA polymerase of the bacteriophage MS2[6] The antibody response to the partial and full-length schistosome proteins fused to MS2 polymerase was evaluated. The availability of a large number of paired sera allowed us to compare reactivities of the antigens in ELISAs[7] before and after chemotherapeutic treatment.

Furthermore, we investigated whether the tertiary structure of native i.e. non-fused proteins can improve the performance of recombinant antigens in diagnostic tests. Sm31 and Sm32 were synthesised by *in vitro* transcription and translation systems[8,9] and human infection sera were found to recognise the *in vitro* translation products via immunoprecipitation[10] more frequently than the fusion products via ELISA . In addition, the gene products were generated in an eukaryotic expression system using the *Autographa californica* nuclear polyhedrosis virus (AcNPV)[11] We were able to demonstrate that the sensitivity of the recombinant products synthesised in insect cells was as high as that obtained with the *in vitro* synthesised antigens.

Materials and Methods

Expression of MS2-schistosome fusion proteins

Partial and full-length cDNA sequences of Sm31 and Sm32 in λgt10 were cloned in pEx34b and expressed as fusion proteins with MS2 polymerase in *Escherichia coli*. The cloning and purification of fusion proteins have been described elsewhere[5,6] Antigens purified to >90% homogeneity were used as probes for measuring the presence of schistosome-specific antibodies.

In vitro translation and immunoprecipitation

Construction of Sm31 and Sm32 cDNA sequences in pSP65/*Nco* I[13] and transcription reactions performed according to Melton *et al.*[8] have been previously described[13,14] Approximately 1μg of the *in vitro* synthesised RNA was translated in rabbit reticulocyte lysates in the presence of [^{35}S]-methionine. Aliquots of the translation products were incubated with antiserum and immune complexes precipitated by protein A sepharose. Immunoprecipitated proteins were analysed by SDS-PAGE and autoradiography.

Expression in *Spodoptera frugiperda* cells

The full-length Sm31 and Sm32 cDNA inserts were cloned into the plasmid transfer vector pBC3[12] derived from pAcJR2[15] Transfer of the schistosome inserts by the chimeric transfer vectors pBC-Sm31 and pBC-Sm32 to the genome of the *A.californica* nuclear polyhedrosis virus (AcNPV) was achieved by cotransfection of

S.frugiperda cells using the calcium phosphate precipitation technique[12] Recombinant viruses were isolated and the corresponding recombinant products were referred to as BC-Sm31 and BC-Sm32.

Infected *S. frugiperda* cells were tested for the presence of recombinant proteins by Western blotting using rabbit antisera raised against the corresponding MS2 fusion proteins. After ammonium sulphate fractionation and purification on preparative SDS-polyacrylamide gels, the recombinant proteins were tested in transferable solid phase(TSP)-ELISA.

Sera

For a large scale testing serum samples were collected initially from 300 school children, both males and females between the ages of seven and 14, attending the primary school in Molodo, Mali. The village is situated eight kilometres from the rice growing district of Niono, endemic for both schistosomiasis mansoni and haematobium. Since sera from *Schistosoma haematobium*-infected patients have been demonstrated to react with the Sm31 and Sm32 antigens of *S. mansoni* [4], persons with *S. haematobium* and mixed *S. mansoni* and *S. haematobium* infections were included in our study. Sera were also donated by the school teachers and their families. At the same time stool and urine samples were examined once for the presence of eggs by faecal smears[16] and by the nucleopore filter method[17], respectively. No measures to control the disease in this village had previously been undertaken.

Serum, stool and urine specimens were reexamined four months and one year after praziquantel treatment (40 mg/kg body weight). Paired sera from a total of 95 individuals were assessed in this study.

Sera selected to form the endemic control group were taken from adults (n=15) and young children aged two to three (n=14), who were classified as uninfected on the basis that they have had no water contact, and showed no clinical signs of *S. mansoni* or *S. haematobium* infections.

TSP-ELISA

The TSP system has been described previously[7] In brief: gel-purified fusion proteins with concentrations of 3 µg/ml in PBS were used to saturate the pins. An antibody dilution of 1:100 in PBS containing 1% BSA and 0.1% Tween 20 was chosen for all human sera. A standard curve was included in each assay to compensate for within-run and run-to-run variation as described by Hancock and Tsang[18] Rabbit antiserum to MS2-SfaN fusion protein served as a reference serum. Different dilutions ranging from 1:2,000 to 1:20,000 were titrated against the fusion protein (3 µg/ml) and 100 ml of the 1:2,000 dilution was arbitrarily designated as 1000 units of activity (U). From the linear range of 40 to 1000 U covered in the standard curve, optical density values were translated into units. All values outside of this range were either counted as <40 or >1000 U. The cut-off value is the mean +2 standard deviation (SD) of 29 endemic control sera, calculated to be 80 U for MS2-NH3, MS2-Sm31 and MS2-Sm32, and 160 U for MS2-SfaN. Ten endemic control sera were used for calculating the cut-offs for BC-Sm31 and BC Sm32, which were found to be 80 and 40 U, respectively. Thus a serum reacting above the cut-off value is scored as positive for the presence of a schistosome infection, while a serum under the calculated value is scored as negative.

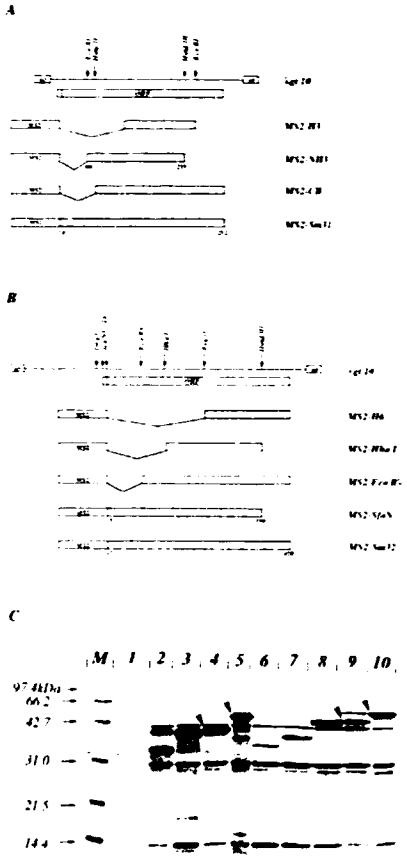

Figure 1 Recombinant gene products of Sm31 and Sm32 expressed in *E. coli*. (a) The cDNA clones of Sm31 are outlined. Adaptor sequences (AD) were used to ligate the complete cDNA sequence to λgt10. The open reading frame (ORF) was deduced from the nucleotide sequence. All MS2 polymerase-schistosome fusion products available to date are schematically presented. (b) Sm32-specific cDNA clones are indicated; use of abbreviations as in (a). (c) Coomassie blue stained SDS-polyacrylamide gel (15%) shows partially purified 7 M urea fractions containing the above MS2 fusion products. Each fusion protein band was excised after separation on preparative SDS gels and electro-eluted. Lane 1 shows non-fused MS2 polymerase fragment as a control. The following lanes show (2) MS2-H3, (3) MS2-NH3, (4) MS2-CB, (5) MS2-Sm31, (6) MS2-H6, (7) MS2-*Hha* I, (8) MS2-*Eco* RI, (9) MS2-SfaN and (10) MS2-Sm32. Arrowheads indicate the fusion proteins used in this study. Molecular weight markers are on the left.

Results

Expression of Sm31 and Sm32 sequences in *Escherichia coli*

The application of *S. mansoni* Sm31 and Sm32 in immunodiagnostic tests was explored in the form of recombinant antigens. The sequences were expressed as hybrid proteins fused in frame with the first 99 amino acids of the RNA polymerase gene of bacteriophage MS2[6]. Various molecular constructs covering different regions of the sequences are depicted schematically in Figure 1. Two each of Sm31 and Sm32 fusion proteins were chosen for further study. MS2-NH3 covers amino acids positioned at 60 to 235, while MS2-Sm31 corresponds to MS2 fused to the complete coding sequence with the exception of the first 13 amino acids (amino acid positions 14 to 252) (Figure 1a). MS2-SfaN carries Sm32-specific amino acids from positions 15 to 334. MS2-Sm32 represents the almost full-length fusion protein from amino acids 15 to 428 (Figure 1b).

The fusion proteins were isolated from the insoluble pellet of lysed cells by solubilisation in 7 M urea and purified on preparative SDS-polyacrylamide gels as described previously[6]. Sm31 fusion proteins were 40 kDa (MS2-NH3) and 48 kDa (MS2-Sm31) in size. The molecular weights of MS2-SfaN and MS2-Sm32 were 46 kDa and 56 kDa respectively (Figure 1c).

Sensitivity of ELISA using recombinant antigens

The degree of sensitivity of the recombinant antigens in TSP-ELISA was calculated based on the number of persons with positive antibodies as a percentage of subjects positive for eggs[19]. Out of the 95 individuals tested, 79 had eggs in their stool or urine (Table 1). Among the egg positive cases, 45 were antibody positive (i.e. "true positive") for MS2-NH3, and 54 for MS2-SfaN, whereas 65 of them were antibody positive in ELISA for either of the partial fusion proteins. We also examined the full-length fusions to see whether immunoreactivities can be increased. We observed that 55 persons had antibody titres to MS2-Sm31, while 54 individuals recognised MS2-Sm32. A total of 70 persons out of 79 had positive ELISA reactivities to MS2-Sm31 or MS2-Sm32. Sensitivities of the ELISA technique utilising partial and full-length recombinant antigens were calculated to be 82.2% and 88.6%, respectively. It therefore appears that the full-length recombinant products show an improved sensitivity compared to the partial proteins.

Table 1 Sensitivity of ELISA using recombinant antigens

	ELISA positive					
(n=79)	MS2 NH3	MS2-SfaN	MS2-NH3 or MS-SfaN	MS2-Sm31	MS2-Sm32	MS2-Sm31 or MS2-Sm32
Egg positive	45	54	65	55	54	70
Sensitivity (%)	56 9	68 3	82 2	69 6	68 3	88 6

Antibody response to recombinant antigens

The seroreactivity to the recombinant proteins was calculated using sera from a total of 95 subjects, instead of using sera only from egg excretors. Interestingly, the number of individuals with antibodies to the fusion proteins calculated as a percentage of the total number of subjects tested did not differ greatly from that calculated as a percentage of those who have eggs in their stool or urine. This is presumably due to the immunological and parasitological techniques being insensitive at low antibody titres and low egg output, respectively. Using MS2-NH3 it was found that 59% of the individuals reacted above the cut-off value of 80 U (Figure 2a and Table 2). Immunoreactivitiy to MS2-SfaN (cut-off value 160 U) was measured in 66% of the individuals (Figure 2c and Table 2). The value calculated for ELISA positivity for MS2-NH3 or MS2-SfaN was 81% (Table 2).

Immunoreactivity to MS2-Sm31 increased to 68% (Figure 2b). Notably with this antigen, the number of individuals reacting with >1000 U is significantly higher than with the corresponding partial fusion product. The percentage of individuals who were found to react positively for MS2-Sm32 (cut-off >80 U) remained at 66% (Figure 2d). Taken together, however, ELISA positivity for both antigens showed a slight increase to 85%. This led us to conclude that the complete coding regions, at least of Sm31, were more reactive in our test system than the corresponding partial fusion protein.

Table 2 Percentage of total ELISA positivity obtained pre- and post-chemotherapy

	ELISA positive					
(n=95)	MS2-NH3	MS2-SfaN	MS2-NH3 or MS2-SfaN	MS2-Sm31	MS2-Sm32	MS2-Sm31 or MS2-Sm32
Pre-treatment	59	66	81	68	66	85
Post-treatment	37	34	51	70	67	85

Effect of chemotherapy

One year after treatment with a single dose of praziquantel, the presence of antibodies to the fusion proteins was measured. As a result of drug treatment, we observed that a large proportion of seropositive reactions to MS2-NH3 and MS2-SfaN partial fusion products became seronegative. Thus the number of patients with ELISA positivity of >1000 U decreased, while those with reactivities below the cut-off value increased (compare Figures 2a and 2c with 2e and 2g, respectively). Overall, the percentage of individuals with positive antibodies directed against MS2-NH3 before and after administration of praziquantel was seen to drop from 59% to 37% (Table 2). Immunoreactivity to MS2-SfaN decreased from 66% pretreatment to 34% post-treatment. Antibodies to both recombinant antigens were measured in 81% of the individuals, and following chemotherapy in only 51% (Table 2).

This post treatment decrease observed in ELISAs to partial fusion proteins is consistent with a reduction in egg excretion, which is a marker of effective

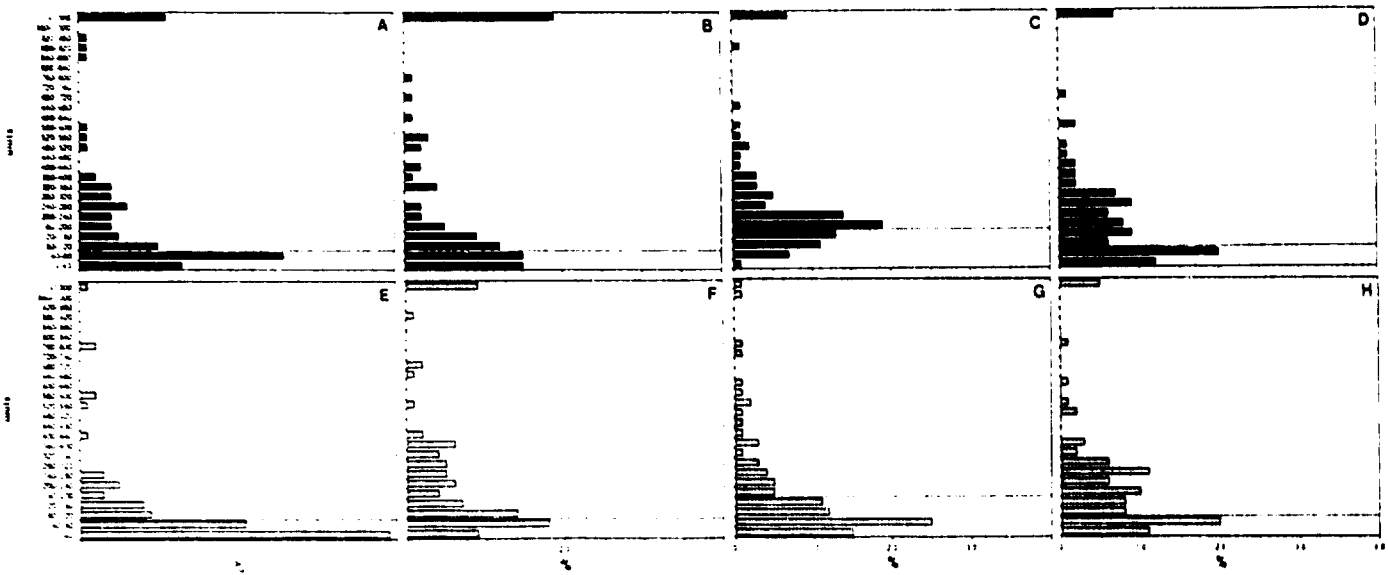

Figure 2 ELISA reactivity on MS2 fusion proteins. The distribution of OD values expressed as units of activity is shown for (a) MS2-NH3, (b) MS2-Sm 31, (c) MS2-SfaN and (d) MS2-Sm32 before the administration of praziquantel to the patients. Endemic control sera were used for calculating the cut-off values, which is the mean ±2 SD. Values >80 units for MS2-NH3, MS2-Sm31 and MS2-SfaN and >160 U for MS2-SfaN were scored positive. The same analysis was performed with paired sera (n=95) to (e) MS2-NH3, (f) MS2-Sm31, (g) MS2-SfaN and (h) MS2-Sm32 one year after drug treatment.

chemotherapy. Before treatment the mean number of *S. mansoni* and *S. haematobium* eggs was 598.4 ±868.2 eggs/g stool and 19.4 ±35.8 eggs/10 ml urine. After treatment mean *S. mansoni* and *S. haematobium* eggs were reduced to 68.2 ±215.2 and 0.4 ±4.4, respectively.

The antibody response was further studied using full-length fusion proteins. Despite the observation that the number of individuals with high ELISA reactivities to MS2-Sm31 decreased after chemotherapy (compare Figures 2b and 2f), the overall positivity calculated was not found to change (Table 2). As can be seen in Figures 2d and 2h, pre- and post-treatment reactivities to MS2-Sm32 were largely similar. Therefore, in contrast to the partial fusion proteins, we were unable to demonstrate a post-treatment decrease in antibody levels to the full-length recombinant products. However, a prerequisite for the utility of an immunodiagnostic test based on the detection of antibody is a post-treatment drop in antibody titres. In the further development of an antibody-based assay with full-length proteins, it will therefore be important to investigate whether the full-length recombinant proteins in particular Sm32 carry epitopes to which long-lasting antibodies are raised and if so, it will be interesting to analyse the different antibody isotypes involved in the immunoreactivity.

Influence of protein conformation on immunoreactivity

Our results so far show that the presence of eggs in stool or urine specimens is not correlated with a positive ELISA. The finding that sera of some non-egg excretors were found to be immunologically reactive to the fusion proteins is most likely due to the insensitivity of the parasitological test based on only one egg count. A possible explanation for the finding of egg excretors with no antibody response is that antibody titres are too low to be detected.

In order to investigate whether low reactivity is partly caused by conformational changes as a result of the presence of the carrier protein MS2 polymerase, we analysed the performance of non-fused recombinant antigens, first of all using *in vitro* translation products.

For this purpose Sm31 and Sm32 cDNAs were subcloned in an RNA expression vector pSP65/NcoI[13] *In vitro* derived Sm31 RNA[8,9] directed the synthesis of two proteins with molecular masses of 37.5 and 31.5 kDa (Figure 3a, lane IVT). In previous work we have shown that the former protein corresponds to a precursor molecule, while the latter represents a product translated from an internal initiation codon in the same reading frame[13] Sm32 RNA translated *in vitro* yielded a 47 kDa product, the molecular mass of which is consistent with the total coding capacity calculated from the deduced amino acid sequence (Figure 3b, lane IVT).

The full-length proteins as represented by the *in vitro* translation products were subjected to immunoprecipitation by human infection sera. The results from sera of 40 egg-positive patients are shown in Figure 3.

