The enzyme-linked immunosorbent assay (ELISA)*

The enzyme-linked immunosorbent assay " ELISA " developed in recent years represents a significant addition to existing serological tools. Encouraging preliminary results obtained through its application to a number of parasitic diseases during the last two years indicate the value of further investigations and trials which will permit a true evaluation.

Although the technique is easy to perform and quite sensitive, there are certain problems to be solved before it becomes widely usable. In the present Memorandum the technical details are given and the advantages and shortcomings of the procedure are discussed. Present applications and future prospects are reviewed.

A number of serological techniques are currently being applied to parasitic diseases. Interpretation of the results obtained, however, is rather difficult, especially when specific and well-defined antigens cannot be used. At present the lack of specific antigens is a serious handicap, but with progress in research towards defining the characterizing parasite antigens and making them available, serology will become a more useful tool.

Attention was drawn to this problem during a recent WHO consultation in Geneva, at which various aspects of parasite antigens were discussed and collaborative programmes were set up among laboratories engaged in work on antigen isolation. It is expected that in the near future specific antigens, particularly of *Plasmodium*, *Schistosoma*, *Onchocerca*, will become available. Such antigens will be submitted to field trials in which several established serological methods will be applied in parallel and the results compared with those of concurrent parasitological examinations. These trials will permit an evaluation not only of the antigens but also of the serological techniques. It is only through parallel studies of this kind that serology can be placed in its right context.

Efforts in several laboratories in recent years have led to the development of promising new serological techniques. The enzyme-linked immunosorbent assay (ELISA), and in particular its application to parasitic diseases, which is the subject of this Memorandum, represents a significant addition to existing serological tools. The method seems practical, easy to perform, and quite sensitive. As with any new technique, a number of problems must still be solved before ELISA becomes widely usable.

Encouraging results have been obtained with ELISA in preliminary collaborative studies and in some field applications in malaria, trypanosomiasis, schistosomiasis, and trichinosis.

DESCRIPTION AND REVIEW OF ELISA

PRINCIPLE (*8*, *9*, *21*)

Immunoenzyme methods having been successfully applied to the localization of intracellular antigens both at the light and electron microscope level, the same general principle was employed to detect soluble antigens and antibodies in body fluids. Immunoenzyme assays were therefore developed as alternatives to radioimmune assays.

Specific antibodies can be estimated quantitatively by ELISA. After incubating the test serum in an antigen-coated polystyrene tube or plate, enzyme-labelled anti-immunoglobulin is added and the enzyme then remaining in the tube or plate after washing provides a measure of the amount of specific antibody in the serum. The test relies on the insolubilization of antigens by passive adsorption to a solid phase, e.g. the polystyrene surface.

The same approach may be used for the detection of antigens in body fluids. Antigens present in the

* This Memorandum was drafted by the signatories listed on page 136. Reprints can be obtained from the Division of Malaria and Parasitic Diseases, World Health Organization, 1211 Geneva 27, Switzerland. A French translation of the Memorandum will be published in a future issue of the *Bulletin*.

test solution may then be detected by performing one of the following assays:

(1) A competitive assay. Using tubes coated with antiserum, a known quantity of enzyme-labelled antigen, employed as reference antigen, is mixed with the unknown sample and the decrease in reaction product is proportional to the antigen present in the test solution.

(2) A double-antibody ELISA. After coating the tube wall with antibody, the test serum is added followed by conjugate consisting of the initial antibody labelled with the enzyme. The reaction product is proportional to the amount of antigen in the test fluid.

(3) An inhibition assay. The test fluid containing antigen is incubated with the standard antiserum. The level of the remaining antibody is then measured by performing ELISA in antigen-coated tubes or wells.

Although the use of ELISA for antigen detection holds some promise for diagnostic purposes in parasitic diseases and although the method's potential for clinical application is recognized, this report will primarily deal with its use for the detection of serum antibodies and will emphasize its application in seroepidemiological studies.

