SERODIAGNOSIS OF PARASITIC INFECTIONS BY ELISA WITH DIFFERENT ANTIGENS

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INTRODUCTION

The enzyme-linked-immunosorbent assay (ELISA) has some advantages in comparison with other serological techniques. Only a small amount of reagents are required and the results can be read automatically and computerized. Many specimens can be tested in one run and the costs for one test are relatively low. Most of the techniques described are for one antibody or antigen only. The problem for many laboratories also outside endemic areas is that specimens from other countries are requested for different parasitic antibodies. Single test-runs in parallel for different antibodies are not practical. Speiser (1982), developed a multi-antigen ELISA for tissue dwelling parasites as a screening test. With the testing of a single serum dilution against several antigens in the same plate the reactions with them can be compared and the cross-reactions will become obviously seen.

The aim of our studies was to establish an ELISA with different antigens for routine-tests parallel to the indirect immunofluorescence tests (IFT). Besides this we tried to use microtitration strips instead of plates because of variations in the number of sera.

An ELISA was established for amoebiasis, schistosomiasis, echinococcosis and filariasis and the results were evaluated on the sensitivity, specificity, reproducability and practicability of the test.

MATERIALS AND METHODS

The ELISA described herein has been established for human serum antibodies in cases of amoebiasis, schistosomiasis, echinococcosis and filariasis.

The following sera were selected from the serum bank:

(I) Amoebiasis : 20 sera from patients with well documented amebic liver abscesses or titer in the indirect immunofluorescence test (IFT) of 1:80 or more which is considered as significant for extrain testinal amoebiasis.

(II) Schistosomiasis: 16 sera from parasitologically confirmed cases (12 *S. mansoni*, 3 *S. haematobium*, 1 *S. japonicum*) and 3 sera with titer in the IFT of 1 : 640.

(III) Echinococcosis: 20 sera from parasitologically confirmed cases.

(IV) Filariasis : 4 sera from parasitologically confirmed cases (2 *Onchocerca*, 1 *Loa*, 1 *Dracunculus*) and 16 sera with IFT-titer of 1:40 or more which indicates a filarial infection.

(V) Negative controls: 48 sera from European blood donors who have never been in endemic areas of amoebiasis, schistosomiasis, filariasis, and for echinococcosis there were no clinical symptoms. All sera were non-inactivated and stored at –70°C.

The technique is based on the ELISA methods described previously (Janitschke *et al.*, 1981; Janitschke and Bode, 1982).

The ELISA was adapted for the application of the above four parasitic infections as follows;

Antigens : For amoebiasis freeze-dried commercial antigen (HK 9) (ICN Medical Diagnostic Product, California USA) was used. One bottle was dissolved in 1 ml coating buffer (carbonate, bicarbonate buffer pH 9.6) and diluted with this buffer 1 : 200.

For Schistosoma antigen, a commercial product (Billarzia Antigen, Institute Pasteur, Paris) was used. The content of one bottle was dissolved in coating buffer and diluted to 1 : 100.

For echinococcosis also a commercial product (Antigene Hydatique, Institute Pasteur, Paris) was used and prepared as above.

The filariasis antigen was prepared from freeze-dried *Dipetalonema viteae*, kept in *Mastomys* in the laboratories of Bayer AG, Germany. 500 mg worms were homogenized with 10 ml coating buffer, centrifugated at 800g for 15 minutes and thereafter the supernatant was centrifugated at 100,000g at 4°C for 1 hour. The final supernatant was ampouled in aliquots of 0.5 ml, freeze dried and stored at –20°C. The content of one ampoule was dissolved in 1.0 ml coating buffer, diluted 1:200 and used for the tests.

The microstrip wells (Flow 78-592-C 5) were put on a combi frame (Flow 78-594-01) and fixed with a strip retainer (Flow 78-555-02). 200 µl antigen were put into each well, stored over night, emptied, dried at room temperature, sealed separately in plastic bags and stored at –20°C.

Each antigen, serum and the conjugate were tested in four dilutions (made with incubation buffer pH 7,6) by chequerboard titration in order to select the dilution which allow the best differentiation between a positive and a negative control serum. The values were tabulated, and mean ($\bar{X}$), standard deviation (SD) and correlation coefficient variant (CV%) were calculated. The combinations with highest $\bar{X}$ and lowest CV % and best practical dilution were selected as working dilutions : antigen, serum 1:80, conjugate (Peroxidase Anti-Human IgG H & L chain goat, Miles) 1:1500. The substrate was composed of: Orthophenylendiamine (Merck 7243) in methanol, peroxide (Merck 8597), sodium phosphate buffer pH 5.0, H_2SO_4 (8 n) was used for stopping the reaction (after 4 minutes) and the test was read by multiscan (Titertek) at 492 mm.

In all the tests the enzyme reaction was stopped according to the extinction of a reference serum. The amoebic reference serum was selected and 200 µl of each was put into a whole strip. The reaction was stopped at an extinction rate between 1.2 and 1.4. All the following tests were stopped in this range, which was reached between 1 and 3 minutes.

For determination of the cut off lines between positive and negative extinction rates 48 negative control sera were tested with the four antigens. The mean–extinction plus two standard deviations for each antibody reaction was determined and calculated in percent according to the following formula :

$$\frac{(\bar{X} + 2\ SD) - \bar{X}\text{neg. control serum)}}{\bar{X}\text{ pos.reference serum} - \bar{X}\text{ neg. control serum}} \times 100 = \%$$

The cut off-values were calculated as follows : amoebiasis 19%, schistosomiasis 3%, echinococcosis 24%, filariasis 11%.