The 37.5 kDa or both 37.5 and 31.5 kDa Sm31 *in vitro* translation products were strongly recognised by 17 out of 40 sera, and weakly by 12 others (Figure 3a). In comparison, Sm32 *in vitro* translated *in vitro* was clearly immunoprecipitated by 37 out of 40 infection sera. Sensitivity of approximately 60-70% for Sm31, 92.5% for Sm32 and 97.5% for Sm31 or Sm32 can be calculated. This high sensitivity clearly has to be confirmed by testing a larger number of sera.

Two serum samples out of the endemic control group were positive, one each for Sm31 and Sm32. Interestingly, it was also observed that these sera reacted positively in ELISA to the recombinant fusion proteins. Despite care in choosing the endemic control persons, these findings strongly suggest that we are dealing with infected persons.

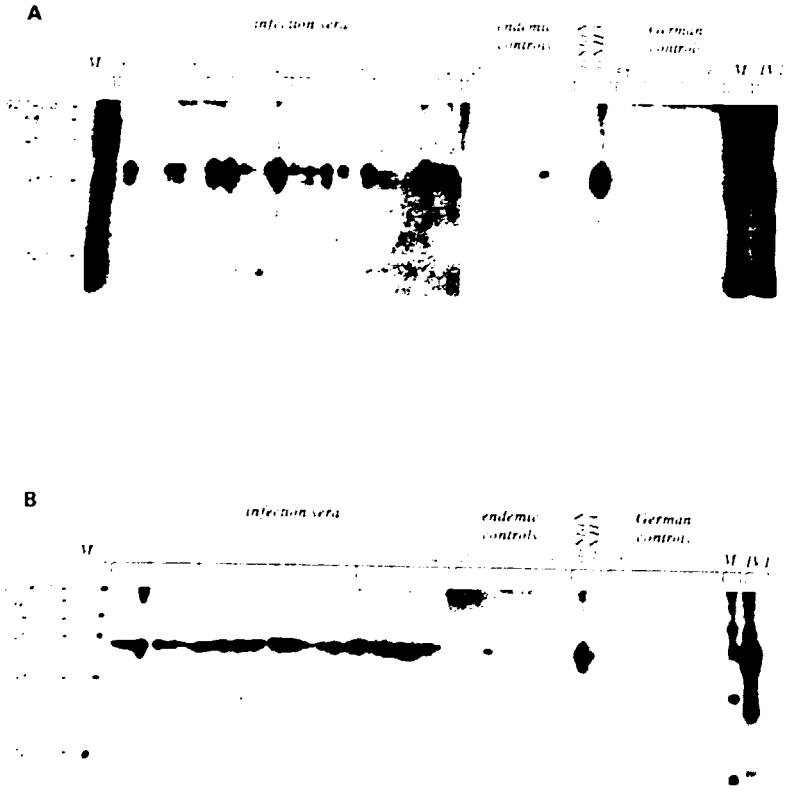
A
infection sera
endemic controls
German controls
M
M
B
infection sera
endemic controls
German controls
M
M

Figure 3 [illegible]

From the above results we believe that a high sensitivity of immunoprecipitation of *in vitro* translation products close to 100% can be achieved. Despite superiority of this technique, it is most unlikely that the use of radiolabelled *in vitro* translation products will be applicable for field study.

Expression in insect cells

Next, we proceeded to clone and express cDNAs of Sm31 and Sm32 in insect cells, where proteins produced are known to be immunologically and functionally similar to their authentic counterparts. Following infection of insect cells with recombinant baculoviruses, total cell extracts were verified for the presence of recombinant BC-Sm31 and BC-Sm32, respectively by SDS-PAGE and Western blotting (data not shown). After ammonium sulphate fractionation and purification on preparative SDS polyacrylamide gels, the recombinant proteins were assessed in TSP-ELISA.

Antibodies to BC-Sm31 with >80 U were detected in 29 out of 40 sera tested, and antibodies to BC-Sm32 with >40 U were measured in 37 out of 40 sera. These results demonstrate that the ELISA test done with recombinant antigens synthesised in the baculovirus system is as effective as the *in vitro* translation system for detecting antibodies in sera from infected humans. As mentioned above, the high degree of sensitivity will have to be confirmed on a greater number of patient sera.

Discussion

In this study, we addressed the question of whether a standardized diagnostic assay using cloned *S. mansoni* antigens Sm31 and Sm32 can be developed. We assessed the performance of recombinant Sm31 and Sm32 expressed in *E. coli* as MS2 polymerase-schistosome fusion proteins. The finding that the full-length MS2-Sm31 fusion is more sensitive than the partial MS2-NH3 fusion can be simply explained by the presence of additional immunoreactive epitopes on the complete sequence. In contrast, there is no improvement in sensitivity with Sm32 and it appears that the antigenicity is attained on both Sm32 partial and full-length fusions.

The possibility that recombinant Sm31 and Sm32 molecules mimicking "*in vivo*" conformation may yield higher sensitivities led us to express the antigens in eukaryotic systems, whereby conformational determinants of the native proteins can be accurately reproduced. Using *in vitro* translation products of Sm31 and immunoprecipitation with human infection sera or baculovirus-synthesised Sm31, sensitivity was 72.5%, which is not significantly higher than the result obtained with full length Sm31 fusion protein. This is suggestive of the absence of additional epitopes on the protein .

It is notable that we have obtained a higher sensitivity of 92.5% using recombinant Sm32 produced in either rabbit reticulocyte lysates or in insect cells. This shows that the recombinant gene products synthesised in these systems are immunologically very similar to their authentic proteins.

Our results show that we can detect antibodies to distinct recombinant *S.mansoni* proteins based on TSP-ELISAs and immunoprecipitation. Although the latter technique is highly sensitive, the involvement of radioactive material and the fact that the method is both expensive and labour intensive make it inappropriate for field use. Disadvantages of the baculovirus expression system are not only difficulties in setting up the system but also the low yields (<1% of total proteins) and laborious purification protocols.

Nevertheless, we have provided evidence that both the creation of conformational epitopes on Sm32 and the adoption of full-length sequence of Sm31 do

significantly improve the performance of recombinant antigens in diagnosis. It is therefore worthwhile to develop a strategy for the expression of non-fused Sm31 and Sm32 in *E. coli*, and to test their suitability as diagnostic probes.

We have followed the effects of chemotherapy on the antibody responses to the fusion proteins. In conjunction with the drop in mean egg counts one year after treatment, we have observed a decline in antibody titres to partial fusion products but not to full-length fusions. In this context it may be worthwhile to investigate which epitopes induce long-lived antibodies.

The specificity of the assays in terms of cross reactivity with other parasites has not been addressed in this study. Previous work, however, has shown that these antigens do not cross-react with sera from patients with other parasitic diseases[4]. We found an alarmingly high number of "false positives" in our study group, i.e. those with antibody titres but negative for eggs. The main reason for this apparent lack of specificity may be due to inaccurate parasitology as determined by a single stool/urine examination. Alternatively, the technique has enabled us to detect individuals early in the course of acute infection before eggs are produced.

In conclusion, we have demonstrated that partial and full-length fusion proteins are sufficiently sensitive to diagnose schistosome infections. However due to our observation that the antibodies to the latter do not decline after treatment, it seems reasonable to suggest the use of TSP-ELISA based on partial fusion proteins for diagnosis. Since it is doubtful to date that serology would completely replace parasitological diagnosis in schistosomiasis, this novel antibody-detection technique could be useful in complementing stool and urine examinations.

Acknowledgements

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Alternative Preparation of *Schistosoma* Egg Antigens for Circumoval and Intraoval Precipitin Tests for Use in Endemic Areas

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Summary

Several simple methods for preparation of schistosome eggs for the circumoval precipitin (COP) test for the diagnosis of schistosomiasis are described. Eggs prepared by these methods would provide favourable alternatives to lyophilized eggs, the preparation of which is not necessarily easy in endemic areas.

Investigations into effects of temperature and duration of storage on schistosome egg antigens indicated that the antigens involved in the COP reaction are heat stable. In the methods described for preparation of eggs for the COP test: preservation in paraformaldehyde, 95% alcohol or acetone, or drying at 37 °C, the robust nature of the egg antigens is exploited.

In addition, a novel method for diagnosis of schistosomiasis, the intraoval precipitin (IOP) test, using formalin-fixed liver-egg sections from infected animals, is described.

Introduction

Since the establishment of the circumoval precipitin (COP) reaction using *Schistosoma mansoni* fresh eggs[1], a more simplified procedure for the COP test has been developed using lyophilized eggs[2,3]. Although lyophilized eggs have been extensively employed in most laboratories for this routine assay, the preparation of the eggs is not always easy in the endemic areas, where there is a great need for the COP test[4].

Since the COP test can be easily performed with minimal equipment, it is now considered to be the serodiagnostic method of choice for schistosomiasis in local, endemic areas. Furthermore, the sensitivity and specificity of the test are much better than other methods such as ELISA or radioimmunoassay[5]. On the other hand, differences in results between laboratories using somewhat different techniques[6,7], indicate that the question of reproducibility needs to be further investigated.

Variations in antigenicity of the egg-batches used in the assay might be a possible cause.

With these considerations in mind, alternative methods of preparation of *Schistosoma* egg antigens were investigated, such as eggs preserved in formalin, alcohol or acetone and air-dried eggs, with a view to making the COP test more useful in the endemic field. In addition, the IOP test, a novel method for detection of antibodies in schistosomiasis is described.

The Nature of Schistosome Egg Antigens

Heat stability

Although it has been supposed that ambient conditions, such as temperature during the preservation period, may significantly affect their antigenicity, few studies have been carried out on the nature of schistosome egg antigens for the COP test with respect to storage and other conditions.

Eggs kept at 4 °C exhibited the highest antigenicity after eight months of preservation. Eggs preserved at room temperature showed reduced antigenicity. Incubation of eggs at a stable temperature of 60 °C for up to eight months did not result in rapidly decreased antigenicity. Surprisingly, eggs still retained antigenicity in the COP test after incubation at 200 °C for one hour (Table 1). This

Table 1 Influence of temperature on lyophilized egg antigen(s) of the COP test for schistosomiasis japonica[10]

Temperature of preservation (°C)	Duration of treatment	Percentage of COP reactive eggs	COP index§
4 (control)*	10 months	33.3	28.8
Room temperature (0-30)†	8 "	23.0	19.2
30	8 "	28.0	22.6
37	8 "	24.0	22.0
60	8 "	23.5	18.1
80	1 hour	30.7	28.6
100‡	1 "	37.3	36.4
120	1 "	29.5	25.2
	12 "	27.5	22.7
	24 "	17.0	12.2
150	1 "	17.7	13.7
	6 "	14.8	9.8
	12 "	5.4	2.5
200	1 "	8.0	4.2
	6 "	0	0

The lyophilized eggs were distributed into small vials and kept in the dark with silica gel except for the eggs of the "Room-temperature" group.

* Original antigenicity of eggs used in the experiment.

† The eggs were placed in a brown bottle with silica gel to prevent the effect of ultraviolet rays and kept in the laboratory.

‡ Many eggs were ruptured

§ Calculated by the formula cited in Tanaka[20]

Figure 1 Specific fluorescence detected in the space between the autofluorescent eggshell and the miracidium in the indirect fluorescent antibody test, using formalin-fixed liver-egg sections; incubated in the serum from a patient with schistosomiasis japonica[11].

evidence suggests that the antigens involved in the COP reaction are heat-stable polysaccharides or glycoproteins[8,10] Since variation in temperature induces a gradual decrease in antigenicity, lyophilized eggs should be preserved under stable temperature conditions.

Locality of antigenic substances in the egg

The heat stable nature of *S. japonicum* lyophilized egg antigens for the COP test[10] allowed the successful application of the indirect fluorescent antibody (IFA)[9,11] and indirect immunoperoxidase (IIP)[12] tests using formalin-fixed liver-egg sections embedded in paraffin for the diagnosis of schistosomiasis japonica. These results clearly showed the location of heat-stable intraoval antigens in the space between the vitelline membrane and miracidium. Furthermore, Periodic Acid Schiff (PAS) staining showed that PAS-positive areas corresponded well with those of specific fluorescence observed in the IFA test (Figure 1).

Alternative Methods of Preparation of Schistosome Eggs for the COP Test

The finding that the heat-stable *S. japonicum* egg antigens were responsible for the COP reaction[10] encouraged us to develop other methods for preparing eggs for the COP test, as alternatives to lyophilization.

Paraformaldehyde (PFA)-fixed eggs

Although our previous studies[11,13] suggested the possibility of using such formalin-fixed eggs in the COP assay for the diagnosis of schistosomiasis japonica, eggs prepared in this way have been shown to exhibit unexpectedly low reactivity, as reported previously by Yogore *et al.*[3]. Our recent findings indicate that a reduced COP reactivity in the case of formalin-fixed eggs is probably attributable to the nature of the eggshell structure. Since it is well known that antigenic substances responsible for the COP reaction leak through micropores in the eggshell[14-17], leakage of such substances localized between the vitelline membrane and the miracidium could be blocked in formalin-fixed eggs by some unknown mechanism. In fact, the function of the micropores might be damaged due to formalin- or PFA-fixation of the eggshell, which has been shown to be composed of protein[18]. This supposition is clearly supported by the observation that sonication, which produces artificial eggshell cracks, distinctly enhances the COP reactivity of PFA-fixed *S.mansoni* and *S. japonicum* eggs (Tables 2 and 3). The frequent occurrence of COP reaction products at these cracks as well as at the surface of the free miracidium also supports this notion (Figures 2 and 3)[19].

It is also likely that the COP test with sonicated eggs has only qualitative significance in the diagnosis of schistosomiasis, since no direct relationship could be noted between the intensity of infection and percentage of COP-positive eggs as shown in murine schistosomiasis mansoni (Table 3). Despite numerous advantages, and although many attempts have been made to quantitate the assay[5,20-22], the COP test has been shown to have an essential defect in this aspect of evaluation.

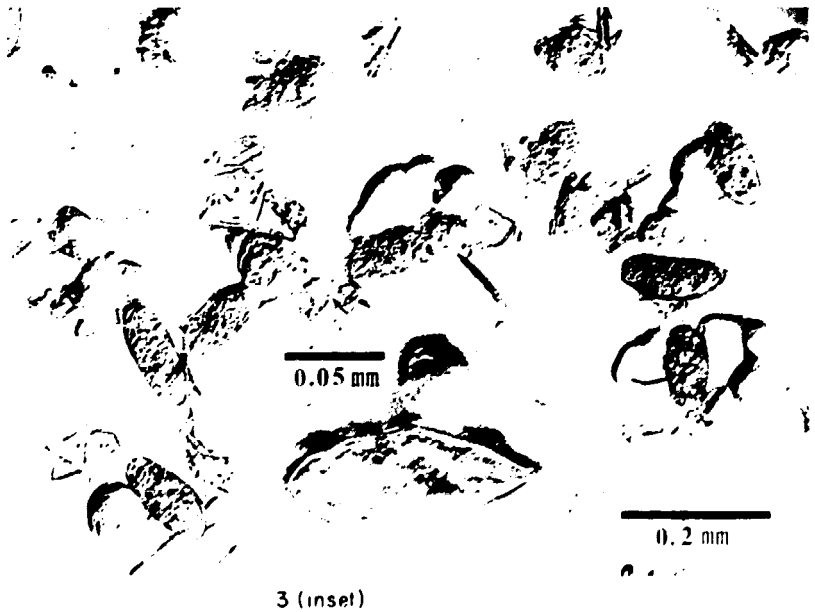

Figure 2 Large and large beaker-shaped COP precipitates at the cracks of sonicated PFA-fixed *S. mansoni* eggs. Note the numerous empty eggshells[19].

Figure 3 High magnification of a large precipitate on a free miracidium of *S. mansoni* after sonication[19].

Table 2 Results of the COP reaction using sera from schistosomiasis japonica patients and PFA-fixed *S. japonicum* eggs, with and without sonication[19]

Type of eggs used	Number of patient sera	Mean percentage of COP-positive eggs ± SD (range)
Lyophilized*	20	20.5±8.8 (8.0-40.9)
Sonicated-I†	20	6.2±3.2 (1.4-11.6)§
Sonicated-II‡	20	22.2±10.2 (7.0-39.6)

* Harvested from the livers of infected mice and kept at 4 °C for four years
† Sonicated at 115 W for 3 minutes
‡ Sonicated at 115 W for 1.5 minutes
§ This value significantly differed from those of the "lyophilized" and "sonicated-II" groups at the *P* value less than 0.001, as assessed by Student's *t*-test

Table 3 Results of the COP reaction using animal sera and PFA-fixed *S. mansoni* eggs, with and without sonication[19]

Serum pool number*	Mean no. of paired flukes recovered	% COP-positive eggs†	
		Not sonicated	Sonicated‡
0§	Not done	0	0
1	3.8	1.0	41.4
2	12.9	2.4	44.7
3	23.0	1.8	43.1
4	33.7	1.3	52.0
5	42.4	1.6	49.2
6	65.2	1.8	51.4

* From groups of infected ddY mice.
† Eggs were preserved in 0.2% PFA for three months at 4 °C.
‡ Sonicated at 115 W for 1.5 minutes.
§ Sera of 10 normal ddY mice.

The use of PFA-fixed and sonicated eggs instead of fresh or lyophilized ones in the COP assay would provide a partial solution to the problem of egg supply for the diagnosis of schistosomiasis in local, endemic areas.

Eggs preserved in alcohol or acetone

S. mansoni and *S. japonicum* eggs preserved in 95% ethanol or acetone were assessed in the COP assay. Approximately 200 eggs suspended either in ethanol or in acetone were added to glass slides and left for one hour prior to use, to allow evaporation of the fixatives. The slides were then subjected to the same routine procedure as adopted for the COP test. Although the reactivity was lower than that of lyophilized eggs (Tables 4 and 5)[23], the egg antigen was found to retain its reactivity for the diagnosis of both schistosomiasis mansoni and japonica (Figure 4).