MATERIALS USED AND STEPS TO BE FOLLOWED [a]

The antigen [b]

Standardization. Antigens used for ELISA are soluble, but can be made insoluble by adsorption to a solid phase. Satisfactory antigens can be found only by performance testing against reference sera. Stable antigen preparations should be used in order to ensure optimal reproducibility. In general, standardization of the following factors should be considered:

(*a*) species, strain, and developmental stage of parasite;

(*b*) species and strain of animal if a maintenance host is used, or if not, the *in vitro* conditions employed;

(*c*) post-infection period before recovery of parasites;

(*d*) isolation method;

(*e*) method of preparing the soluble antigens; [c]

(*f*) quality control.

Adsorption to solid phase. Most antigens adhere to polystyrene surfaces by physical adsorption. At present it is not known what part of the antigen is preferentially bound to the solid phase. Coating of the surface seems to depend on the quality of the polystyrene surface, among other factors. Although the use of physical adsorption as a means of binding antigens to a solid phase seems attractive, its drawback is that no precise information on the binding itself can be obtained. Therefore, the use of various activation methods or specific " spacers " has been studied in order to ensure a more standardized antigen binding procedure. Attention is currently focused on the use of different polystyrene surfaces. The binding capacities of new batches of polystyrene tubes or plates can be tested in a checker-board titration using standard antigens, antisera, and conjugates.

Apart from the nature of the surface material, adsorption of antigen to a solid phase is also dependent on time, temperature, and pH. Prolonged adsorption procedures (overnight) at 4°C seem to give a more uniform coating. Originally alkaline conditions were employed, but, in some applications, coating at pH 7 was found to be equally satisfactory. Coating should preferably be done immediately before assay, it being preferable to avoid storing coated tubes or plates containing the antigen solution.

Concentration. For each application, the optimal antigen concentration should be established by checker-board titration.

Lyophilization. In some cases lyophilization does not affect the antigen activity, and the same batch of antigen can then be used over a long period.

The washing fluid

Washing is performed: (i) after coating the surface with antigen directly before the assay, (ii) after serum incubation, and (iii) after incubation of the conjugate. Phosphate-buffered saline (0.01 mol/litre PBS, pH 7.2) with 0.5 g/litre of polysorbate-20 (e.g., Tween 20) can be used as washing fluid. Tap water may be substituted for the PBS, provided it has a neutral pH and a low chlorine content. Washing

[a] See Annex 2.

[b] See also the Memorandum entitled " Parasite antigens ", *Bull. World Health Organ.*, **52**, 237-249 (1975).

[c] Descriptions of antigen preparations could include details such as protein content and chromatography patterns. The results of other serological tests using soluble antigens may also be valuable for standardization purposes.

should consist of rinsing the tubes or plates three times for 3–5 min each. Washing times may be reduced to 1 min if tubes are flushed with an excess of washing fluid under pressure (1–2 atm).

Dilution of antiserum

Serum dilutions may be made in PBS containing 0.5 g/litre of polysorbate-20. In some instances the addition of 5 g/litre bovine serum albumin (BSA) decreases non-specific (i.e., background) staining.

When ELISA is performed by testing at one serum dilution only, the appropriate dilution is found by carrying out a preliminary test with positive and negative reference sera, and the dilution that is found to give maximum differences in extinction values between the known positive and negative sera is then used. On another hand, when ELISA is used for determining endpoint titres, several dilutions of the test sera should be made.

The conjugate (1, 13)

In the preparation of conjugates the following information should be taken into account:

(*a*) *Immunoglobulin preparation*

(i) the type of immunoglobulin preparation used for immunization, e.g., IgG (H+L), IgG (H);

(ii) the animal species used for immunization, e.g., sheep, rabbit;

(iii) the immunoglobulin fractions from hyperimmune serum used for labelling Ig, IgG, and immunospecifically purified IgG.

(*b*) *Enzymes*. Details concerning:

(i) the alkaline phosphatase (*8, 9*);

(ii) the horseradish peroxidase (*21*);

(iii) the glucoseoxidase.