Routine testing : Patients sera were collected as described before and put into micronic tubes (Flow 61-228-C2) which allows an easy pipetting with multichannel pipettes and stored at-20°C. Microstrips were taken from the deep freeze according to the number of patients sera, fixed in the frames and washed three times with washing solution (NaCl-Tween). 200 µl serum in optimal dilution (1:80) was filled into each well and was incubated for 2 hours at 37°C.

A reference plate was added to each test run. This consists of 1 microstrip with amoebic reference-serum for the reaction stop and 4 wells for each antigen filled with positive and negative reference sera (4 wells each).

Plates were washed three times and 200 µl conjugate in optimal dilution (1:1500) was added and stored for another 2 hours at 37°C. After three times washing 200 µl substrate was filled in each well. The reaction was stopped according to the reference extinction rate of the amoebic reference-serum. The test results were calculated in percentages according to the extinction rates of each positive and negative reference sera.

$$\frac{\text{Patient's serum} - \text{neg. ref. serum}}{\text{Pos.ref. serum} - \text{neg. ref. serum}} \times 100 = \%$$

RESULTS

Sensitivity and specificity of ELISA was observed as follows :

Sera from 20 patients with amoebiasis were tested by ELISA, 17 reacted positive, 3 were negative while all 20 control sera were negative.

Sera from 20 patients with schistosomiasis tested were found positive by ELISA, all 20 negative controls were negative.

Out of 20 sera from confirmed cases of echinococcosis, 18 reacted positive and 2 negative. The 20 negative controls were negative.

All the 20 sera from filaria patients reacted positive but 2 out of the negative controls had false positive reactions.

Reproducability was studied by 33 test-runs which have been performed since the establishment of this ELISA technique. In each test run the same positive reference sera and negative controls were used. The reproducability between the test runs were calculated by the means and standard deviation:

ELISA for antibodies	Positive reference $\bar{X}$	SD	Negative control $\bar{X}$	SD
Amoebiasis	1.441	0.196	0.182	0.057
Schistosomiasis	1.912	0.131	0.161	0.074
Echinococcosis	1.438	0.473	0.133	0.049
Filariasis	1.707	0.310	0.285	0.105

On the practicability of this ELISA, the test-run can be performed (1 plate patients sera) within 5 hours.

DISCUSSION

Speiser (1982), who described a multi-antigen ELISA for screening motivated us to establish an ELISA for quantifying antibodies. The techniques of both tests are not quite different. We also use only one serum dilution (for the four antigens), but the optimal dilution was selected by a chequerboard titration, whereas Speiser (1982) stated that testing with one serum dilution would be a compromise and therefore not optimal for each serum dilution. The same chequerboard titration was used for the selection one optimal dilution for conjugate, but different dilutions were chosen for the antigens.

Speiser's (1982) screening method is for patient's sera with 8 antigens. The advantage of this technique is the detection of cross reaction with the antigens. but it is too mechanically designed and positive reaction may sometimes give misleading results. The testing of sera according to the clinical data of a patient, when requested for tests is preferable.

The sensitivity of our test system was excellent for schistosomiasis and filariasis but not for the other two parasites. Three confirmed cases of amoebiasis did not react in ELISA. We could observe false negative reactions also in our routine tests for amoebiasis. Also Speiser (1982) found 3 sera out of 29 with no reaction.

Two of our 20 parasitologically confirmed cases of echinococcosis showed false negative reactions. One of these was a case of orbital infection with IFT of 1:160 but negative reactions in indirect haemagglutination test (IHA) and latex agglutination which may show insufficient amount of circulating antibodies in this case. The other negative had titers of 1:160 (IFT) and 1:64 (IHA), but was negative in latex agglutination. It is well known that in some echinococcosis cases (Janitschke *et al.*, 1985) antibodies can be detected by no other means, but in the above mentioned case two other techniques gave positive results, which shows that the ELISA can be false-negative as a single test. In summary we believe that our ELISA-system has a sufficient sensitivity for quantitative routine tests, and we use this test parallel to IFT which will give more confirmed results.

The specificity of the described ELISA was evaluated by testing sera from patients who presumably never had the above mentioned parasitic infections. The only false positive reaction could be observed in two cases with filarial antigen. One serum reacted with 11% at the cut off-line (= 11% means positive), the other serum reacted with 44% and no explanation can be given. Above all our ELISA test system has a good specificity, only with filarial antigen some false-positive reactions can be expected.

The reproducability was acceptable. Not a single serum produced controversial results.

The practicability of the described ELISA does not differ from known techniques. The method of Speiser (1982) is however much faster because of a high concentrate substrate-indicator solution which we also used according to Speiser, but our incubation periods for serum and conjugate are much longer and they should be shortened. Our system of dispensing, storing and diluting sera with multi-channel-pipettes and semi-automating washing the plates seems practicable and reliable, therefore we can recommend this multiantigen ELISA for routine work.

This test system can be extended to more than 4 antigens. In preliminary tests we were successful to test sera also for antibodies against *Angiostrongylus*, *Trichinella* and *Gnathostoma* which offers a good tool for a broader serodiagnosis.

SUMMARY

An enzyme-linked immunosorbent assay is described in which four antibodies (amoebiasis, schistosomiasis, echinococcosis and filariasis) can be tested at once. Because of the sensitivity, specificity, reproducability and practicability this test system can be recommended as a quantitative routine test.

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