Table 4 Results of the COP reaction using schistosomiasis japonica patient sera and *S.japonicum* eggs, lyophilized or preserved in ethanol or acetone for one month[23]

Patient serum no.	% COP-positive eggs*		
	Lyophilized	Ethanol†	Acetone‡
1	40.9	12.4	25.3
2	37.1	10.4	20.9
3	27.9	12.0	18.4
4	27.6	11.4	13.6
5	26.2	8.2	11.4
6	24.9	11.0	12.1
7	24.6	7.2	15.7
8	22.7	2.3	11.1
9	20.7	10.9	10.8
10	18.9	6.5	11.2
Mean	27.2	9.2	15.1

* From ddY infected mice.
† Preserved in 95% ethanol for one month.
‡ Preserved in acetone for one month.

Table 5 Results of the COP reaction using animal sera and *S. mansoni* eggs preserved in ethanol or in acetone for one month[23]

Serum pool number	Mean no. of paired flukes recovered	% COP-positive eggs*		
		Lyophilized	95% ethanol†	Acetone‡
1	3.8	46.5	23.0	17.4
2	12.9	50.6	31.3	21.6
3	23.0	51.0	32.2	23.4
4	33.7	50.0	37.7	23.2
5	42.4	50.4	34.4	24.3
6	65.2	51.7	33.8	25.8
Mean		50.0	32.1	22.6

* From infected birds.
† Preserved in 95% ethanol for three months.
‡ Preserved in acetone for eight months.

These alternative methods for preparation of eggs for the COP test have the advantage that eggs are put straight into the fixative soon after purification. The evaporation may cause eggshell cracks, further facilitating the reaction. It should be emphasized that eggs preserved in alcohol or acetone still retain antigenicity after

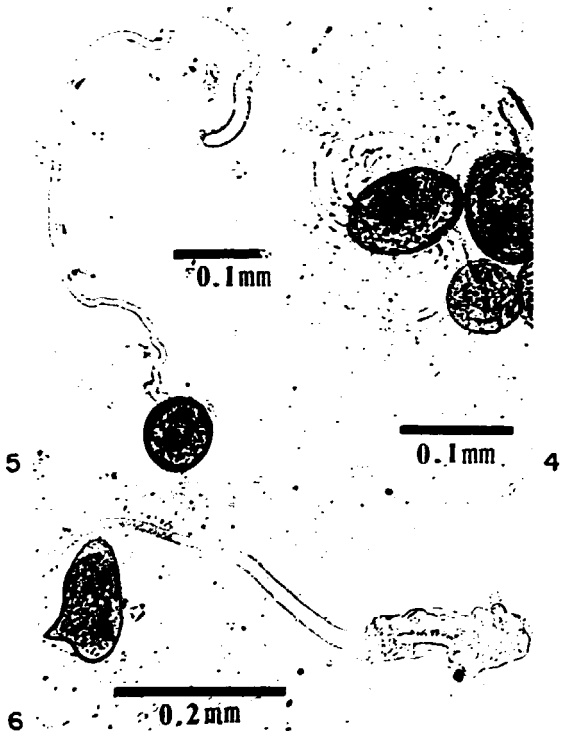

Figure 4 COP on *S. japonicum* eggs preserved in 95% ethanol[23].

Figures 5 and 6 COP on air-dried *S. japonicum* (5) and *S. mansoni* (6) eggs[24].

more than six years' preservation. These findings might be useful with respect to utilizing the COP test in the field.

Air-dried eggs

Since egg antigens responsible for the COP reaction are naturally extremely robust, it was considered worthwhile investigating the possibility of using air-dried eggs in the COP test. The advantage of being able to use air-dried eggs rather than those processed by lyophilization or preservation fixatives, would be considerable.

Air-dried eggs were collected by the digestion procedure[4] and washed in cold distilled water. One drop of water, containing approximately 200 eggs, was placed on a glass slide, in the dark, to prevent them from hatching. Following evaporation of the water, the slide was left at 37 °C overnight in a drying oven. These slide preparations could be preserved in a refrigerator with silica gel without change of antigenicity.

It was demonstrated that air-dried eggs of both *S. japonicum* and *S. mansoni* could be successfully employed for the COP test (Tables 6 and 7; Figures 5 and 6)[24]. The usefulness of this method and also the reproducibility of the reaction were evaluated[25]. This variation of the COP test using air-dried eggs would be applicable for the immunodiagnosis of schistosomiasis in local, endemic areas.

Table 6 Comparison of reactivity between lyophilized and air-dried eggs of *S. japonicum* in the COP test using positive patient sera[24]

COP type*	Lyophilized eggs	Air-dried eggs
Negative	0	5
Type I†	10	7
Type II‡	13	12
Type III§	13	12
Total	36	36

* Criteria by Yokogawa *et.al.*[28]:
† Small globules of precipitates;
‡ Medium-sized globules of precipitates and/or short segmented precipitates;
§ Large globules of precipitates and/or long and segmented precipitates.

Table 7 Comparison of reactivity between fresh, lyophilized and air-dried eggs of *S.mansoni* in the COP test using positive sera from jirds[24]

COP type*	Number of cases forming COP with		
	Fresh eggs	Lyophilized cells	Air-dried eggs
Type II†	8	7	10
Type III‡	22	23	20
Total	30	30	30

* Criteria by Yokogama *et al.*[28]
† Medium-sized globules of precipitates and/or short segmented precipitates.
‡ Large globules of precipitates and/or long and segmented precipitates.

The IOP Test with the Application of Formalin-Fixed Liver-Egg Sections

As it had been previously established that formalin-fixed eggs retain their antigenicity[9,11,13,19], the principle of the COP reaction was applied to formalin-fixed liver-egg sections of animals infected with *S. japonicum*. The sections were prepared according to a routine pathological tissue sectioning process. Serum was added to each liver-egg section slide and cover-slips placed on top. The edges of the cover-slips were then sealed with vaseline and the slides were incubated in a moisture chamber at 37 °C for 48 hours[25]. Intraoval precipitation was clearly observed between the eggshell and the miracidium in the eggs. The antibody binding site has been demonstrated previously (Figures 7 and 8)[9,11,12].

Since a large number of liver-egg sections are available from each liver of an infected animal, preparation of the antigen for the IOP test is less expensive than for other methods. Another merit is that the sectioning process exposes the antigens in the eggs, which should result in increased sensitivity and reproducibility of the reaction.

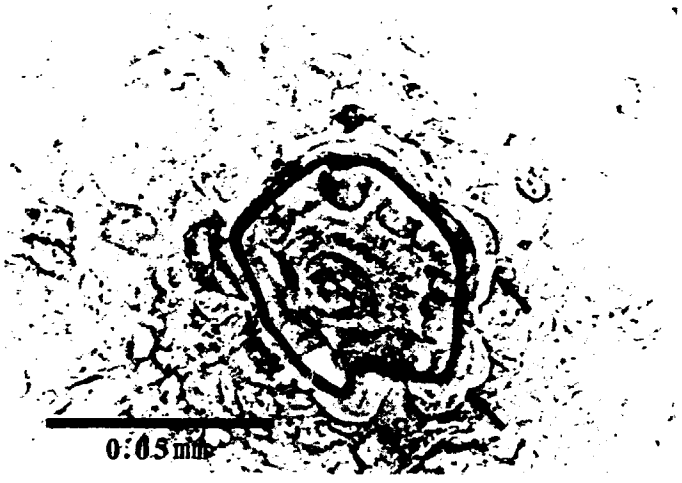

Figure 7 Distinct IOP reaction (↑) in *S. japonicum* eggs incubated with serum from a schistosomiasis japonica patient. The section was made from a *S. japonicum*-infected mouse liver, fixed in paraformaldehyde.

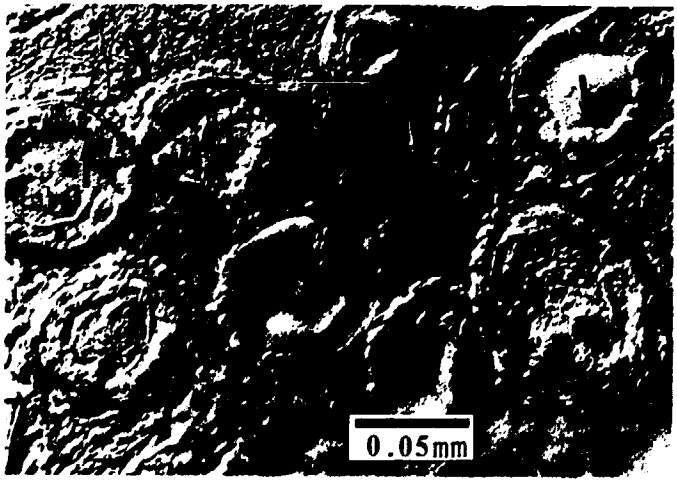

Figure 8 Differential interference photomicrograph of a typical intraoval precipitate (↑) in *S. japonicum* eggs incubated with serum from a schistosomiasis japonica patient.

Cross-Reactivity with other Helminths

Although the COP test has long been recognized as a highly specific reaction[3,5,27], cross-reactivity was observed in sera from persons infected with *Trichinella spiralis* and *Paragonimus westermani*. This finding should be taken into consideration when using the COP test in endemic areas where these helminth species are also prevalent.

Conclusions

Two methods for diagnosis of schistosomiasis in endemic areas, the COP and the IOP tests were investigated with particular regard to alternative methods of preparing the egg antigens used. The applications described provide an alternative to lyophilized eggs and thus offer a partial solution to the problem of egg antigen supply in endemic areas.

Since egg antigens associated with the reaction might be adversely affected by purification or lyophilization, eggs prepared by these alternative methods also contribute to the standardization and reproducibility of the COP test as undertaken by Garcia *et al.*[29].

Furthermore, the IOP test should be further developed, since it has many advantages for immunodiagnosis of the infection in the field. These approaches may introduce a new facet in the immunodiagnosis and control work of schistosomiasis.

Acknowledgements

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Immunodiagnosis Based on Enzyme Markers

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Summary

A revision of the various *Schistosoma mansoni* enzymes that are recognized as antigens by infected hosts and a brief evaluation of their usefulness as markers of infection (and/or resistance) is made. The catalytic property of the antigens has allowed the development of direct solid phase assays where Protein-A bound polyclonal IgG antibodies from infected patients are able to specifically capture the enzymatic antigens from crude parasite preparations; a positive capture of anti-enzyme antibodies is revealed by subsequent hydrolysis of the specific substrate. By using this type of assay, it has been shown previously that a high proportion of sera from *S. mansoni*-infected patients but not from healthy donors or people infected with other parasites, possess anti-alkaline phosphatase (AKP) antibodies (AKP immunoassay or APIA). The appearance of circulating anti-AKP antibodies seems to be associated in time with the appearance of mature parasites; this response is not related, however, to sex, age or clinical form of the disease. Differences in the intensity of the response among individuals might depend on a differential genetic capacity to recognize and respond to the antigen, to the production of more or less inhibitory antibodies and/or the competition within the assay for binding sites by other non anti-AKP IgG antibodies. The lack of cross-reaction found so far between *S.mansoni* and other host or parasite AKPs and the simplicity of APIA supports its

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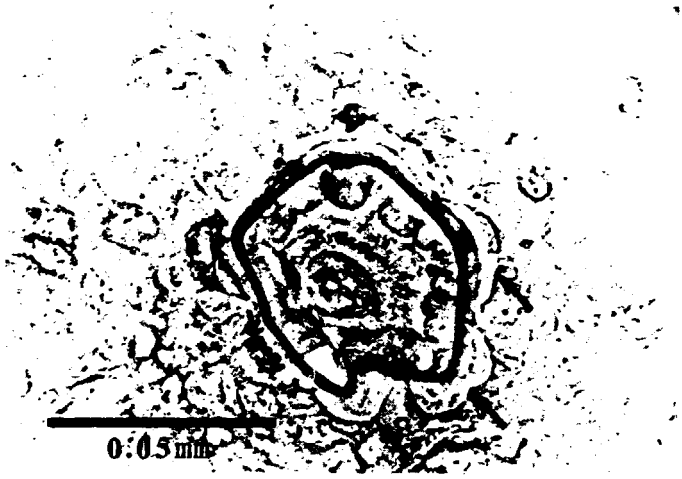


Figure 7 Distinct IOP reaction (↑) in *S. japonicum* eggs incubated with serum from a schistosomiasis japonica patient. The section was made from a *S. japonicum*-infected mouse liver, fixed in paraformaldehyde.

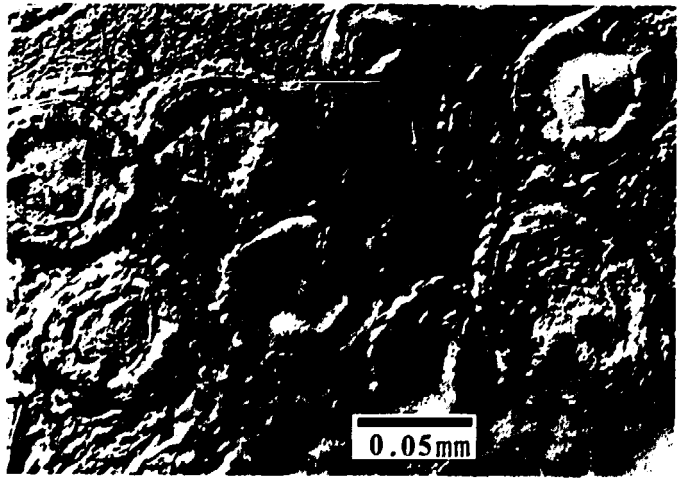


Figure 8 Differential interference photomicrograph of a typical intraoval precipitate (↑) in *S. japonicum* eggs incubated with serum from a schistosomiasis japonica patient.

possible trial in serological surveys in endemic areas for schistosomiasis mansoni. Preliminary assays to detect the presence of antibodies to the adult *S. mansoni* type I phosphodiesterase by using a similar methodology have, so far, given similar results to APIA. The advantages and limitations for the use of these assays is discussed.

Introduction

The identification of vital processes in parasites have led to the definition of molecular targets for immunological recognition and attack. The knowledge on the location, synthesis and function of parasite antigens is rapidly growing. Some have been identified as enzymes and may be responsible for an important part of the immune response against parasites[1-4] Furthermore, a few antigenic enzymes of *Schistosoma mansoni* have been already used in experimental immunoprophylaxis studies[5,6] or as tools for immunodiagnosis[7,8] The knowledge on the function of an antigen might be relevant to:

a) a better understanding of the host-parasite relationships;
b) the development of improved diagnostic assays;
c) a better understanding of the immunoresistance mechanisms;
d) the development of new strategies for immunoprotection;
e) the development of new strategies for pharmacological attack.

The parasitologic diagnosis of schistosomiasis mansoni is usually done by the microscopic identification of eggs in the faeces of the patient using the Kato and/or the modified Kato-Katz technique. These are specific tests but their sensitivity depends mostly on the intensity of infection which is related to the number of productive worm pairs and to the size of the faecal sample[9] They are useful in regions of high disease prevalence where most people eliminate more than 100 eggs per gram of faeces. However, when the intensity of the infection is low, as in Venezuela, these tests are insensitive and a high number of false negative results are produced. They can therefore no longer be used efficiently as tools for diagnosis in the epidemiological surveillance by national control programs.

Immunodiagnostic tests may play an important role as alternative tools for diagnosis. They offer indirect evidence on the presence of parasites in a patient and exhibit a variable degree of sensitivity and specificity but these two parameters depend greatly on the methodology and the quality of the antigens used.

Most of the methods that have been traditionally used for immunodiagnosis of schistosomiasis such as the intradermal test, complement fixation, *cercarien-hüllen reaktion* (CHR), indirect haemagglutination, immunoprecipitation in gel, circumoval precipitation (COP), indirect immunofluorescence, radioimmunoassay and enzyme-linked immunosorbent assays (ELISA) are based upon the detection of circulating anti-parasite antibodies in the infected host[10] Excepting perhaps the COP and the CHR, these methods do not generally discriminate between a past experience of the disease and an active infectious process because many antibodies persist even after parasitologic cure (disappearance of eggs in the faeces) following chemotherapy. The COP test[11] has been reported to show high sensitivity (that may reach 95%) and specificity (96-100%)[10,12,14] unfortunately it depends on a supply of eggs, is time-consuming and is not possible to automate.

However, more recently developed ELISA techniques use specific polyclonal or monoclonal antibodies to detect and quantify seric immune complexes or free circulating antigens in the blood (antigenemia) and in the urine[10,15,17] These

methodologies suggest the actual presence of parasites in the patient and offer information on the efficiency of the treatment; furthermore, a quantification of the captured antigen may provide an estimate of the intensity of the infection[16,18,19]. These techniques may exhibit elevated sensitivity and specificity. However, high sensitivity is usually achievable in patients with moderate or high levels of faecal egg excretion and might be low when there is low elimination of eggs; it might also be affected when circulating antibodies compete with the antigen-capturing antibody in the assay[20]. Nonetheless, pretreatment of biological fluids with protein-precipitating agents allow these techniques to detect < 1 ng of polysaccharide antigen per ml of sample[15,16,21].

Criteria to base decisions on when to apply mass chemotherapy in communities of endemic regions relying merely on immunological tests are not yet well established; it is important then to increase and to accumulate comparative field experience with different immunological techniques to develop such criteria.

Unfortunately, some good techniques are not suitable for automation or are not easily implemented in the field and others are too expensive or laborious to be used in massive immunodiagnosis campaigns. Sensitivities and specificities vary from one test to another. Antibody-detecting assays depend on a parasite cycle-dependent supply of antigen whereas antigen-detecting assays on the limited availability of specific antibodies.

An interesting view advanced by some authors deals with the idea that the possible refinement of diagnosis (for instance, differentiation between recently and chronically infected patients, evaluation of treatment effectiveness) may depend more on the measurement of antibodies against defined antigens[22]. From this viewpoint, immunological tests based on antigenic functional markers might be promising.

Excretory/Secretory (E/S) Antigens

Schistosomes may live in the host bloodstream for many years in the presence of a hostile immune response which is evaded by mechanisms mostly related to the surface of the parasite tegument[23]. The tegumental surface is highly invaginated and delimited by a membrane with a double ultrastructural appearance that accomplishes important absorptive functions of nutrients and that expresses, among other antigens, exclusive parasite components which may be susceptible to immunological and/or pharmacological attack[23,24].