(*c*) *Coupling procedure*. Details of:

(i) the glutaraldehyde methods (chemical binding);

(ii) the oxidation of enzyme by sodium periodate (chemical binding);

(iii) the peroxidase-antiperoxidase complex (immunological binding).

Alkaline phosphatase and horseradish peroxidase have usually been used as the marker enzyme. Commercially available conjugates are prepared using the glutaraldehyde coupling method with peroxidase. So far, no specifications for conjugates in terms of enzyme/protein ratio, absence of unlabelled immunoglobulin, free enzyme, and/or free glutaraldehyde, have been laid down.

The optimal conjugate dilution to be used in the test should be determined by checker-board titration. In order to obtain maximum differences between positive and negative sera, the extinction values of the " conjugate control " (i.e., antigen coated tube or well, incubated with conjugate and substrate) should be negligible. One way of decreasing background staining is by adding a BSA solution (40 g/litre) to the polysorbate-20 as diluent for the conjugate.

The substrate

4-Nitrophenylphosphate, which is the substrate used for alkaline phosphatase, is safe to use. However, 5-aminosalicylic acid, which is the substrate currently used for the visualization of horseradish peroxidase, may be carcinogenic, though much less so than 3,3′-diaminobenzidine, which is routinely used for the visualization of peroxidase in light and electron microscopy; although normal handling of the material does not constitute a health hazard, it is advisable to continue the search for possible alternative substrates.

Incubation time. The enzyme–substrate reaction should be stopped within the linear phase of the reaction curve. When large numbers of tests are carried out simultaneously, an incubation time of at least 30 min should be allowed, since this reduces timing errors. For qualitative purposes, a standard incubation time can be introduced, but for quantitative purposes, it is better to follow the reaction kinetics.

Reading and assessment of the results

When ELISA results are based on several dilutions, endpoint titres are determined and reactions may therefore be read visually; but, when results are based on one dilution only, readings should be performed with a spectrophotometer.

ELISA results may be expressed in the following ways:

(*a*) Extinction value. This is based on one serum dilution only. The significance of a certain extinction value should be assessed against known positive and negative reference sera. The extinction values of the negative sera may be used to set certain confidence limits.

(*b*) Endpoint titre.

(*c*) Percentage of a positive reference serum. This is based on one dilution of the test serum only.

(*d*) Probability percentage. This applies when either endpoint titres or extinction values are beyond the normal range.

Comments

For some purposes tests should be performed in duplicate or triplicate. ELISA performed in tubes clearly requires a larger volume of reagent than does the same assay in plates, but in either case only small volumes of test material are used. Blood samples collected in capillaries can be put on filter paper (see Annex 2).

ADVANTAGES AND DISADVANTAGES OF THE TECHNIQUE

In Table 1, the advantages and disadvantages of ELISA, radioimmune assay (RIA), and the immunofluorescence (IF) test, are compared.

Although the RIA has not been widely applied to the serology of parasitic diseases, it was thought important to compare various aspects of this technique with ELISA because of their close resemblance. However, for practical purposes the comparison with IF is more relevant, as the technique has been widely used.

One of the advantages of ELISA is the possibility that it may be used for the detection of circulating antigens. Although RIA and IF can also be used for this purpose, the DASS (defined antigen substrate spheres) system must usually be applied at the same time (*6*).

With ELISA it is easier than with IF to screen sera simultaneously for the presence of antibodies to various infective agents. This is especially important for certain field applications such as for establishing epidemiological profiles.

As mentioned in Table 1 (see also page 131) quality standards for conjugates are still under evaluation; continued research in this area is necessary.

To evaluate the reliability of the test it is essential to have both known positive reference sera, and a stock of sera from non-infected persons from the same area. These sera should be of known status with regard to the possible presence of antibodies to parasites other than the species under study.

Detection of free (circulating) antigens (*5*)

As mentioned above, ELISA may be used for the detection of antigens in body fluids in general. This seems particularly useful as a means of discriminating between the early phases of a disease and a past experience. Its use towards this end will depend, however, on the availability of highly specific reagents.

Table 1. Comparison of ELISA with other indirect antiglobulin tests (RIA and IF)

Criterion	ELISA	RIA [a]	IF
Sensitivity	high	high	usually lower
Specificity	depends on antigen preparation	depends on antigen preparation	high
Reproducibility	acceptable	acceptable	acceptable
Reading	objective	objective	usually subjective
Antigen preparation	can be complicated	can be complicated	easy
Conjugate	under evaluation	quality standard	quality standard
Feasibility of performance under field conditions	easy	not easy	intermediate
Mechanization of assay	possible	possible	difficult
Relative cost per test	low	high	high
Shelf-life of reagents [b]	long	short	long
Health hazards for laboratory personnel [b]	none or minor	present	none or minor

[a] RIA is not widely used for the diagnosis of parasitic diseases.

[b] Should be regarded as a limiting factor.

APPLICATION OF ELISA TO PARASITIC DISEASES

MALARIA (*22*, *23*)

Using a heterologous antigen (e.g., *Plasmodium knowlesi* from monkeys) it has been possible to discriminate between sera from endemic and non-endemic areas. In another study, using *P. falciparum* as antigen, ELISA tests were applied to human sera from South America and it was possible to identify the groups of people from a malaria endemic area and those from an area where malaria had been eradicated.

In comparing ELISA results with those of other serological tests, a somewhat better correlation was obtained with the passive haemagglutination test using the same antigen than with the IF test using the whole malaria parasite as antigen. A possible advantage of ELISA is its potential for large-scale application in seroepidemiology.[a]

Present activities and future prospects

At present the use of ELISA for malaria is being evaluated at the National Bacteriological Laboratory, Stockholm, the Nuffield Institute of Comparative Medicine, London, and the School of Public Health, Teheran. The Nuffield Institute of Comparative Medicine, London, and the National Bacteriological Laboratory, Stockholm, are also conducting field evaluations in Africa, South America, Papua New Guinea, and Sri Lanka. It may also be possible to incorporate ELISA in a WHO project in Benin, Nigeria. The future use of ELISA may, however, be limited by the availability of the malaria antigen now employed. Infected *Aotus* monkeys are at present the only source of a suitable malaria antigen, but a regular supply of these animals cannot be guaranteed and soon antigen from this source will probably no longer be made available by the Nuffield Institute of Comparative Medicine. In view of this situation, other sources of antigen should be explored.

TRICHINOSIS

ELISA has been used for the detection of serum antibodies to *Trichinella spiralis* in pigs and in man. In order to test the system currently in use, the sensitivity of ELISA was assessed in pathogen-free pigs experimentally infected with *T. spiralis*, as well as in conventionally raised pigs, experimentally or naturally infected with this parasite. Of particular interest was the fact that the presence of antibodies was demonstrated before day 17 post-infection, i.e., before the number of days required for muscle larvae to become infective for another possible host. Furthermore, ELISA proved to be much more sensitive than the classical diagnostic method, trichinoscopy. ELISA compared favourably with other serological tests such as IF, immunodiffusion, and counter-current electrophoresis. Using a crude saline extract of muscle larvae as antigen, it is possible to discriminate between sera from infected and non-infected conventionally raised pigs.

ELISA is now used routinely in screening for the presence of *T. spiralis* infections in pigs for slaughter on a random sampling basis. The assay has already been mechanized and, under present conditions, up to 4000 sera daily can be tested by micro-ELISA (*7*, *14–20*).

Present activities and future prospects

ELISA is being routinely used in for trichinosis testing at the National Institute of Public Health, Bilthoven, The Netherlands. In attempts to improve the technique, the use of more specific antigens is being studied and conjugates prepared in various ways are being tested. Moreover, a comparative programme aimed at standardization of the technique is being conducted by the European Economic Community countries and is co-sponsored by WHO.