The different forms of the parasite that are in contact with the definitive host (cercariae, schistosomula, adults, eggs/miracidia) are thought to excrete into the host variable amounts of different E/S products such as proteins, glycoproteins proteoglycans, polysaccharides, hormones and components of various molecular weights. Many E/S products may originate by turnover from the parasite surface or emerge from within the parasite through natural pores (glandular conducts, oral region, genital apertures, excreting orifices, etc.) in the form of small membranous vesicles or of soluble compounds[25-27]. In schistosomula and adult worms, the oral region is ends in a blind gut with a syncytial absorptive epithelial surface or gastrodermis. The absence of an anal orifice obligates these trematodes to regurgitate their gut contents every three to four hours to liberate themselves of undigested residual blood products[28] which enter the host circulation together with the parasite digestive enzymes[29]. In eggs, the E/S products produced by enclosed miracidia, emerge to the host tissues through micropores present in the egg shell[30].

The release of E/S products into the incubation medium by schistosomes has been assessed *in vitro*. Adult male and female *S. mansoni* worms maintained in MEM for up to 16 hours at 37°C excrete and/or secrete to the medium various soluble and membrane-bound polypeptides of molecular mass in the range of 15-100 kDa[25,26].

Many components which perform different cytoskeletal and enzymatic functions have been detected, as for instance, paramyosin, actin, 70 kDa heat shock protein(hsp70), glutathione S-transferase (GST), haemoglobinase (HGase, pH 3.8), acid phosphatase (ACP, pH 5.2), azocoll protease (pH 5.5), mannosyl/glucosyl transferases (pH 7.4), leucine aminopeptidase (LAP, pH 8.0) and surface membrane-associated alkaline activities (pH 9.5) like alkaline phosphatase (AKP), type I phosphodiesterase (PDE) and a Ca-stimulated adenosine triphosphatase (Ca-ATPase) among others[25,27,29,31] Most of the above enzymatic activities were detected in the medium after three hours of incubation and the specific activities of some of them (ACP, azocoll protease, LAP, AKP, PDE, Ca-ATPase) were much increased after 16 hours of incubation suggesting that they were accumulated in the medium by the living worms[25,26] The *in vitro* detected enzymes might be similarly excreted or secreted by schistosomes *in vivo*.

Surface enzymes may have an important participation in the host-parasite interaction. The AKP, PDE and Ca stimulated ATPase are integral glycoproteins of the tegumental surface membrane which expose probably a glycosylated site on the parasite surface whereas the active site seems to be buried in the internal part of the membrane[32-35] Turnover and shedding of the double surface membrane of adult schistosomes is probably the mechanism by which particulated antigens from this membrane are put into circulation *in vivo* or are released *in vitro* [36,37]

Different E/S enzymes are probably responsible for the parasite metabolism, invasion and/or migration through the host tissues, and others may participate in pathogenic events seen in this disease (anaemia, tissue damage, etc.) A secreted cercarial protease of 28 kDa promotes skin penetration and schistosomular transformation[38] Other enzymes seem to be critical for the parasite evasion of the immune response, as for instance, the schistosomulum proteases hydrolyzing surface bound antibodies[39] or the above-mentioned 28 kDa protease that cleaves protein components of the alternative pathway of the complement cascade[38]. Detoxifying enzymes, such as GST and superoxide dismutase (SOD), are thought to be important as defense mechanisms against antihelminthics and/or the host cellular immune response[40,41].

Many schistosome enzymes have been found to be able to induce host humoral (Table I) and/or cellular immune responses supporting the argument that whole enzyme molecules or their subunits are probably excreted and/or secreted by the parasites into the host circulation at a certain stage in their development. The mechanisms that regulate the release of these parasite products remain largely unknown but they might be, in some cases, partially conditioned by the presence of a specific host immune response.

The immune response against some of the E/S products shows a remarkable specificity and in some cases the response may be protective[6,42] Paramyosin is able to induce a humoral as well as a cell-mediated immune reponse which have been found to be protective in mice; a vaccine using this antigen has been proposed[42] Apparently, resistent individuals in endemic areas would have elevated serum antibodies against this molecule[43]

Enzymes as Possible Markers of Infection

Numerous schistosome enzymes have now been reported to be antigenic in infected hosts (Table I). The intestinal adult *S. mansoni* enzyme that digests host haemoglobin upon its release from ingested red cells (HGase), induces a rapid histaminic response, showing the diagnostic potential of a parasite enzyme, when purified and injected in the skin of infected patients and experimental animals[44-46] High IgM and IgG titres against this enzyme were found in *S. mansoni*-infected mice three weeks post-

infection and high IgE titres after four weeks of infection[47,48]. Immunoblot analyses revealed that sera from patients infected with *S. mansoni* and *S. japonicum* reacted specifically with polypeptides of 31 and 32 kDa [49], both thought to be proteolytic enzymes. The genes that code for each of these proteins have been cloned and sequenced[50-52]: the 31 kDa protein has been reported to have a sequence similar to thiol-proteinase or cathepsin B [51], whereas the 32 kDa protein seems to have a unique sequence so far unknown for a protease[50,52]. These proteinases are antigens of major importance which probably start to circulate prior to egg excretion[48]. While antibodies to cathepsin B remained elevated for several months after chemotherapy, anti-32 kDa antibodies were seen to decrease[53]. A molecule with a sequence similar to the 32 kDa protein was found in the preacetabular glands of cercariae where its function remains to be investigated[52]. Host haemoglobin is then probably degraded by more than one digestive enzyme, including enzymes from adult *S. mansoni* worms that are not thiol-dependent[54,55].

An acid S-protease of molecular mass 25-27 kDa from the *S. mansoni* egg was found associated with the penetration glands of the enclosed miracidium. It did not bind to Con A and was immunogenic in mice[56,57].

An IgM immune response against the elastase (serine-protease) of molecular mass 30 kDa secreted from the preacetabular glands of the cercariae was detected in *S. mansoni*-infected humans and mice; the response against this enzyme was specific for cercariae[58] and it provided high sensitivity when used as antigen in antibody-detecting ELISA [59].

IgG antibodies against the LAP of the adult and the schistosomulum forms of the parasite were found after four weeks of infection in sera of *S. mansoni*-infected rats; infected humans also possessed anti-adult LAP antibodies[60]. On the other hand, the LAP from eggs, a glycoprotein of molecular mass 140 kDa, possibly involved in the egg hatching process[61], is able to induce IgM and IgG antibodies in *S. mansoni*- or *S.japonicum*-infected mice[62]. IgG antibodies against this enzyme were also found in *S.mansoni*-infected patients[26]. Interestingly, the anti-egg LAP antibodies decreased in the sera of experimental animals after treatment with praziquantel[62].

Malate dehydrogenase (MDH) is a major enzymatic component of schistosomes[5,63]. The enzyme is found in the cellular layer of the schistosome gut and it is an E/S product. Two isoenzymes (pI 7.1, pI 8.5) of this enzyme (60 kDa) have been described as important immunogens[5]. The pI 7.1 isoenzyme is a circulating antigen able to induce early specific antibodies in humans and in experimental infections; it has been found to be a partially protective antigen in experimental infections[5]. The reported enzyme is a dimer of two similar polypeptides which do not dissociate in the presence of SDS. By immunodiffusion, 24/30 patients were found to have anti-MDH antibodies. On the other hand, the purified *S. mansoni* mitochondrial 65 kDa MDH, an antigen which cross-reacts with other human schistosomes, conjugated to Sepharose-4B beads (DASS test), was also able to detect anti-MDH antibodies in infected human sera and experimentally infected mice[64]. The anti-MDH antibodies did not inhibit the enzymatic activity. However, low sensitivity (2/19 positive patients) was found by DASS and by immunoelectrophoresis with this antigen[64].

Simurda *et al.* [40] found that the dimer (40 kDa) and the 20 kDa monomer of the anti-oxidant enzyme superoxide dismutase (SOD) secreted by *S. mansoni* were immunoprecipitated by antibodies present in the sera of infected humans. This enzyme may be essential in the prevention of oxygen toxicity or in mechanisms of defense by the parasite against the cellular immune response. It shows 40-45% homology with the human sequences for Cu/Zn SOD.

In humans infected with *S. mansoni* or with *S. japonicum* it is possible to detect a dominant antibody response against a parasite protein of 70 kDa which is

Table 1 Antigenic enzymes of *Schistosoma mansoni*

Parasite form	Enzyme	Molecular mass (kDa)	Origin	Isotype (host)	Reference
Cercaria	Elastase	30	Preacetabular glands	IgM (human, mouse)	Pino-Heiss *et al.*, 1986[58] McKerrow and Doenhoff, 1988[59]
Schistosomulum (transient)	Leucine-aminopeptidase	-	Tegument	IgG (human, rat)	Damonneville *et al.*, 1982[60]
	Glutathione-S-transferase	-	Tegument	IgG (baboon, hamster, mouse, rat)	Balloul *et al.*, 1987[6]
	Glyceraldehyde-3P-dehydrogenase	-	Tegument	IgG (human)	Goudot-Crozel *et al.*, 1989[72]
Adult	Haemoglobinase	27-32	Gut	IgM, IgG, IgE (human, baboon, mouse	Senft and Maddisson, 1975[49] Zerda *et al.*, 1987[75] Chappell *et al.*, 1988[48]
	Malate dehydrogenase	60	Gut	IgG (human, mouse)	Bout *et al.*, 1978[5]
	Malate dehydrogenase	65	Mitochondria	IgG (human, mouse)	Rotmans, 1978[64]
	Alkaline phosphatase	260	Tegument	IgG (human, mouse)	Cesari *et al.*, 1981[32]; 1987[26] Pujol and Cesari, 1990[67]

	Leucine-aminopeptidase	-	Tegument	IgG (human, rat)	Damonneville *et al.*, 1982[60]
	Acid phosphatase, Phosphodiesterase, Ca-ATPase	-	Tegument	IgG (human, mouse)	Cesari *et al.*, 1987[26]
	Cathepsin B	31	Gut	IgM, IgG, IgE (human, mouse)	Ruppel *et al.*, 1987[49] Klinkert *et al.*, 1989[51]
	Glutathione-S-transferase	28	Parenchyme	IgG, IgE (human, mouse, rat, baboon, hamster)	Balloul *et al.*, 1987[6] Holy *et al.*, 1989[78]
	Superoxide dismutase	40	E/S	IgG (human)	Simurda *et al.*, 1988[40]
Egg/miracidium	Thiol-proteinase	25-27	Hatching fluid	IgM (mouse)	Dresden *et al.* 1983[57]
	Leucine aminopeptidase	140	Hatching fluid	IgM, IgG (mouse)	Xu *et al.*, 1988[62]
				IgG (human)	Cesari *et al.*, 1987[26]

localized in the tegument, the nervous system of the worms and in the hatching fluid of mature eggs; this protein has been found to be similar to hsp70 present in most types of cells in different organisms where they perform several important functions such as ATP-dependent unfolding of proteins or prevention of aggregate formation[53]. In general, hsp70 may also exhibit an ATPase activity. Since there are few amino acid differences between the hsp70 of schistosomes and that from their hosts, a limited structural diversity seems to be sufficient to induce an antibody response able to discriminate between these two evolutionarily conserved proteins[65], a property which might provide a specific diagnosis. Anti-hsp70 antibodies have been detected in a significant number of individuals suffering from chronic schistosomiasis mansoni but not in patients with recent infections; these antibodies were not detectable until 5-6 weeks post infection in experimental mice[53]. The *S. mansoni* hsp70 is immunologically distinct from that of *S. japonicum* and does not cross-react[65], although it cross-reacts with the hsp70 of *S. haematobium*. On the other hand, cross-reacting antibodies to the *S. mansoni* hsp70 have been found in donors infected with filariasis and malaria[53].

The IgG fraction from chronically *S. mansoni*-infected CBA mice but not that from uninfected mice, inhibited up to 25% the AKP activity of a detergent-solubilized tegumental membrane preparation; however, the same IgG fraction did not inhibit the host (mouse, hamster) intestine or liver AKP activities. Another two phosphohydrolytic activities, type I PDE and Ca-ATPase, were not significantly inhibited by this IgG fraction[12]. Sera from chronically infected C57BL/6 mice inhibited up to 35% the AKP activity[66] and sera from *S. mansoni*-infected patients up to 20%, independently of the presence of a high or a low titer of anti-AKP antibodies in the individual (as measured by other serological tests)[56,67]. These results suggested the antigenicity of the parasite AKP in infected hosts.

The *S. mansoni* AKP has a broad substrate specificity and exhibits mainly 5'-nucleotidase activity; it is generally accepted as a plasma membrane marker[32] that favours surface exchange of nutrients by its dephosphorylating action on external nucleotides and sugars. An IgG fraction prepared against mouse liver 5'-nucleotidase had no effect on the parasite AKP although these antibodies inhibited the mammalian membrane-bound 5'-nucleotidase[32]. Lack of cross-reaction of mouse anti-parasite AKP with a preparation of mouse liver or intestinal AKP was confirmed by Pujol and Cesari(1990)[67]. Altogether, these studies suggest that there is no apparent immunological cross-reaction in exposed epitopes between the host AKPs and an enzyme that performs similar biochemical functions in the parasite.

Use of Parasite Enzymes for Detection of Host Antibodies

Preliminary studies using the Protein A-Sepharose capture of seric IgG, subsequent capture of enzyme antigen and detection of the activity with specific chromogenic substrates[60] or by colored reactions, made it possible to establish that the AKP, ACP, PDE and Ca-ATPase from an adult worm preparation and the LAP from eggs were all specifically recognized by antibodies present in pooled sera of infected patients[26]. Results from this experiment also indicated that some immunocaptured enzymes showed up more readily than others. The degree of activities was as follows:

AKP (adult) > LAP (egg) > PDE (adult) > ACP (adult) > ATPase (adult)

The AKP thus emerged as a candidate to be tested in immunocapture assays despite the fact that a partial inhibition of activity was previously demonstrated with

some sera[32,66,67] This fact indicates that inhibition by antibodies do not greatly block the development of the reaction. The same is probably true for the PDE and the Ca-ATPase activities suggesting that the epitopes recognized by the polyclonal antibodies on these enzymes are at a different position than the catalytic site, this site being probably buried within the membrane domain under natural conditions[32-35] The native AKP is an integral surface membrane glycoprotein of an apparent molecular mass of 260 kDa, composed of four subunits of 65 kDa each[33] and linked to the membrane via a glucosylphosphatidylinositol residue[68] This enzyme may be extracted from the parasite tegumental membranes and subsequently stabilized in solution by a suitable n-butanol-detergent procedure before its use as an antigen (Cesari *et al.*, in preparation).

In order to facilitate the evaluation of the appearance/disappearance and the presence of circulating anti-AKP antibodies in the sera of human populations from the endemic areas, a simple solid phase assay on microtitre plates was developed[8] (Figure 1). Briefly, the assay consists of fixing the IgG fraction from 1:100 diluted sera onto Protein A-coated wells. If anti-AKP antibodies have been adsorbed, they will capture the enzyme from the butanolic extract and subsequent addition of substrate (p-nitrophenyl phosphate) will lead to the catalytic release by the antigen of the colored (p-nitrophenol) product (positive) as compared to unhydrolyzed substrate in absence of enzyme capture (negative)[8]

Using the AKP immunoassay (APIA), IgG antibodies against this enzyme were detectable after four weeks of experimental infection[67] The testing by APIA of 56 sera of parasitologically (Kato-Katz), COP and/or ELISA (egg antigen) *S. mansoni*-positive individuals, 16 sera of *S. mansoni*-negative uninfected donors and 24 sera of patients infected with other parasitic diseases including amoebiasis (three), paragonimiasis (one), trichuriasis (one), ascariasis (one), trichuriasis/ascariasis (one), strongyloidiasis (one), hydatidosis (one), taeniasis (two), cysticercosis (two), fascioliasis (one), leishmaniasis (four), malaria due to *Plasmodium falciparum* (four) and schistosomiasis due to *S. haematobium* (two), indicated that an elevated number (93%) of the tested *S. mansoni*-infected patients possessed anti-AKP antibodies, whereas none of the *S. mansoni*-negative patients tested exhibited reactivity (100% specificity)[8] It was concluded that the AKP could be possibly used as a marker of infection for schistosomiasis mansoni.

The statistical analysis on a small sample (n = 44) of parasitologically (Kato-Katz) and COP-positive (cut-off = 10%) *S. mansoni*-infected Venezuelan patients (Table 2) showed a frequency distribution of eggs/g of faeces typical for other samples of patients from the Venezuelan endemic region, that is, exhibiting low intensities of infection (<100 eggs/g faeces) (Figure 2). The frequency distribution of the COP-positive sera values is also reported in Figure 2. An analysis by the Spearman's rank correlation test indicated that the values obtained for eggs/g of faeces and those for the COP test were strongly correlated ($p = 0,001$). APIA results obtained with these sera showed that 40/44 individuals were positive by this test (cut-off = 0.079 O.D. units at 405 nm in this experiment); most of the positive individuals exhibited a low to moderate response to AKP, with a few exhibiting high anti-AKP responses (Figure 2). The anti-AKP response did not correlate with sex, age or the clinical status (Table 2) of the patients. On the other hand, APIA values showed a positive correlation with those of eggs/g faeces ($p = 0.026$) but not with those of the COP test.

A more recent evaluation of APIA using sera of 45 different COP positive Venezuelan patients from the endemic zone (stools were not analysed for eggs) led to similar results as shown in Figure 2 (data not reported); 40/45 patients were positive for APIA (cut-off = 0.009 O.D. units at 405 nm in this experiment). Again, no Spearman's correlation was found between the APIA and the COP values. However,

Table 2 Description of *S. mansoni*-infected patients

Number		44
Males/Females		28/16
Mean age ± SD		19 ± 11
Clinical status:	Asymptomatic	5
	Intestinal	10
	Hepato-intestinal	28
	Hepato-splenic	1

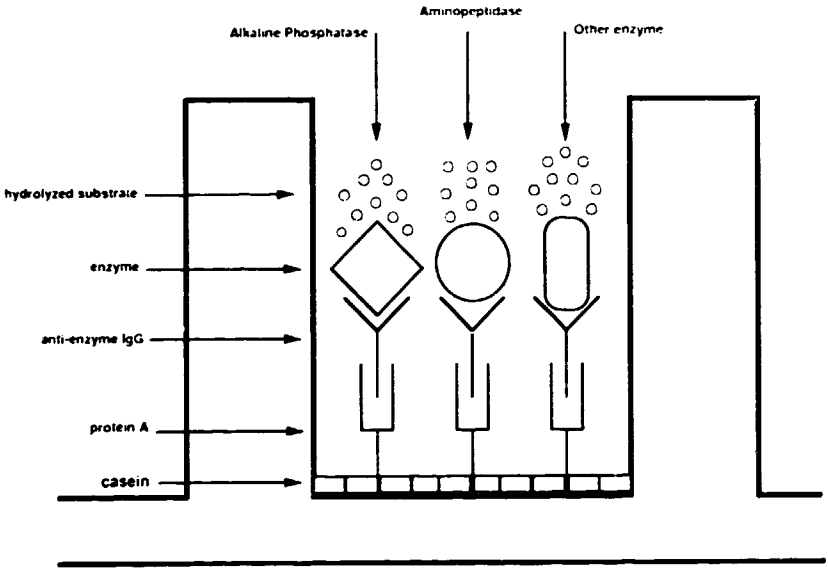
a positive correlation ($p = 0.01$) between these two tests was found when data from the two surveys (corrected for their cut-off values) were pooled ($n = 89$).