SCHISTOSOMIASIS

Sera from individuals infected with *Schistosoma mansoni* were compared with sera from European and African controls. ELISA was performed using both a crude adult antigen and a purified egg antigen. The extinction values of the patients infected with *S. mansoni* were higher than those of the non-infected controls, particularly when the purified antigen was used. However, some false negative and false positive results were obtained, but this also occurs in all the other serological tests used up to now in schistosomiasis. In general, patients with

[a] It may be noted that the general applications of serology to malaria including antigen preparation have already been discussed in a published Memorandum: " Serological testing in malaria ", *Bull. World Health Organ.*, **50**, 527-535 (1974).

S. mansoni infection show higher titres than those with *S. haematobium* infection (*5*, *12*).

In the passive haemagglutination test, *S. mansoni* and *S. haematobium* antigens will detect both homologous and heterologous antibodies; this will probably also be the case with ELISA.

The application of ELISA for the detection of free (circulating) antigens in schistosomiasis may be important.

Present activities and future prospects

ELISA is being used at the National Bacteriological Laboratory, Stockholm, the Institute of Parasitology, University of Leiden, The Netherlands, the Laboratory of Parasitology, Faculty of Medicine, Lille, France, the Nuffield Institute of Comparative Medicine, London, and the London School of Hygiene and Tropical Medicine. At present various crude and purified antigen preparations are being used in ELISA and it is suggested that, in order to improve the technique, the efficacy of the various antigens should be compared.

The evaluation of ELISA for seroepidemiological purposes could be linked with existing WHO programmes in Africa, in particular a WHO project on research on the epidemiology of schistosomiasis in man-made lakes. As for the evaluation of ELISA for clinical use, three lines of research are envisaged: (i) longitudinal studies in hospitalized patients in endemic areas; (ii) longitudinal studies in hospitalized patients in non-endemic areas (imported schistosomiasis); and (iii) longitudinal studies of schistosomiasis patients undergoing chemotherapy.

TRYPANOSOMIASIS

The results obtained with ELISA have been satisfactory for both African trypanosomiasis (sleeping sickness) and American trypanosomiasis (Chagas' disease) (*24*, *25*). Wide cross-reactions between *Trypanosoma cruzi*, *T. brucei*, and *Leishmania* were observed. Acceptable correlations were found between the IF test and ELISA. By using ELISA it was possible to distinguish between groups of individuals with and without trypanosomiasis, although some overlap (i.e., false negatives and false positives) was observed.

An alternative to *T. brucei* antigen seems feasible, since each trypanosome strain exhibits many antigenic types (e.g., more than 100 antigenic types for one defined strain). A small number of these types are common to several and maybe to all strains. Preparations containing the greatest number of immunogenic determinants of these common antigenic types should preferably be used as the standard antigen (*11*, *24*, *25*).

Present activities and future prospects

(*a*) *African trypanosomiasis.* With the collaboration of WHO and the Ahmadu Bello University, Kaduna, Nigeria, the Nuffield Institute of Comparative Medicine, London, and the National Institute of Public Health, Bilthoven, The Netherlands, are evaluating ELISA in the screening of sera for trypanosomiasis. For these tests *T. brucei* is used as antigen. These institutes maintain contacts with institutions of veterinary parasitology, such as the Centre for Tropical Veterinary Medicine, Edinburgh, Scotland, and the Institute for Tropical and Protozoan Diseases, Veterinary Faculty, State University of Utrecht, The Netherlands, with which they exchange information, with special reference to the use of ELISA for the detection of trypanosomiasis in cattle. With a view to improving the technique, the Institut national de la Santé et de la Recherche médicale, Bordeaux, France, is willing to collaborate with other laboratories dealing with the serology of trypanosomiasis to study and evaluate newly developed antigens. At a later stage, ELISA field studies might be introduced in a WHO trypanosomiasis project in Upper Volta.

(*b*) *American trypanosomiasis.* ELISA is being used at the Nuffield Institute of Comparative Medicine, London, and at the London School of Hygiene and Tropical Medicine. ELISA can be evaluated in the field projects of the Evandro Chagas Institute, Belem, Brazil; and the Faculty of Medical Sciences, Federal University of Brasilia, may be interested in cooperating in field tests using the possibilities offered by the project in Recife. Brazilian groups working on Chagas' disease would be willing to discuss the possibility of collaborative efforts in the future.

FILARIASIS, INCLUDING ONCHOCERCIASIS

Experience with ELISA in filarial infections is still limited. Cross-reactions occur between *Ascaris suum*, *Dipetalonema*, *Onchocerca gutturosa*, *Litomosoides carinii*, and *Brugia pahangi*. *O. volvulus* antigen from human sources cannot be used because it is difficult to obtain a pure antigen free from host material. However, with a crude extract of *O. gutturosa* it was possible to differentiate between sera from Europeans, sera from filariasis-negative Africans, and

sera from patients with *O. volvulus* infection. More specific antigen preparations are being developed in various laboratories. The use of immunohistological techniques may prove to be important in determining specific antigens in the filarial worm (*2*).

In filariasis, ELISA could be used for sero-epidemiological studies, special attention being paid to the 5–10 year age group, as well as to the determination of antibody levels before and after chemotherapy and the detection of free (circulating) antigens.

Present activities and future prospects

ELISA is being evaluated at the Nuffield Institute of Comparative Medicine, London, and the London School of Hygiene and Tropical Medicine. In order to improve the technique for application to filarial infections, a high priority is being given to making antigen supplies available and to the comparison of antigens; this type of work is being carried out by the Minerva Institute for Medical Research, Helsinki, and the London School of Hygiene and Tropical Medicine. *Litomosoides carinii*, which constitutes one raw material source for antigen preparation, is being made available by the Institute of Parasitology and Animal Parasitic Diseases, Justus Liebig University, Giessen, Federal Republic of Germany.

ELISA can be evaluated in filariasis and onchocerciasis field programmes in various representative endemic areas, in particular through links between Nuffield Institute of Comparative Medicine, London, and various programmes in Africa, Papua New Guinea, and India, and through links between the Institute of Tropical Hygiene and Public Health, Heidelberg, Federal Republic of Germany, and programmes of the Coal Mines Labour Welfare Organization, Dhanhad, India, and of the Calcutta School of Tropical Medicine, India. The use of ELISA in the WHO Onchocerciasis Control Programme in the Volta River Basin is being considered, and its application for multipurpose seroepidemiological and clinical studies is contemplated in areas that are also endemic for malaria, trypanosomiasis, and schistosomiasis. ELISA field studies could also be made in areas of Sudan, Tanzania, and American Samoa where WHO programmes are in operation or being planned.

OTHER PARASITIC INFECTIONS

Toxoplasmosis

Variable results have been obtained with ELISA for the detection of antibodies against *Toxoplasma gondii*. Most investigators have reported a rather unsatisfactory correlation with the IF and dye tests and a better correlation with the passive haemagglutination test when the same antigen was used. Some satisfactory results have been obtained with ELISA, however, and in particular good correlations have been established with the clinical status of the patient. These variable results might be attributed to differences in the antigen preparations. Since antibodies to cell membrane are the first to be formed, antigens used in ELISA should contain this type of material if a good correlation between ELISA values and the clinical picture is to be obtained. Such antigens should also be used if ELISA is to be applied to the mass screening of high-risk groups, such as pregnant women (*5*, *26*).

Hydatidosis

Good results have been obtained with ELISA in the detection of antibodies in hydatidosis (*5*, *10*).

Amoebiasis

Promising results have been obtained with ELISA in the detection of antibodies in amoebiasis (*3*, *4*).

DETECTION OF FREE (CIRCULATING) ANTIGENS IN PARASITIC INFECTIONS

In a number of parasitic infections, particularly those in the blood, free antigens have been detected, using various immunological tests. ELISA, because of its high sensitivity, may be very suitable for the detection of such antigens. It is important to relate the presence of free (circulating) antigens to the clinical status of the patient, as these may be a better indicator of his actual disease state than the presence of serum antibodies. In filariasis, particularly, antigen detection could be advantageous.

TRAINING

In specialized laboratories

The laboratories where ELISA is now being applied will accept trainees with some experience in serology provided they receive adequate financial support. When selecting the laboratory for training in the ELISA techniques, the interests of the trainee

should be considered from the following viewpoints: (*a*) clinical diagnosis; and (*b*) seroepidemiological studies. A few weeks should be adequate for such training; WHO could help in making such arrangements.