Sera of a small number($n = 20$) of COP-negative healthy donors from non-endemic sites were all APIA-negative (data not reported), further supporting the elevated specificity of the test. However, in a different survey, 3/29 COP-negative but suspected sera from a zone of disease transmission were found to be positive by APIA. In this zone, endemic also for *Ascaris lumbricoides* and *Trichuris trichiura*, *S.mansoni*-negative patients excreting eggs of these two parasites, were found to be serologically negative by APIA (unpublished). This would exclude the possibility that the APIA-positive sera which were negative in the COP test were cross-reacting with the AKP of other helminths in the region. These cases may thus be explained by the fact that patients were either truly infected with *S. mansoni* and cured thereafter, unisexually infected with this parasite, or cross-reacting with an as yet unknown agent. A deeper analysis of these cases has, however, to be carried out.

A solid phase assay similar to APIA was preliminary used to look for antibodies against the adult PDE. This was done in a sequential manner after APIA; thus, after washing away the products of the AKP reaction, a PDE substrate was added and the plate incubated for a longer period of time (16-18 hours due to the intrinsic kinetic properties of this parasitic enzyme). Although the kinetic parameters to optimize this immunoassay have not yet been worked out, it was possible to detect positive patients in a manner similar to using APIA (data not shown) and a strong positive correlation ($p = 0.001$) was seen with this assay. Most individuals which were positive or negative in APIA were also positive or negative in the PDE immunoassay; some individuals were only positives in the PDE immunoassay whereas others only in APIA.

Thus, the enzyme immunoassays described here allow the detection of antibodies, in the same individual, to more than one antigenic enzyme present in the preparation, opening the possibilities to measure the response to more than one antigen using the same initially adsorbed antigenic material (Figure 1). Individual differences in response to these antigens might be due to genetic restriction for the immune recognition and/or response to particular enzyme epitopes.

Since IgG antibodies against AKP start to be detected in experimental animals 30 days post-infection[6,7] and last for several months after successful chemotherapy[8], APIA is probably not useful for discriminating between a recent and a chronic infection or for providing information about the outcome of medical treatment. Nonetheless, our results emphasize some advantages of this technique. In terms of simplicity, cost and the colour reaction, APIA resembles the antibody-detecting ELISA



1 Coat well overnight (4 C) with protein A solution (10 µg/ml) in carbonate buffer (0.05 M, pH 9.6)

2 Saturate wells with 1% casein in carbonate buffer (0.1M, pH 9.6)

3 Add serum diluted 1:100

4 Add enzyme-enriched parasite fraction

5 Add specific chromogenic substrate, wait for optimal color development and evaluate reaction

Figure 1 Enzyme antigen immunoassay

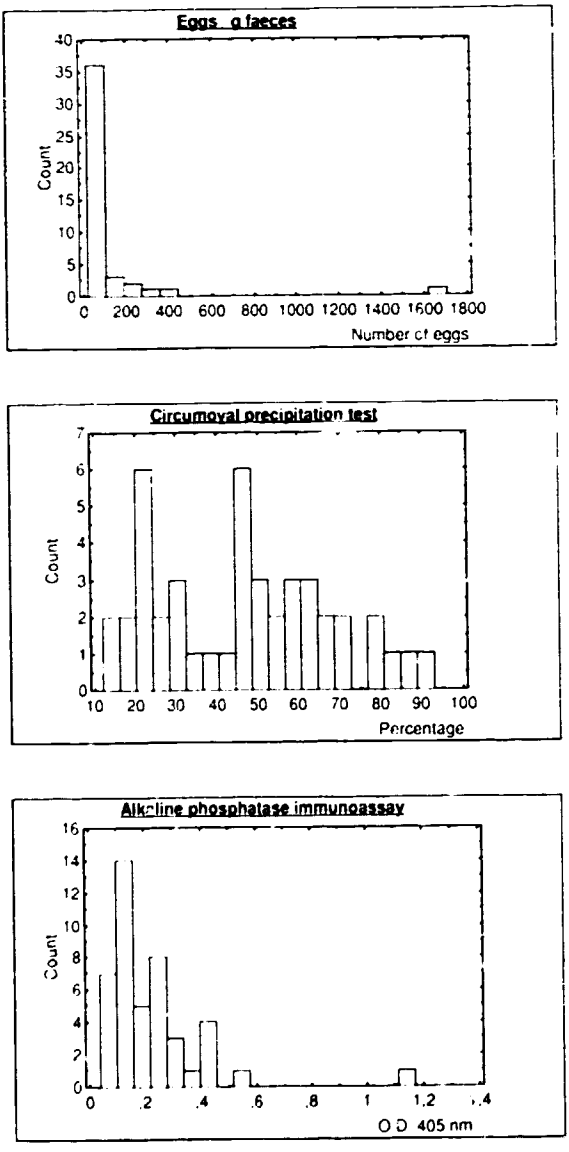


Figure 2 Frequency distribution of diagnostic parameters

and may be applied for massive screening of populations; however, it is far more specific than ELISA with crude adult or egg soluble antigens. It detects the presence of heterogeneous antibodies to one single, defined antigen using small amounts (µg) of crude antigenic material per well. APIA needs small amounts of diluted serum or plasma (50 µl/well of a 1:100 dilution) that may be easily obtained from a single drop of blood after finger puncture[69]. Despite the above-mentioned limiting factors, the highly elevated specificity of APIA would permit determination of the immune status of individuals (responders and non-responders) in a population and enable perhaps immune mapping of sites in endemic regions, which, together with the availability of safe chemotherapy, would help to take decisions on mass treatment.

Further field studies with a greater number of sera should substantiate this contention for patients from other geographic areas, with recent or very long-standing infections and with infections of other schistosome or parasite species. More research is also needed to identify other antigenic enzymes of this parasite, in particular stage-specific enzymes with high substrate turnover, to develop adequate solid-phase assays that may provide an answer to the above unsolved diagnostic problems.

Besides adults, the AKP is also present in cercariae, schistosomula and eggs[33]. However, it may be differently expressed in these stages; it is for instance paradoxically available for surface iodination on schistosomula but not on adult worms[33]. On the other hand, antibodies in sera from mice immunized with highly irradiated cercariae did not inhibit the parasite AKP activity[32]. The importance of this molecule in relation to immune protection mechanisms or responses with different isotypes remains an open question.

Enzymes as Possible Markers for Diagnosis of Immune Resistance

Some enzymes are targets of protective immune responses and the question can then be put whether antibodies directed against an antigen with important biological functions can have an important effect on worm load or worm metabolism[5].

Previous studies immunizing experimental animals with the pI 7.1 isoenzyme of the MDH partially answered this question in that a partial protection of about 30-40% against a challenge infection could be achieved[5].

Immunization with, or presence of, antibodies to tegumental and sub-tegumental GST (the p28-1 antigen) has been described to be associated with partial protection (about 40% or more) against an infectious challenge with cercariae in rats, mice, hamsters and baboons[6]. Glutathione S-transferases are a group of multifunctional proteins involved in the detoxification of harmful compounds such as lipid hydroperoxides[41]. Host immune defense mechanisms against this enzyme may involve inhibition of the GST activity by antibodies; an anti-GST (p28-1) IgM monoclonal antibody was apparently able to reduce *in vitro* and *ex vivo* worm fecundity[70], and immunization of baboons with GST could reduce female schistosome fecundity and egg-induced pathology[71].

Sera from subjects with low susceptibility to *S. mansoni* infection reacted with a major larval surface antigen of 37 kDa against which sera of susceptible individuals showed little reactivity. This antigen shows similarity to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPD), a highly conserved protein (72,5% of positional identity with human GAPD)[72].

Enzyme immunoassays such as those discussed above[8] and the use of monoclonal anti-enzyme antibodies in assays for capturing protective circulating enzyme antigens (for instance, the Sj26: a GST from *S. japonicum*)[73] might be of potential interest as indicators of resistance in humans.

Final Comments

Parasites release *in vitro*, and also conceivably *in vivo*, many E/S products that possess defined functions for the physiology of the parasite such as cytoskeletal proteins, various enzymes, enzyme inhibitors, hormones, etc. A number of these E/S products are proteins highly conserved through evolution which may, however, still be major targets of host immunity. Responses against highly conserved molecules in schistosomes have been found to be very specific, for example, those against SOD [40], hsp-70 [65] and GAPD [72]. Genetic restriction of the immune response against these antigens may occur in heterogeneous human populations because of a limited number of non-self epitopes carried by these homologue proteins. The latter authors hypothesize that such genetic defects might allow the maintenance of parasite transmission through low or non-responder (susceptible) individuals, a hypothesis that remains to be tested and confirmed.

Immuno-inhibition of an enzyme's activity may in some cases be associated with resistance to reinfection by means of interference with the physiology of the parasite, as seems to be in part the case for the GST. In these cases, however, immunological assays based on the inhibitory properties of the antibodies might be useful tools in detecting resistance or susceptibility to infection in endemic populations.

From the diagnostic point of view, the use of antigenic enzymes as markers of infection would be favoured if:

a) the enzymes are present only in the parasite and absent in the mammalian host (for instance, glucanase of yeasts, ß-lactamase of bacteria);
b) the parasite enzymes are sufficiently different in antigenic structure or in assay conditions (substrate concentration, ionic strength, pH) to the host enzymes;
c) the parasite enzymes exist in sufficient quantity to be detected by the assay;
d) the enzymes are sufficiently stable to be isolated and processed as antigen through the immunoassay; and
e) antigen-antibody complex formation does not interfere completely (as in the case of AKP) or at all (as in the case of PDE, Ca-ATPase) with the binding of substrate and enzyme.

Various effects can theoretically occur when antibodies interact with an enzyme target, namely:

a) inhibition (e.g. if hosts produce neutralizing or inhibiting antibodies);
b) enhancement;
c) change of properties that require new assay conditions (substrate concentration, ionic strength, pH); and
d) no apparent effect.

Combined effects may be expected with heterologous populations of antibodies.

A limitation of the immunocapture assays would certainly occur when antibody inhibits the activity. Enzyme antigens in which the active site and the immunological epitope are closely located or coincident, might not show enzymatic activity after immune complex formation because of shielding of the active site by the bound

immunoglobulin. If this does not occur, the degree of assay sensitivity with a determined enzyme might be limited by:

a) a host genetic restriction to respond to the available enzyme epitopes;
b) the amount of enzyme captured;
c) antibody isotype; and
d) the turnover number of the enzyme.

Finally, besides detection of anti-enzyme antibodies, monoclonal antibodies may be raised against relevant antigenic enzymes[58,73,74] which may be used in capture assays of circulating enzymes[8,73].

Immunoscreening based on antigenic enzymes may help to ascertain population exposure to parasites in endemic regions. Appropriate studies on enzyme antigens, anti-enzyme (mono- and polyclonal) antibody isotype analysis, longitudinal evaluation on the time of the appearance/disappearance (kinetics) of specific anti-enzyme antibodies during infection and/or after chemotherapy, and stage-specific enzymes of schistosomes are needed.

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Antigen Detection in Urine Samples for the Diagnosis of Schistosomiasis

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Summary

A monoclonal mouse antibody of the IgM class, raised against an epitope of the gut epithelium of the adult schistosome worm but also present in the egg in relatively large amounts, was selected for its capability to detect a parasite antigen circulating in the host and excreted in the urine. The antigen is thermostable, soluble in trichloracetic acid, not hydrolysed by proteinase K but destroyed by metaperiodate, suggesting a polysaccharide constitution. The antigen was shown to be *Schistosoma* genus-specific and was detected in the urine by means of passive haemagglutination inhibition, reverse passive haemagglutination or sandwich ELISA.

When used in Central Africa for the study of prevalence in foci of schistosomiasis due to *S. mansoni*, *S. haematobium* and *S. intercalatum*, good correlation with the parasitological examinations was found. Excretion of the antigen decreased after treatment with praziquantel.

In 1958 Okabe and Tanaka[1] had already tried to utilize the detection of specific antigens in the urine of patients with schistosomiasis japonica in Japan and China. But they had little success, probably because they used crude antibodies in their precipitin reaction.

Circulating Antibodies for Diagnosis

Frozen sections of adult worms used as antigens in seroimmunological tests have a doubtful specificity because of the complexity of the worm antigens. The implementation of fixed sections (Rossman fixative), destroying the proteins and selectively preserving the polysaccharide structures of the gut epithelium, allows a better specificity of the test [2]. This specificity, when the test is performed on fixed sections, is excellent in untreated patients[3], but in treated patients, looking for circulating antibodies offers only moderate advantages because of the long persistence of the antibodies after treatment. Hence the advantage of detecting antigens for diagnosis.

Circulating Antigens

In 1959, Timms and Bueding[4] demonstrated the existence of circulating antigens, originating from the epithelium of the adult worm, similar to the one later described by Capron *et al.*[5]

A thermostable, non-dialysable antigen was found in the serum and in the urine of infected hamsters by Berggreen and Weller[6]. Concentration of the antigen has been shown to be proportional to the parasitic load[7], and the antigenic structure is located in the gut epithelium[8]. It is a proteoglycane and corresponds to the CAA (circulating anodic antigen) of Deelder and Eveleigh[9]

A circulating antigen of cathodic mobility, first described by Carlier *et al.*[10] was subsequently demonstrated and named CCA (circulating cathodic antigen) by Deelder *et al.*[11]. This antigen, a thermostable polysaccharide, purified by extraction from the soluble trichloracetic acid fraction, was demonstrated in the serum and urine of experimentally infected animals, in patients with schistosomiasis and in the milk of women with the disease[12]. The titre in the urine was proportional to the number of eggs found in the stools and inversely proportional to the age of the patient, consistent with the view that the antigen was of parasitic origin. Enumeration of worms found *post-mortem* demonstrated that the number of worms diminished with increasing patient age[13]. As opposed to anodic antigen, the cathodic antigen has a positive load at pH 8 [14] like the circulating anodic antigen, it has an heterogenous molecular mass due to its polymeric structure and the tendency of the molecules to form aggregates[15]. Furthermore, like the anodic antigen, the cathodic antigen has been demonstrated in the gut epithelial cells of the adult schistosome[16].

Other polysaccharide antigens have since been described. Qian and Deelder[17] studied the antigenic components of a trichloracetic acid soluble fraction of *S.japonicum* adult worms by means of electrophoresis, and compared them with *S.mansoni* antigens. Among the eight trichloroacetic acid soluble antigens found in *S.japonicum*, five have an immunological similarity with *S. mansoni* antigens. Five antigens with an anodic electrophoretic mobility have been identified as circulating antigens in the sera of hamsters and rabbits infected with *S japonicum*: the

principal circulating antigen is CAA. Two other antigens, with a cathodic mobility, including CAA, have been demonstrated as being circulating antigens in *S. mansoni*, but not in *S. japonicum* infections. Most of these circulating antigens have been defined as being associated with the gut intestinal epithelium of the adult worm.

Such antigens are important for immunodiagnosis because the detection of circulating antigens indicates an active infection. In contrast, circulating antibodies persist in the body long after elimination of the parasite. Detection of circulating antigens is preferable in monitoring chemotherapeutic efficiency because the amount of circulating antigen is related to the parasitic load.

Advantages of Producing Monoclonal Antibodies for Antigen Detection

A monoclonal antibody is an antibody produced by only one clone of B-lymphocytes. All molecules of this antibody have the same sequence of amino-acids and the same binding capacities. The monoclonal antibodies are directed against one determinant only of the antigen, while crude antisera contain a pool of antibodies, with different specificities and affinities.

Taking into account the difficulties of conventional serodiagnosis, we prepared a monoclonal antibody directed against an antigenic structure located in the intestinal epithelium of the adult schistosome, capable of allowing detection of circulating antigens in infected hosts. Deelder[18] showed that the titres of the detected antigens give good correlations with the elimination of eggs, contrary to that which occurs with antibodies.

The techniques used in our laboratory for producing monoclonal antibodies are described in detail elsewhere[19].

B-lymphocytes were obtained from immunized animals, having been infected and treated, thus producing large amounts of antibodies. The clones selected were those secreting antibodies directed against the polysaccharide antigen of the gut epithelium.

Among the 480 supernatants tested by immunofluoresce, 14 gave a positive reaction and 11 reacted well. The 11 hybridomas were cloned and the hybridoma Sm 10 was then used for the following study.

Characteristics of Antibody Secreted by the Hybridoma and of the Antigen Excreted in the Urine

The mouse monoclonal antibody Sm10 is of the IgM class, type Lambda. It is directed against an epitope of the intestinal epithelium of the adult worm. The antigen found in the urine of the host is thermostable, soluble in trichloracetic acid, destroyed by metaperiodate and resistant to the effect of proteinase K, suggesting that the antigen is a polysaccharide. The detected antigen is related to other schistosomal antigens earlier described, in the structure and location in the intestinal epithelium of the adult worm. It differs, however, from them in its location in large amounts in ovular extracts.

Three techniques have been used for the detection of the polysaccharide antigen in the urine of laboratory animals or naturally infected humans: the passive haemagglutination inhibition assay (IPHA); the reverse passive haemagglutination assay (RPHA) and the sandwich-ELISA.