Field training

The specialized laboratories now using ELISA can provide " on the spot " training in field situations. Such training should be sponsored by WHO. Indeed, it is felt that this programme is highly relevant to the efforts of WHO in the field of parasitic diseases.

* * *

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Annex 1

ELISA METHODS

ELISA may be performed in tubes (macro-assay) or in microplates (micro-assay), depending on the reagents available. Both methods are described below.

MACRO-ASSAY

The following method is applicable to *Trichinella spiralis* infection in pigs (*17*, *18*).

Immediately before the assay, disposable polystyrene tubes (50 mm high and 11 mm wide at the base)[a] are coated with antigen as follows: 1 ml of the antigen solution, containing 5 μg of protein per ml, dissolved in sodium carbonate buffer (0.1 mol/litre; pH 9.6) with sodium azide (0.2 g/litre), is placed in each tube. The tubes are incubated in a water bath at 37°C for 30 min. They are then washed 3 times with 2 ml per tube of phosphate-buffered saline (PBS—0.01 mol/litre; pH 7.2) containing 0.5 g of polysorbate-20 (Tween 20) per litre, each washing lasting 5 min. Sera are diluted 1 to 10 with PBS containing 5 g per litre of bovine serum albumin (BSA) and 0.5 g per litre of polysorbate-20. One millilitre of a serum dilution is added to each tube.

The tubes are incubated under rotation at 37°C for 30 min. They are then washed 3 times, for 5 min each time, with PBS containing 0.5 g of polysorbate-20 per litre. One millilitre of conjugate (anti-species IgG–horseradish peroxidase),[b] diluted to an optimal dilution in a 40 g/litre BSA solution containing 0.5 g of polysorbate-20 per litre, is added to each tube. The tubes are again incubated under rotation at 37°C for 30 min and washed 3 times, for 5 min each time, with PBS containing 0.5 g polysorbate-20 per litre. Finally, the amount of enzyme bound to the tubes is determined. The specific substrate used is 5-aminosalicylic acid (5 AS) and hydrogen peroxide. This substrate is prepared by dissolving 80 mg of 5AS in 100 ml of distilled water at approximately 70°C. Just before this stock solution is used, its pH should be brought to 6.0 with 1 mol/litre NaOH; 1 ml of a 0.5 ml/litre solution of hydrogen peroxide is added to 9 ml of 5AS.

[a] LKB-Producter AB, Stockholm, Sweden.

[b] Institut Pasteur, Paris, France.

Incubation is performed at toom temperature while the tubes are shaken gently. The reaction is stopped after 1 h by the addition of 0.1 ml of 1 mol/litre NaOH. The reaction product, which is brown in colour, is poured into a cuvette. The density is measured in a spectrophotometer at 449 or 450 nm. The substrate control (antigen-coated tubes incubated with the substrate only) is used to obtain the zero level of the spectrophotometer.

MICRO-ASSAY

This method is applicable to malaria (*22*).

Sensitization of plates

Polystyrene micro-haemagglutination plates with wells (Cooke Microtiter [a] M2 9AR) are used as the carrying surface for the antigen. Each well is sensitized with 0.3 ml of the soluble antigen diluted in 0.05 mol/litre carbonate buffer, pH 9.6. After sensitization, which is achieved overnight at 4°C or in 1 h at 37°C, the plates are washed 3 times for a total of 15 min in saline containing 0.5 g of polysorbate-20 per litre. The plates are then shaken dry and are ready for immediate use.

Performance of the test

1. The test sera are diluted in PBS (pH 7.2) containing 0.5 g of polysorbate-20 per litre.

2. After adding 0.3 ml of each appropriately diluted serum to separate wells in the sensitized micro-haemagglutination plates, the plates are incubated in a humid atmosphere for 2 h at room temperature of 1 h at 37°C.