Antigen Detection in the Urine of Laboratory Animals

The monoclonal antibody of the IgM class selected for the study reacts with the polysaccharide antigen excreted in the urine of laboratory animals experimentally infected with *S. mansoni*. In mice, the excreted antigens disappear after treatment with praziquantel. Antigen concentration in the urine decreases slowly, over a period of several months, before it finally disappears completely. This phenomenon is probably due to sequestration of the antigen in the liver. The elimination curve from this organ runs parallel to the one of the urine antigen[20].

Antigen Detection in Human Schistosomiasis Mansoni

The mouse monoclonal antibody was first used in the field for the detection of the homologous antigen *S. mansoni* in the intestinal schistosomiasis focus of Djohong (Adamawa - Cameroon)[21]. In Djohong, the prevalence of intestinal schistosomiasis is 26,9% by stool examination[22], and 46,8% by antigen detection in urine. The prevalence by age and sex measured by antigen excretion in urine is similar to that measured by elimination of *S. mansoni* eggs in the stools, but the antigen detection is more sensitive than parasitological examinations of stools by means of direct smear and enrichment by the formalin ether technique[23]. In Djohong, children excreted antigen very early while very few of them eliminated eggs of *S. mansoni* in the stools.

The haemagglutination inhibition test was used to evaluate the prevalence in another focus of schistosomiasis mansoni in Cameroon in Nalassi-Emana[24], where results similar to those observed in Djohong were obtained for the detection of the homologous *S. mansoni* antigen with the monoclonal antibody.

Nine months after antihelminthic treatment with praziquantel in Nalassi-Emana, inhabitants excreting antigen in the urine diminished markedly, while circulating antibody levels remained high. The test for detecting the antigen in urine seems to be the most efficient way of monitoring the effect of mass treatment in intestinal schistosomiasis[25].

Antigen Detection in a Schistosomiasis Haematobium Focus

Since the antigen detected is characteristic for the genus *Schistosoma*, the test has also been used in Cameroon in the schistosomiasis haematobium focus of Barombi Kotto[24]. In Kotto, 21,8% of the inhabitants were voiding eggs in their urine, (filtration technique of Plouvier *et al.*[26]), where the polysaccharide antigen was found in 46,7% of the examined inhabitants. The test is also very sensitive in schistosomiasis haematobium and is more often positive in subjects voiding eggs than in those where such eggs have not been found.

Antigen Detection in Mixed *S.mansoni-S.haematobium* Foci

When used in Cameroon for the study of prevalence in a focus of schistosomiasis due to both *S. mansoni* and *S. haematobium* (Tala Mokolo) in the Mandara Mountains, the haemagglutination inhibition test for detecting the antigen in urine gives good correlations with parasitological examinations for detecting eggs in faeces and urine[27]. Since the antigen is characteristic for the genus *Schistosoma*, the test can be used for the study of mixed infections caused by both *S. haematobium* and *S.mansoni*.

Antigen Detection in Schistosomiasis Intercalatum Foci

The detection in urine specimens of a sample of the inhabitants of Edea (Cameroon) of the polysaccharide antigen characteristic for the genus *Schistosoma*, with the monoclonal antibody by means of the inhibition of a passive haemagglutination test, shows that this technique is very sensitive for measuring prevalence of schistosomiasis due to *S. intercalatum*. In Edea, examination of stool specimens for eggs gives a low prevalence rate for the disease because of the low parasitic load in the area. The prevalence by age, according to the voiding of eggs, parallels the excretion of antigen[28]. Similar results have recently been obtained in the schistosomiasis intercalatum focus of Anayabo in Gabon[29], by means of the haemagglutination inhibition test but also by other techniques for antigen detection in urine, the reverse passive haemagglutination test (RPHA) and the sandwich ELISA test. The specifity and the sensitivity of the three tests seems to be very similar, but the sandwich-ELISA test is the easiest to use in the laboratory.

Discussion

The aim of this article is to compare the results of a test for the detection of a *Schistosoma* specific antigen excreted in the urine with the data obtained by microscopic examination of the stool or urine specimens in patients with schistosomiasis mansoni, haematobium or intercalatum.

In the host, the antigen excreted by schistosomes in the circulating blood is concentrated in the urine. It is a polysaccharide specific for the genus *Schistosoma*. The antigen is found at all stages of the life cycle and particularly in the egg where it is found in large amounts. Detection of the antigen can be effected by means of the passive haemagglutination inhibition test (IPHA), the reverse passive haemagglutination test (RPHA) or the sandwich-ELISA test[29]. In epidemiological surveys there is a fundamental advantage in detecting the metabolic antigen excreted by schistosomes instead of looking for circulating antibodies. The antigen is directly released by the parasite itself, in contrast to antibodies, which are produced by the host and in a manner that varies from one individual to the next. Collecting urine specimens is, for field workers, easier than obtaining blood from the inhabitants. The procedure for detection of the antigen in the urine is simplified by the fact that the antigen is concentrated by the kidney and in the urine is uncontaminated by antibodies, contrary to the case with the blood. Subjects voiding eggs in the stools or urine excreted the antigen in 60% of cases in Djohong, 72% of cases in Nalassi, 73% in Barombi and 75% in Edea. These percentages increased when overnight urine specimens were collected in the early morning. Early morning specimens are more concentrated and contain more antigen, as has been demonstrated in a study carried out in Djohong[10].

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Detection of the Circulating Anodic Antigen for Immunodiagnosis of *Schistosoma* Infections

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Summary

The diagnosis of schistosome infections is still mainly based on the detection of the parasite's eggs in the patients' faeces or urine. However, the strong fluctuation in the egg excretion necessitates repeated examinations, in order to get a reliable impression of the intensity of infection. In light infections the number of eggs excreted is often very low, so that even with repeated microscopical examination, the diagnosis cannot be confirmed.

Numerous studies have been undertaken to find alternative assays for the diagnosis of infections with *Schistosoma*. Many efforts have been given to the development of tests in which host (patient) antibodies directed against the parasite are detected. Although a number of such assays is being used widely in countries where schistosomiasis is not endemic (e.g. Europe, United States), these methods have not shown to be an acceptable alternative for the commonly applied parasitological methods in epidemiological studies of schistosomiasis and/or in schistosomiasis control and eradication programmes.

The detection of circulating antigens in principle offers a number of advantages over the detection of antibodies. First, antigenemia indicates an active infection. Second, a good correlation is generally found between antigen concentration in serum or urine, and the worm burden (the number of parasites as expressed by egg output). The detection of circulating antigens in serum and/or urine thus allows the follow-up of drug treatment.

The use of monoclonal antibodies has led to both increased sensitivity and specificity in comparison with the results obtained by using polyclonal antibodies. The lower detection limit of indirect haemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA), and time-resolved immunofluorometric assay (TR-IFMA) for determination of circulating anodic antigen (CAA) was 5, 0.2, and 0.02 ng AWA-TCA (trichloroacetic acid soluble fraction of adult worm antigen) per ml,

respectively. The specificity of the ELISA was 100%. The performance of the ELISA was compared with data from literature. The ELISA and TR-IFMA showed the highest detectability for the quantitation of CAA. The possible application of the ELISA for the quantitation of CAA in epidemiological studies or schistosomiasis control programmes is discussed.

Introduction

The development of an assay for the quantitative detection of schistosome circulating antigens is justified by the shortcomings of the techniques currently in use for the diagnosis of schistosomiasis.

Often used in epidemiological surveillance are direct microscopical examinations of faeces preparations or urine filtrates[1]. Longitudinal studies on egg output have shown long term stability in egg output[2-6], and the distribution of eggs in the faeces to be even[7,8]. However, there is a considerable day to day fluctuation in the egg excretion[9,10]. Moreover, many light infections are missed with the commonly applied stool examination techniques[11]. This may be explained by the fact that only a small proportion of the total daily egg production is found in a single stool or urine examination. It has been estimated that in infections with *Schistosoma mansoni* or *S. japonicum* a typical 50-100 mg stool sample contains only 0.3-1.0% of the daily egg excretion[12]. A 10 ml urine sample, collected during the time of peak egg excretion for *S. haematobium*, is considered to contain 2.5% of the total daily egg output[13].

Hatching assays, demonstrating the emergence of miracidia from stools or urine, are an alternative for the demonstration of schistosome infections, but these techniques can not be well standardized[14,15].

Examination of biopsy specimens or endoscopy may have their clinical value for the confirmation of active infections, but they can not be used as a routine screening method for large populations.

Many techniques have been developed for the quantitation of anti-schistosome antibodies, but antibody detection in general does not give sufficient information about intensity of infection, duration of infection, or the efficacy of drug treatment[16].

An accurate test for the quantitation of the worm burden by the detection of parasite antigens would allow the assessment of chemotherapeutic efficacy, the impact of environmental measures in *Schistosoma* transmission, or the efficacy of a (future) vaccine. The possibility of measuring antigens would also provide a tool in studies on the host-parasite relationship, for example studies on development of immunity in long-term infections, or on the induction of tolerance or sensitization by the passing of antigens from infected mothers to their newborn children.

Although the possible advantages of antigen detection are obvious, the number of studies undertaken to develope sensitive assays in schistosomiasis is limited. Moreover, a number of studies describing the detection of schistosome circulating antigens report the use of radiological techniques[17-19]. The health and environmental hazards associated with radioisotopes, together with their short half-life, and high costs of technique and equipment, make these techniques definitely inappropriate for use in large scale epidemiological studies or routine diagnosis, especially in developing countries.

We recently described sensitive techniques for the quantitative determination of the two major circulating antigens: circulating anodic antigen (CAA)[20,21] and circulating cathodic antigen (CCA)[22]. Here the applicability of techniques for the detection of CAA is evaluated.

Circulating anodic antigen (CAA)

CAA is also known as GASP (gut-associated schistosome proteoglycan). This antigen was described first by Berggren and Weller[23] and it has been further characterized by Gold *et al.*[24], Nash *et al.*[25,26] and Deelder *et al.*[27,28]. CAA is named after its electrophoretic mobility; it is a proteoglycan with a molecular mass of approximately 70 kDa[29] and a strong negative charge at neutral pH. It is heat stable and trichloroacetic acid (TCA) soluble. It consists mainly of N-acetylgalactosamine and D-glucuronic acid[26]. Double-diffusion and immunoelectrophoresis studies have clarified that CAA is genus-specific[25,30,31]. The antigen is released into the host's circulation in relatively large amounts. De Water *et al.*[32] have studied the ultrastructural localization of CAA in the digestive tract of *S. mansoni* using immunogold labelled monoclonal antibodies. In 3.5 week-old *S. mansoni* worms CAA was demonstrated in the Golgi apparatus, in cytoplasmic vesicles, and in the luminal surface coat of the gut epithelium. No antigen was found in the parasite's oesophagus. CAA-positive lysosome-like bodies were found caudally in the gut. CAA found in the lumen of the gut of adult worms was associated with host leukocytes and with a thick layer covering the gut epithelium. Deelder *et al.*[33] reported a steady state serum level of 500 ng CAA per worm in infected hamsters. Using the defined antigen substrate spheres (DASS) and IHA techniques, free CAA in the serum of infected hamsters could be shown in detectable quantities from 28 days post infection. The biological function of the antigen is unknown, but it has been suggested that it protects the gut against its own proteolytic secretion or host antibodies. CAA provokes a strong immune response in infection[34], and it is thought to be involved in the genesis of glomerulopathy in schistosomiasis[35], although the antigen is not very immunogenic. CAA has been reported in kidney eluates of infected hamsters[28], and in the glomeruli of *S. mansoni*-infected mice and hamsters[28,36]. The antigen has been found in glomerular deposits with immunoglobulins and complement[37], indicating the involvement of the antigen in immune-complex formation. Immune complexes containing CAA were shown to be present in kidney deposits and in Kupffer cells in the liver of infected mice[33,39]. Sobh *et al.*[40] showed the presence of granular deposits of CAA in kidney biopsies of schistosomiasis patients with renal failure.

Nash[41,42] studied the rate of removal of CAA in mice. The antigen appeared to be taken up by the liver, released, and eventually excreted in the urine. The rate of removal from the circulation appeared to be dependent on the size of the immune complexes formed. Large CAA-containing immune complexes were removed more quickly than small immune complexes, the latter being formed in high antigen access[29]. At that time, using an inhibition assay, CAA could not be detected in concentrated urine from heavily infected mice or men. Reports on the relationship between worm burden and CAA concentration in the serum have been conflicting. A close correlation has been reported in *S. japonicum*-infected experimental animals[31,43], but not in hamsters heavily infected with *S. mansoni*[28,33], except in early infections[44].

Assays for detection of CAA

Techniques developed for the quantitative detection of CAA are: an indirect haemagglutination assay (IHA)[20], an enzyme-linked immunosorbent assay (ELISA)[21] and a time-resolved immunofluorometric assay (TR-IFMA)[45]. These three techniques are all non-radioisotopic. The IHA, although simple and attractive from the point of view of possible field applicability, lacks sufficient sensitivity. The ELISA and the TR-IFMA are the most sensitive assays described for the quantitative

determination of schistosome circulating antigens. The TR-IFMA offers a high sensitivity and broad measuring range, but the assay requires an expensive measuring apparatus. Therefore, mainly the ELISA was used to evaluate the determination of circulating anodic antigen as a diagnostic marker and to assess chemotherapeutic efficacy[46-51].

Discussion

Sensitivity of assays

The use of monoclonal antibodies not only provides an enhanced specificity when compared to the use of polyvalent antisera, but also gives a significant increase in the sensitivity of assays for the demonstration of schistosome circulating antigens. Deelder and Eveleigh[52] described an IHA for the detection of CAA, using polyclonal antibodies, with a lower detection limit of 50 ng AWA-TCA per ml. The IHA described described by Deelder *et al.*[20] showed a lower detection limit of 5 ng AWA-TCA per ml.. A similar improvement in assay performance was observed in the detection of circulating schistosome antigen (CSA) by radio-immunoassay where Feldmeier *et al.*[19], using a monoclonal antibody, reported an increased assay sensitivity compared to assays in which polyclonal antisera were used[18,53]. The ELISA described by Deelder *et al.*[21] is of high sensitivity, only surpassed by a fluorescence-based assay system: the time-resolved immunofluorometric assay (TR-IFMA)[45].

A number of assays for the quantitative detection of circulating schistosome antigens has been developed in the last decades[17,23,24,26,28,31,33,44,52,54,65]. Table 1 lists the lower detection limit of a number of assays detecting schistosome circulating antigens. Different unities for the measurement of the lower detection limit are sometimes used in the various studies. The detection limit for the assays described by our group is given in concentration AWA-TCA per ml. Based on immunoadsorption studies (data not shown), this antigen preparation is considered to contain approximately 10% CAA. The detection limit of the ELISA described by Deelder *et al.*[21] was 0.8 ng AWA-TCA per ml. However, due to further optimization of the conjugation procedure and a combined incubation of samples and conjugate in the assay, the lower detection limit of the CAA ELISA is now 0.2 ng AWA-TCA per ml.

Thus far, our research has mainly been focussed on detection of circulating antigen in *S. mansoni* infections[20,21,45-47]. Although CAA could be detected successfully in infections with other schistosome species e.g. *S. haematobium*[48,51], *S. intercalatum*[49] and *S. japonicum* (unpublished data), the antigen concentrations appeared to be of different levels in the various schistosome species. Studies on the antigen production by distinct schistosome species in experimental animal models would allow a proper interpretation of the values found.

Specificity of assays

A good diagnostic test not only requires a high sensitivity (i.e. a low lower detection limit), but also high specificity (i.e. absence or low frequency of cross-reactions).

To study the specificity of the ELISA (and the TR-IFMA, for which the same monoclonal antibody was applied in a similar sandwich assay) for the quantitative determination of CAA, several hundreds of negative control sera have been tested. These samples included control individuals (with repeated negative stool examinations) from areas where schistosomiasis is endemic and from areas where the disease is absent[20,21,45,46]. High prevalences have been reported in the negative control group from Zaire for *Ascaris lumbricoides*, hookworm, *Strongyloides stercoralis*, *Onchocerca volvulus*, and *Dipetalonema perstans*, while infections with

Table 1 Lower detection limit of assays detecting schistosome circulating antigens

Reference	Assay*	Antigen†	Lower detection limit (ng per ml)
Berggren and Weller, 1967[23]	IEP	CAA	?
Gold *et al.*, 1969[24]	ID	?	?
Bawden and Weller, 1974[44]	CF	CSA	?
Houba *et al.*, 1976[54]	CCIEP	CSA	?
Nash *et al.*, 1977[26]	IEP	CAA	?
Madwar and Voller, 1977[55]	ELISA	AWA	?
Deelder and Eveleigh, 1978[52]	IHA	CAA	20
Deelder *et al.*, 1978[33]	DASS	CAA	50
Santoro *et al.*, 1978[58]	RIPEGA	CSA	?
	RIPEGA	Ag '4'	?
Ferreira *et al.*, 1979[56]	ELISA	polysacch.	3000
Carlier *et al.*, 1980[17]	SPRIA	CSA	50
Deelder and Dozy, 1980[57]	SPIA	CAA	1-10
Qian and Deelder, 1983[31]	ELISA	CAA	100
Weltman, 1982[59]	ELISA	CSA	?
Abdel-Hafez *et al.*, 1983[60]	IELISA	CSA	10
Abdel-Hafez *et al.*, 1984[61]	IELISA	CSA	10
Nogueira-Queiroz *et al.*, 1986[64]	IRMA	CCA	5-10
Feldmeier *et al.*, 1986[19]	IRMA	CCA	150
	ELISA	CAA	125
Hayunga *et al.*, 1986[63]	IELISA	Cerc.Ag.	100
Ripert *et al.*, 1988[65]	IPHA	?	20
Deelder *et al.*, 1989[20]	IHA	CAA	5
	IHA	CCA	64
Deelder *et al.*, 1989[21]	ELISA	CAA	0.8
De Jonge *et al.*, 1989[45]	TR-IFMA	CAA	0.02

* CCIEP = counter current immunoelectrophoresis; CF = complement fixation; DASS = defined antigen substrate spheres; ELISA = enzyme-linked immunosorbent assay; ID = immunodiffusion; IELISA = inhibition ELISA; IEP = immunoelectrophoresis; IHA = indirect haemagglutination assay; IPHA = indirect passive haemagglutination; IRMA; immunoradiometric assay; RIPEGA = radioimmunoprecipitation (polyethylene glycol) assay; SPIA = sol particle immunoassay; SPRIA = solid phase radioimmunoassay; TR-IFMA = time-resolved immunofluorometric assay

† Ag'4' = Antigen 4; AWA = adult worm antigen; CAA = circulating anodic antigen; CCA = circulating cathodic antigen; Cerc.Ag = cercarial antigen; CSA = circulating schistosome antigens; polysacch = polysaccharide; ? = not reported

Trichuris, and *Loa loa* were also common[66]. None of the many negative control sera tested were positive for CAA. We further tested 260 serum samples from the WHO filariasis serum bank including patients with *Wuchereria bancrofti*, *Brugia malayi*, *Onchocerca volvulus* and *Loa loa*, as well as 10 controls from Flores (Indonesia), where schistosomiasis is absent. All but five out of these 260 cases were negative for CAA, but the positive cases were likely to be schistosome infected, as could be

concluded from their positivity in the immunofluorescence assay (IFA) for the detection of specific anti-schistosome IgM and from their origin (Côte d'Ivoire and The Philippines). Because well documented serum samples from humans infected with other trematodes (e.g. *Fasciola, Clonorchis, Paragonimus*) were not available to us, we have not yet studied possible cross reactions, caused by epitopes of these related organisms which are shared by schistosomes. However, the fact that CAA could not be detected in serum of experimental animals heavily infected with *Fasciola hepatica* (unpublished data) indicates that a possible cross-reactivity will not be a great problem in diagnosis.