3. The wells are emptied and the plates are washed with three changes of saline containing 0.5 g of polysorbate-20 per litre, for a total of 15 min. The plates are then shaken dry.

4. To each well is added 0.3 ml of the optimally diluted conjugate and the plates are then incubated for 3 h at room temperature or 1 h at 37°C.

5. The wells are emptied and the washing is repeated as in step 3.

6. To each well is added 0.3 ml of enzyme substrate and the plates are then incubated at room temperature.

7. The reaction is stopped after an appropriate time with 0.05 ml of 2 mol/litre NaOH.

8. Absorbance of the contents of each well is read in a spectrophotometer at 400 nm (=ELISA value).

The conjugate used in this test is alkaline phosphatase coupled with antihuman globulin, and the substrate is 4-nitrophenyl phosphate (1 g/litre) made up in diethanolamine buffer (pH 9.8) containing 0.5 mol/litre of magnesium chloride.

[a] Dynatech Laboratories, Alexandria, VA, USA.

Annex 2

COLLECTION OF SERUM AND PLASMA SAMPLES [a]

Serum and plasma can both be used in ELISA. In many instances where venipuncture is inadvisable, it is possible to collect blood by finger-pick into heparinized capillary tubes. Wherever possible, the capillary tube method should be used for the collection and transport of samples, although under certain conditions the only practical way may be to use the filter-paper technique.

SERUM

In order to obtain serum, the blood collected should be coagulated at room temperature within a few hours and not at 4°C, otherwise significant quantities of IgM, and to a lesser extent of IgG, may be lost.

PLASMA

Capillary tubes

The finger is cleaned with an alcohol-moistened swab and is allowed to dry in order to prevent haemolysis of the sample. A disposable lancet is then used to prick the finger and the first drop of blood is wiped off, then one or more labelled heparinized

[a] Refer also to a Memorandum already published: " Serological testing in malaria ", *Bull. World Health Organ.*, **50**: 527-535 (1974).

capillary tubes (with a minimum internal diameter of 1.1 mm and a length of 75 mm), preferably containing dried thiomersal, are filled. The capillary tubes are then sealed at one end, either by plugging with plasticine or by heat, and centrifuged within a few hours. An electrical centrifuge or effective alternative should be used. The capillaries are then cut at the plasma–cell interface. The plasma section is again sealed at both ends with plasticine and stored.

Filter paper

Under field conditions, the blood samples collected into capillary tubes may be absorbed on to filter paper from which the serum can be eluted prior to serological testing. In this case, it will be necessary to absorb precisely measured quantities of blood on to the filter paper, e.g., using capillary tubes. The impregnated filter papers must not come into contact with fixing agents and should be dried as soon as possible, avoiding temperature exceeding 56°C. Although these dried blood samples on filter paper are known to withstand normal environmental temperatures in subtropical conditions for at least a fortnight without loss of seroreactivity, it is advisable to store them as soon as possible at −20°C, at which temperature, experience indicates, they can be preserved for at least a year.

For the tests, the eluate is used. If the blood sample is 70 μl, as is the case with the standard haematocrit capillary tubes, each filter paper is placed in 0.7 ml of buffer solution of pH 7.2 and is left overnight at a temperature of 4°C. This corresponds to a dilution of $^1/_{20}$ on an assessed haematocrit value of 50%. Even when the haematocrit values are very low, overestimation of the titre value is not great.

Recent investigations suggest that absorption of blood on the filter paper may destroy the reactivity of IgM antibodies. Therefore, for seroepidemiological studies of infections in which such antibodies are of diagnostic importance, fresh plasma samples should be used.

STORAGE OF SERUM

There appear to be very few reports on the stability of parasite antibodies in human serum during storage, but general experience suggests the following:

1. Ideally, tests should be carried out on fresh serum.

2. Sera should be stored in small aliquots and preferably at a temperature of −70°C or lower.

3. Bacterial growth should be avoided. This can be achieved by handling the serum so as to avoid gross contamination and by the addition of sodium azide or thiomersal to the blood or serum sample.

4. Delipidization may be preferable in certain cases.

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