Diagnostic performance

There are different ways to compare the performance of diagnostic tests with known sensitivity and specificity. Diagnostic tests can be characterized by Youden's J-index[67]; an alternative approach is to calculate the predictive values of positive and negative test results and the efficiency of a test[68]. Unfortunately, few studies give enough data for a proper analysis of predictive values of test results and efficiency or J-index.

Four studies on predictability of assays (detecting anti-schistosome antibodies) are reported in the literature[69]. Table 2 shows a modification of the table given by Maddison[69].

Superior performance of serological assays in comparison with parasitological examinations have only been reported by a number of investigators. Ruiz-Tiben *et al.*[70] reported the application of a serological assay (Circumoval Precipitin Test (COPT)[71]), which showed a greater sensitivity for the detection of *S. mansoni* infection than a single stool examination by the modified Ritchie formol-ether concentration technique (MRCT)[72]. Yogore *et al.*[73] and Lewert *et al.*[74], using an ELISA with crude *S. japonicum* soluble egg antigen (SEA), also reported better results for serology in comparison to routine stool examinations, for determining the prevalence and incidence of infection with *S. japonicum*. Mott[75] reported high sensitivity and specificity of immunodiagnostic assays using crude *S. japonicum* antigens. Less satisfactory results were found by Polderman and Deelder[76], who compared stool examinations and serological data obtained with ELISA, DASS, IHA and IFA for a *S. mansoni* population in Ethiopia. It was concluded that these serological tests were lacking in specificity and sensitivity. Long *et al.*[77] made a comparison of the sensitivity and specificity of four diagnostic tests for *S. mansoni* infection. In their study an individual was regarded as infected if positive in one or more of both a parasitological and immunological test, establishing a diagnostic reference based on a combination of parasitological and serological results. Tests compared were the Bell filtration technique[78,79], the Kato thick smear[80], radioimmunoassay (RIA) for the detection of major surface antigen (MSA_1)[81] and an ELISA using SEA[82]. The Kato direct smear test applied in their study showed a sensitivity of 93% and a specificity of 95%. Overall reliability (measured by Armitage's 'J' index, which is identical to Youden's J) was 85%. The reliability of the Bell filtration technique was 64%, of the ELISA 68%, and of the RIA 78%[77]. We previously concluded that the CAA-ELISA was at least as sensitive as the Kato-Katz thick smear[46,83].

Care should be taken with the interpretation of these data. There is no generally accepted 'golden standard' for the correct identification of infected people. Stool or urine examinations are commonly used as reference test, but various parasitological methods may differ in sensitivity. Sensitivity of these parasitological examinations can be increased by the processing of larger volumes of faeces or urine or by multiple testing. However, even with infinitely repeated parasitological examinations, unproductive or unisexual worm loads will be missed.

Table 2 Studies in which predictability, sensitivity, and specificity of immunodiagnostic assays for schistosomiasis were determined (after Maddison[69])

Reference	Assay (antigen)	Se (%)	Sp (%)	J (%)	Eff (%)
Assays detecting antibodies					
Ruiz-Tiben *et al.* (1979)[70]	COPT	95	96	91	97
	IFA	73	86	59	78
Hillyer *et al.* (1979)[95]	COPT	95	96	91	95
	RIA (MSA_1)	95	79	74	87
Ishii and Owhashi (1982)[86]	ELISA (JEA)	97	91	97	?
	ELISA (JEA-1)	100	100	91	?
Maddison *et al.* (1985)[87]	ELISA (MAMA)	94	99	94	?
Assays detecting circulating antigen					
De Jonge *et al.* (1988)[46]	ELISA (CAA)*	75	100	75	81
		93	100	93	93

* J-index, sensitivity, and specificity determined in two *S. mansoni* study groups where prevalence was 35% and 95%, respectively (based on three times repeated duplicate Kato-Katz stool examinations).

Abbreviations: Eff = efficiency; J = J-index; JEA = *S. japonicum* egg extract; JEA-1 = partially purified fraction of JEA; MAMA = microsomal fraction of adult *S. mansoni*; Se = sensitivity; Sp = specificity.

In addition, Youden's J-index completely ignores the effect of prevalence on the testing situation. The average number of schistosomes harboured by infected residents, however, appears to be related to the overall prevalence of the infection[46,84,85]. The positivity rate which is found for a test is dependent on the study population chosen. The J-index calculated will hence be prevalence-dependent. In comparative studies, therefore, a clear definition has to be given of the study groups tested: what is the reference test to classify individuals as infected or non-infected? What is the prevalence, and what is the intensity of infection? A high J-index, for example, is reported in the study of Ishii and Owhashi[86], but these authors fail to give an indication of the intensity of infection of the *S. japonicum* infected individuals used in their study. Prevalence data, which would allow the calculation of the test efficiency were not given by this group either, nor by Maddison *et al.*[87].

For the reasons given above, the test efficiency (the prevalence dependent proportion correctly classified individuals as infected or non-infected) is also given in Table 2 and was used by De Jonge *et al.*[46].

Non-invasive immunodiagnosis

The high sensitivity of the ELISA described in this study and the finding (due to this lower detection limit) that CAA is also present in the urine of schistosomiasis patients[48] enhances the possibilities of the assay in routine diagnosis of

schistosomiasis. It is not yet clear whether the antigenic material which is detected in the urine consists of intact CAA-molecules or whether antigen fragments are excreted with the urine as is the case in lymphatic filariasis[88].

The use of urine samples for the diagnosis of schistosomiasis offers a number of advantages over the use of serum samples: the diagnosis is non-intrusive, cultural traditions sometimes pose a barrier to obtaining blood and the risk of contamination or infection with pathogens (e.g. Hepatitis B, Human Immunodeficiency Virus) is smaller when urine samples are used. Our concentration procedure as used for concentration of urine (TCA-pretreatment, dialysis, and freeze-drying), however, is not appropriate for use on a large scale. The procedure is laborious and time-consuming, and needs relatively expensive equipment. Alternative techniques (e.g. filtration-techniques) are currently under investigation.

Correlation CAA - parasite burden

The correlation between worm burden and levels of circulating antigen has been studied by a number of authors. Using polyvalent antisera, a significant correlation could be found between antigenemia and parasite burden, as expressed by egg output[24,31,33,53,89]. These findings have later been confirmed by Feldmeier *et al.*[19] and the studies from our group. A strong correlation between CAA concentration and egg output was found for *S. mansoni*[20,21,46] and *S. intercalatum* infections[49], but not for *S. haematobium*[48,51].

The amount of antigen which is found in the host's circulation is not only a function of the number of worms present, but also of the antibody response of the host[41]. The formation of immune complexes may be expected to interfere with the correlation of antigen concentration and worm burden. This is presently under study in a series of worm transfer studies, where adult *S. mansoni* schistosomes are surgically transferred to naive experimental animals. In this way we will be able to study the rate of specific antibody formation, formation of immune complexes, and the relationship between free antigen and antigen trapped in such immune complexes. An optimal diagnostic assay would not only give an estimate of the intensity of infection, but would also allow an estimation of the duration of the infection. However, no such relationship between CAA concentration and duration of infection seems to exist.

The lack of availability of well documented human serum and or urine samples thus far has hampered the study of antigenemia in recent infections. Currently, urine and serum samples of recently infected *S. haematobium* and *S. mansoni* patients from Egypt and Brazil, are being collected in collaboration with researchers from those countries. Qian and Deelder[90] were able to demonstrate CAA in serum of rabbits with a light *S. japonicum* infection from six weeks post infection onwards. In our studies, CAA levels were determined mainly in chronically infected patients.

Application of ELISA for CAA

Possible fields of application for an immunodiagnostic test in schistosomiasis are *i*) epidemiological studies, *ii*) schistosomiasis control programmes and *iii*) individual diagnosis of clinical cases, be it under field conditions in areas where the disease is endemic, or in countries where the infection is seen as an imported disease (e.g. Europe, United States).

Important research topics in the field of epidemiology include the impact of schistosomiasis on public health, how to measure this impact and how this impact is influenced by chemotherapy. The role of circulating antigen measurements as an indicator of morbidity has still to be studied extensively. Future studies will include

an epidemiological analysis of antigen determination in comparison with classical parameters for morbidity.

A proper estimation of the worm load may be also very important for use in schistosomiasis control programmes. Although a nationwide eradication of schistosomiasis does not seem to be a reality, local successes may be made[75]. The ability to find and monitor active infections is of vital importance in end-phase eradication programmes.

In such cases, the identification of the relatively few and probably often subclinical cases which occur in sparsely endemic areas, requires a sensitive and specific diagnosis, in which the determination of schistosome antigens may play an important role. The same holds true for vaccine studies. Although a vaccine for schistosomiasis is not yet even within sight[91], there is hope that in the future such a vaccine will materialize. For vaccine trials, an accurate measurement of active infection will be very important.

A major advantage of the detection of schistosome antigens is the possibility to assess the effect of chemotherapy. The effect of praziquantel on antigenemia in murine schistosomiasis was first described by Weltman[59] and later by Abdel-Hafez *et al.*[60]. In both studies an antigen half life of approximately 72 hours was found. A rapid drop in circulating schistosome antigen was also reported in *S. japonicum* infected rabbits[89] and *S. haematobium*-infected baboons[92]. Studies on antigenemia following chemotherapy in human schistosomiasis, however, have been limited. Feldmeier *et al.*[19] examined paired serum samples of patients with intestinal and urinary schistosomiasis before treatment and four months after treatment. Antigen concentration (CAA and/or CCA) decreased significantly four months after chemotherapeutic intervention. The same conclusions were drawn by De Jonge *et al.*[47,51], where follow-up of chemotherapy was done at six weeks, one month and also in a period of days after treatment. This allowed a much more detailed analysis of the antigenemia following drug treatment.

The search for a diagnostic test which allows the assessment of chemotherapy in schistosomiasis control programmes has been given high priority[93]. Important in this context are the operational aspects of a diagnostic test: its costs, reliability, ease of use, and performance under field conditions. Recently, the CAA-ELISA was tested under field conditions in Kisangani, Zaire (data not shown). It could be concluded that the ELISA for the detection of CAA, performs well under 'field conditions' (high relative humidity, high temperature, tap water, transportation). Although the test might not replace the Kato smear for routine diagnosis, its application in the field as a seroepidemiological tool (e.g. in schistosomiasis control programmes or vaccine trials) raises no problem from a technical point of view.

The possible application of the CAA-ELISA in the immunodiagnosis of schistosome infections in The Netherlands was studied by De Jonge *et al.*[50]. Whether the low positivity rate (approximately 20%), which was found for CAA, was entirely due to the generally low level of infection of the patients studied is not yet clear. Health status, immunogenetic factors or tolerance may play a role in the clearance of CAA containing immune complexes.

Quite unexpectedly, a possible application of immunodiagnosis of schistosomiasis was found in palaeoseroepidemiology. CAA (or at least its specific epitopes) appeared to remain intact for several thousand years in dessicated Egyptian mummy tissue, and (low) levels of CAA could be measured in even non-visceral tissue of Egyptian mummies, while no antigen could be found in similar preserved material from mummies from a non-endemic area in Chile, which could therefore be considered as negative controls. Questions of archaeological importance might be addressed by application of a sensitive technique for the quantitation of CAA[94].

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The Use of Recombinant DNA Methods to Produce Schistosome Antigens in order to Assess their Immunodiagnostic Potential: *Schistosoma mansoni* Tropomyosin an Example

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Summary

With recent advances in recombinant DNA methods, it is now possible to identify schistosome genes that encode antigens of interest, identify the gene product, produce large quantities of the antigen in bacteria, purify the antigen and evaluate its diagnostic potential. Once an antigen has been demonstrated to have diagnostic potential, the epitopes important for diagnosis can be identified and synthesized and eventually used to facilitate diagnosis.

Tropomyosin from adult schistosome parasites is a strong immunogen that is recognized by sera from chronically infected humans and from animals immunized with irradiated cercariae. A cDNA clone from a *Schistosoma mansoni* adult worm cDNA library was identified by hybrid selection of the mRNA which was *in vitro* translated and immunoprecipitated with specific antisera. A λgt11 expression clone which contained an insert close to the full length mRNA was subsequently isolated from a *S. mansoni* cercarial library. The complete sequence of the mRNA was determined by sequencing the insert of this clone as well as primer extension of total RNA. The only open reading frame coding for 284 amino acids in the 1316 nucleotide sequence showed a 44-55% homology with the amino acid sequence of 18 different tropomyosins from various species. Northern and Western analysis showed greater amounts of tropomyosin were detected in extracts from adult worms as compared to extracts from cercariae and egg stages. Immunocytochemical analysis showed that tropomyosin is associated with the tegument of adult worms.

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S.japonicum, parasitic infections other than schistosomiasis, or uninfected reacted with the recombinant tropomyosin. The species specificity of *S. mansoni* tropomyosin suggests that further study of its potential as an immunodiagnostic reagent is warranted.

Introduction

Schistosomiasis, a chronic debilitating disease currently afflicts 200 to 300 million people throughout the world, causing an estimated 800,000 deaths each year (for review)[1]. The primary causative agents are *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. The parasites have multi-stage life cycles. Worms from different life cycle stages differ not only in morphology but also in their antigenicity. These differences probably reflect adaptations to their respective environments. Interest has focussed on antigens of the schistosomula stage as it is a target of immune elimination[2]. However, the schistosomula shares a number of epitopes with other schistosome stages[2,3]. This is not surprising as the concept of concomitant immunity[4], demands that epitopes must be shared between egg laying adult worms and schistosomula. We, among others, have reported on the polypeptide patterns of schistosomula and adult worms of *S. mansoni* [5]. Over 60% of the schistosomula polypeptides were found to be present in adult worms as revealed by silver stained non equilibrium two-dimensional gel electrophoresis (NEPHGE). This included a strong immunogen of about 40 kDa (pI 4.85). This strong immunogen was recognized by antibodies in serum from chronically infected schistosomiasis mansoni patients as well as antibodies in serum from animals immunized with irradiated cercariae. Therefore, it appeared that this 40 kDa antigen which induced a strong antibody response may be useful in diagnosis. Initially, we produced polyclonal antibodies directed against the 40 kDa antigen[5]. This was done by excising silver stained polypeptides from NEPHGE gels and using them to immunize mice and rabbits. The polyclonal antibodies showed specificity as determined by enzyme-linked immunosorbant assay (ELISA), Western blot, and immunoprecipitation of specific in vitro translation products[6]. To identify the cDNA clone encoding the 40 kDa antigen, a pBR322 cDNA library from *S. mansoni* adult worm mRNA was first screened by cDNA probes transcribed from size-fractionated mRNA. The clones obtained in this manner were further analysed by hybrid selection analysis. One cDNA clone designated clone 1, hybrid selected a mRNA, which translated into a 40 kDa antigen that was immunoprecipitated by human chronic infection serum as well as specific antibody[6] pBR322 clone 1, which contained an insert of approximately 200 bp, was labelled and used as a probe to screen a λgt11 cDNA library made from *S. mansoni* cercarial mRNA. Forty independent positive clones were isolated from 120,000 plaques. Nine clones were analysed. Three of the nine cDNA clones were expression clones based on reactivity with rabbit serum made against the 40 kDa antigen. One of these clones, clone 1-22, which contained an insert of 1.2 kb, expressed a polypeptide of approximately 40 kDa, the same size as the native molecule. It turns

out that this was not a ß-galactosidase fusion protein. Translation was initiated from an ATG start codon in the insert. The complete sequence of the mRNA which contained 1,316 nucleotides encoding a hydrophilic polypeptide of 284 amino acids was determined. The deduced polypeptide sequence showed homology to the amino acid sequence of tropomyosin[7], one of the major regulatory molecules in contractile systems[8,9]. Schistosome tropomyosin was also similar to other tropomyosins by several criteria such as computer predicted secondary structure, the typical size; amino acid sequence, lack of proline and tryptophan, and a heptapeptide periodicity, typical for alpha-helical coiled-coil structures. However, no immunological cross-reactivity was detected by Western analysis between chicken gizzard tropomyosin and schistosome tropomyosin despite significant sequence homology between the two molecules. In order to determine the distribution and abundance of tropomyosin within the various parasite stages, we performed Northern and Western blot analysis. Schistosome tropomyosin was found to be developmentally regulated with the mRNA and the protein being more abundant in the adult worm stage compared to the cercariae and egg stage. As tropomyosin was most abundant in the adult stage, we employed specific antibody to immunocytochemically localize the 40 kDa antigen. Tropomyosin was found to be associated with the tegument. In particular it localized within cells associated with the tubercles and the area underlying the tegument. As the adult schistosome membrane is thought to turn over every 3-6 h, this may be the source of antigen for the host immune system. In addition tropomyosin release may result from parasite attrition and continuous tegumental damage and repair.

Thus all our data supported the idea that tropomyosin, a molecule that elicited a strong antibody response, was readily available to the host during infection. To further study the value of this molecule for immunodiagnosis, recombinant DNA methods were used for large scale expression and purification of *S. mansoni* tropomyosin. The purified tropomyosin was then used in ELISA and Western blot assays employing various individual normal and infection sera to demonstrate that *S.mansoni* tropomyosin shows species specificity and thus warrants further study as an immunodiagnostic reagent.

Materials and Methods

Sera

Rabbit and mouse sera against a group of *S. mansoni* proteins including tropomyosin were generated as previously described[6]. Individual sera from patients with chronic schistosome infections were from Egypt (*S. mansoni* and *S. haematobium*) and the Philippines (*S. japonicum*). Sera from individual patients with unrelated parasitic infections, such as *Giardia lamblia*, *Fasciola hepatica*, *Trichinella spiralis*, *Echinococcus granulosis*, *Wuchereria bancrofti* and *Ascaris lumbricoides* were from Egypt and India. Normal human sera were from laboratory personnel. Some of the sera were gifts from Dr. Gene I. Higashi, University of Michigan; Dr. Victor Tsang, Center for Disease Control; Dr. Karim Kamel, NAMRU-3; and Dr. John Cross, Uniformed Services.

Cloning and expression of *S. mansoni* tropomyosin cDNA

pSMTM, a cDNA clone for *S. mansoni* tropomyosin in the plasmid vector pGEM3Zf(+) has been previously described[6]. This clone was partially digested with EcoR1. The 1.2 kb insert was purified from an agarose gel and was ligated into EcoRI digested pGEX-3X vector[10]. The recombinant plasmid was transformed into *E. coli* JM83 cells. Colonies containing the recombinant plasmids of interest were screened

for by a cracking gel assay[11] Clones of interest were inoculated into LB-ampicillin medium and induced with IPTG for protein expression according to a published protocol[10]

To clone the *S. mansoni* tropomyosin cDNA into the pOTSNCO vector[12], DNA from the pGEX-3X expression clone was digested with the restriction enzymes *Sma* I and *Fsp* I. The 2.15 kb fragment containing the insert was purified from an agarose gel and used as an insert for recloning. The plasmid vector pOTSNCO was digested with *Nco* I. The cohesive ends were converted to blunt ends by T4 polymerase repair[11] The linearized vector was dephosphorylated with calf intestine alkaline phosphatase[11] The blunt-ended insert and vector were ligated at 15 °C overnight. For the preparation of plasmid DNA, the recombinant plasmid was transformed into *E. coli* N99Cl+. DNA was analysed by restriction digestion with *Nco* I or *Pvu* II and agarose gel electrophoresis to determine the size and the orientation of the insert.

A recombinant plasmid with an insert in the correct orientation was retransformed into *E. coli* AR120. The transformants were then grown in LB-ampicillin medium at 37 °C. When the OD_{650} reached 0.4, nalidixic acid (60 µg ml^{-1}) was added and the culture was continued for another five hours. To harvest cells, the culture was centrifuged at 5,000 rev./min for five minutes at room temperature. The pellet was resuspended in 1/10 -1/20 volumes of phosphate-buffered saline (PBS) with 1 mM phenylmethylsulfonylfluoride, frozen in liquid nitrogen, thawed at room temperature and sonicated for one minute with a Bronson sonicator. The crude cell lysate was stored at -20 °C before analysis by SDS-PAGE and Western blot.

Purification of the recombinant protein

Proteins in a crude cell lysate were separated by SDS-PAGE. After electrophoresis at a constant 60 volts overnight, strips were cut from both right and left sides of the gel. The gel was wrapped with plastic wrap and stored at 4 °C while the strips were stained with Coomassie Brilliant Blue R250 (0.2% in destaining solution containing 10% acetic acid and 40% methanol). After destaining, the strips were aligned to the unstained gel, and the desired protein band was cut from the gel. To elute the protein, the polyacrylamide gel fragment was cut into small pieces and homogenized in about 15 ml PBS. The homogenized gel suspension was stirred at 4 °C for at least four hours before centrifugation (8,000 rev./min for 10 minutes at 4 °C). The supernatant was concentrated by a Minicon-B15 concentrator (Amicon Division, W.R. Grace & Co., Danvers, MA).

For large scale protein purification, a cell lysate was prepared from a 300 ml culture. The lysate prepared as above was centrifuged at 10,000 rev./min for 15 minutes at room temperature. Ammonium sulphate was slowly added to the supernatant while it was stirred at room temperature. After the desired salt concentrations (20%, 24%, 27% and 36%) were reached, the sample was spun down. Each time, the precipitates were dissolved in 2-5 ml PBS (pH 7.4). The fractions were dialyzed extensively against PBS and analysed by SDS-PAGE and Western blot.

The ammonium sulphate fraction which contained the desired protein was further fractionated by f.p.l.c. (Gradient Programmer GP-250 Plus and Fraction Collector FRAC-100, Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) using a salt gradient (30 to 60% NaCl in 20 mM Tris, pH 8.0) on a DEAE ion exchange column. Forty fractions (1 ml each) were collected and analysed by SDS-PAGE and Western blot. Fractions enriched for the protein were pooled and reapplied to the f.p.l.c.-DEAE column until the protein preparation eluted as a single sharp peak.

Protein concentrations in various preparations were determined by the Bio-Rad protein assay or bicinchoninic acid protein assay following the manufacturer's manual (Pierce Chemical Company, Rockford, IL).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis

Proteins separated by SDS-PAGE were transferred onto nitrocellulose paper (0.45 or 0.20 μm pore size) in a Bio-Rad protein transfer unit as described by Towbin *et al.*[13] Human or animal sera (as described above), diluted 1:500 in most cases were used as primary antibodies. Biotinylated secondary antibodies and Vectastain ABC-AP Kit (AK-5000) were both applied according to the product sheet (Vector Laboratories, Burlingame, CA).

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Becton Dickinson, NJ) were coated with antigens 0.5 μg/well. The protocol of Hillyer and Gomez de Rios[14] was followed. Alkaline phosphatase-conjugated or peroxidase conjugated secondary antibodies appropriately diluted were used in conjunction with specific substrate to detect reactivity. Antibody binding was measured by reading OD_{405}/OD_{540} with a Bio-Rad microtiter plate spectrophotometer for the colour reaction.

Results

Expression of *S. mansoni* tropomyosin in pOTSNCO system

S. mansoni tropomyosin cDNA has been previously expressed in kgt11[6] However, the expression efficiency was not sufficient for large scale production and purification of the recombinant protein. In order to obtain more efficient protein expression, the insert of clone pSMTM was first recloned into pGEX-3X vector[10] A fusion protein of about 66 kDa was expressed and recognized by mouse antiserum on Western blot. Since *S. mansoni* tropomyosin was fused to the carboxy terminus of *S. japonicum* glutathione S-transferase (Sj26), and Sj26 may have cross-reactivity with the sera from *S. mansoni* patients, we were concerned about the possible contamination of Sj26 in *S. mansoni* tropomyosin purified from cleaved fusion protein. The insert from pGEX-3X was therefore recloned into pOTSNCO vector at the unique *Nco* I site[12] Colonies from the cloning were analysed by restriction digestion with *Nco* I or *Pvu* II. Clones that had an insert of the expected size and the correct orientation were chosen for induction. While temperature induction was not satisfactory, chemical induction with nalidixic acid in *E. coli* AR120 transformants gave good results. A 40 kDa band started to appear on SDS-PAGE in samples taken from the expression clone three hours after induction. This 40 kDa band was recognized by specific rabbit antiserum on Western blot. The supernatant and pellet were separated by centrifuging the freeze-thaw lysate that contained the recombinant protein. The 40 kDa band, as analysed on SDS-PAGE, was primarily present in the supernatant and not in the pellet, indicating a high solubility of *S. mansoni* tropomyosin produced from this clone. This was in agreement with a computer generated hydropathic plot showing that the molecule is very hydrophilic.

Purification of the recombinant *S. mansoni* tropomyosin

The recombinant protein was first purified by separating cell lysates by preparative gel electrophoresis, removing the 40 kDa band and eluting the protein from the acrylamide gel fragment. The recombinant protein obtained in this manner was of the expected size and was recognized by specific antiserum.

To purify the recombinant protein on a large scale, cell lysates were fractionated by ammonium sulphate precipitation. Fractions from different salt concentrations were analysed by SDS-PAGE and Western blot. A 40 kDa band was enriched in the fraction precipitated with 20% ammonium sulphate. This fraction contained approximately 1.0% of the total cell pellet weight. The 40 kDa polypeptide was recognized by specific rabbit antiserum.

The 20% ammonium sulphate fraction enriched for schistosome tropomyosin was further purified on a DEAE ion exchange column by f.p.l.c.. The fractions that contained the recombinant protein were pooled together and reapplied onto the column. After a second round of f.p.l.c. fractionation, the recombinant schistosome tropomyosin eluted as a single major peak at a salt concentration of about 40%. The yield of *S. mansoni* tropomyosin from f.p.l.c. was estimated to be 21-22% of the 20% ammonium sulphate fraction.

The purified recombinant *S. mansoni* tropomyosin was used to immunize mice. The resulting antisera reacted specifically with the *S. mansoni* tropomyosin on Western blot as well as in ELISA assays. The sera also reacted with a soluble adult worm antigen preparation (SWAP).

S. mansoni tropomyosin as an immunodiagnostic reagent

The purified recombinant *S. mansoni* tropomyosin was tested as a diagnostic reagent by Western analysis using sera from individual patients with chronic schistosomiasis (*S. mansoni*, *S. japonicum* and *S. haematobium*) as well as sera from individuals with parasitic infections other than schistosomiasis. Rabbit antiserum against tropomyosin and normal human serum were used as controls. Immobilized *S.mansoni* tropomyosin was only recognized by rabbit antiserum and sera from *S.mansoni* infected patients. The purified *S. mansoni* tropomyosin was further tested in ELISA assays with similar results. There is a significant difference of ELISA readings when individual sera from *S. mansoni* patients were compared to sera from patients with *S. japonicum* or *S. haematobium* infections, sera from normal controls and sera from individuals with parasitic infections other than *Schistosoma*. In the latter case, *Fasciola hepatica* and *Trichinella spiralis* are both known to have shared antigens with *S. mansoni* [15 17] Seven of the 50 sera samples from non-*S. mansoni* infected individuals gave borderline readings. By Western blot analysis these seven sera samples showed no specific reactivity against the purified recombinant *S.mansoni* tropomyosin.

Discussion

Schistosomiasis is still increasing in incidence today. The conventional diagnosis of this disease by stool examination is time consuming and lacks sensitivity.

The circumoval precipitin test[18] is still considered to be the standard immunological test although multiple testing is sometimes required. Since the 1950's, various antigen preparations from different schistosome life cycle stages have been tested by immunofluorescence, radioimmunoassay, ELISA or immunoblotting assays to detect specific antibodies present in patient sera(reviewed)[19,20] Among these, microsomal antigens from *S. mansoni* (MAMA) or *S. japonicum* (JAMA) adult worms developed by Tsang *et al.*[21,22] have proven to be specific (although not absolutely species specific), sensitive, stable, and cost effective (see this volume)[20]

Another line of work has been focussed on detecting circulating antigens (see this volume).

In recent years, with the help of the modern techniques such as recombinant DNA and computer analysis, study has also been focussed on the identification and characterization of defined antigens which have potential significance in immunodiagnosis or vaccine development. Among those schistosome antigens that have been cloned, polypeptides of 31 and 32 kDa from *S. mansoni* adult worm digestive tract identified as *S. mansoni* proteinases, cathepsin B and haemoglobinase have been found to be valuable for immunodiagnosis (see this volume). Another well defined antigen is the 70 kDa schistosomal homologue of the major eukaryotic heat-shock protein (hsp70), which also appeared to be useful in immunodiagnosis[23-25]

We demonstrate that the recombinant *S. mansoni* tropomyosin shows species specificity in the limited number of samples tested in both ELISA and Western analysis. It does not cross-react with sera from patients with *S. haematobium* and *S.japonicum* nor other parasitic diseases. This finding suggests that the *S. mansoni* tropomyosin warrants further study into its potential as an immunodiagnostic reagent.

Like heat shock protein, tropomyosin in general is ranked as a highly conserved protein. The *S. mansoni* tropomyosin has significant homology (around 50%) to host molecules[6] Yet, it induces strong immune responses in humans as well as in mice.

Large scale production of the purified recombinant protein described in this paper make it possible to study the role *S. mansoni* tropomyosin plays in the immunology of schistosomiasis mansoni.

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Conclusions and General Recommendations

Immunodiagnostic tests should be developed according to a strategic plan specifying important operational parameters in advance according to criteria established in concert with basic research laboratories, health service laboratories, field epidemiologists, community health workers and industry. It was agreed that batch-testing techniques permit standardized handling of large-scale surveys but are not well suited to field conditions. Although the need for development in this area was acknowledged, the meeting recommended that greater emphasis be placed on developing technologies which can actually be used in the field.

The validation of diagnostic techniques should be defined in terms of sensitivity, specificity, predictive values, cross-reactivity and reproducibility and an index of test performance or test efficiency should be included. The problem of poor serological specificity should receive particular attention and emphasis should be put on quality control of reagents, assays and participating laboratories. Interpretation of results should consider the possibility of immunocompromised subjects, either due either to treatment or other diseases.

Antibody-detection was not seen as suitable for assessment of active infection but it was believed that this approach would yield sufficiently accurate results in controlled areas with a prevalence of less than 3% according to parasitological examination. It was also felt that it could provide useful estimates of the prevalence in populations which had not been previously treated. Standardization would be facilitated if specific antigens, produced by recombinant or synthetic peptide technology, could be developed but, for the time being, standard antigen preparations such as the soluble egg antigen (SEA), produced using standard protocols, were recommended. Useful laboratory-based assays would include the circumoval precipitin test (COPT), complement fixation, different versions of the enzyme-linked immunosorbent assay (ELISA), as well as indirect applications of immunofluorescence, haemagglutination and latex agglutination.

Antigen-detection was deemed useful for assessment of the incidence of new infection in areas recently brought under control. This approach would also be important for evaluating vaccine efficacy following human trials. For reasons of standardized production, it was recommended that monoclonal antibodies or mixtures of such antibodies with different specificities be used as reagents. It was felt that an inventory of available antibody specificities would now be timely.

Although detection of circulating antigens may be carried out in different body fluids, serum tests were seen to have the current advantage. However, further research would be necessary for evaluation of the correlation between worm burdens and antigens, particularly in the urine and other specimens keeping in mind that the intact glomerular membrane filters out large-size antigens. On the other hand, urine specimens are relatively easy to obtain in most cultures and pose less risk for laboratory-acquired HIV or HBV infection. In addition, due to the non-invasive

nature of their collection, accidental spread of these infections will be reduced. It was felt that excretion of antigens in the urine should be investigated also in subjects infected with *Schistosoma japonicum* and *S. mekongi* and it was recommended that diagnostic schistosome antigens be further characterized immunologically and biochemically in order to understand their nature and origin.

It was emphasized that the various approaches to immunodiagnosis should not be seen as competitive ways of assessment of schistosome infection but rather as different applications using complementary techniques. However, it was deemed necessary to directly compare assays for antibody-detection with those for antigen-detection taking into account the role of immune complexes. It was also felt that it might be useful to compare other diagnostic approaches including intradermal testing.

Since the expected prevalence and the diagnostic purpose of testing vary from one locality and situation to another, appropriate test parameters for each case should be specified before studies are undertaken. These should be planned in great detail with carefully chosen patient groups based on well-characterized clinical status including acute, chronic asymptomatic chronic symptomatic and end-stage fibro-obstructive schistosomiasis. Each subject's parasitological status should be defined as the egg excretion class based on multiple specimens. Rectal biopsy was seen as a useful adjunct but was not recommended for application on a large scale. Evaluation of the correlation between worm burdens and circulating antigens should preferably be carried out in young age groups in order to reduce interference with egg excretion by fibrosis. Future collaborative studies should be made flexible enough to allow new assays and additional laboratories to be included, as and when appropriate.

Recombinant DNA probes constitute a sensitive and highly specific means of demonstration of the presence of an infectious agent but are less useful for the diagnosis of multicellular parasites that do not replicate in the host. Although fragments may be found in the blood, this is probably infrequent and mainly associated with treatment, so schistosome DNA in man originates mainly from eggs and miracidia. Considering its cost and sophistication, this technology was not felt to be applicable for diagnostic surveys since it would not offer better results than parasitological methods and since the sensitivity would be greatly affected by the irregular output of eggs and the release of miracidia during hatching. Although the application of the polymerase chain reaction (PCR) for urine or stool examinations might theoretically be useful in improving the sensitivity of techniques based on DNA probes, it was not felt to be a realistic approach that should be encouraged. When applied to the snail intermediate host, however, schistosome DNA probes can be used to identify infection including demonstration of specific strains and the sex of parasites, whilst snail DNA sequences can be used for determination of geographical variations and taxonomic relationships in the intermediate host. In the future, the techniques could be applicable in monitoring the potential of drug resistance in parasites. In order to do this, however, the genetic basis of drug resistance in schistosomes would have to be elucidated and the appropriate DNA sequences identified and used as probes.

Implementation of serological testing for communicable diseases, including schistosomiasis, by national public health laboratories using standardized testing systems would permit large-scale surveys and comparisons between different provinces providing support for decisions regarding national policies. In this connection, it would be useful to identify a limited number of assays that could be developed to test kits for use on a national or regional scale. The meeting recommended that kit development be centralized to a single reference laboratory or to industry.

The development of new assays and kits would require the establishment of a reference specimen bank, preferably with samples from different endemic areas including schistosomiasis due to both the Chinese and to the Philippine strains of *S.japonicum* as well as to *S. mekongi*. Designated centres in different endemic foci should be considered for collection and preservation of significant quantities of clinical specimens in a standardized manner. These would be dispatched to a laboratory in charge of a central specimen bank responsible for quality control, aliquoting, storage, inventory control and distribution. The specific requirements of the collection centres would be to obtain and provide specimens together with essential clinical data. In order to ensure long-term operation of the proposed specimen bank, it was recommended that new specimens be continuously added. Before establishing this facility, practical and administrative matters including financing would need to be addressed in detail.