Circulating gut-associated antigens of Schistosoma mansoni : biological, immunological, and molecular aspects

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Chapter 6

Detection of IgM antibodies directed against the gut-associated circulating cathodic antigen in sera from *Schistosoma mansoni* infected patients

Development and comparison of three enzyme-linked immunoassays

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Abstract

The majority of the human IgM antibodies detected with an immunofluorescence assay (IFA) on adult worms are directed against the gut-associated circulating cathodic antigen (CCA). In order to study this phenomenon further we developed and evaluated three related ELISA methods to specifically detect IgM antibodies against purified CCA. The assays employed 1) direct coating of CCA, 2) indirect coating of CCA via a monoclonal antibody, and 3) IgM antibody-capture by rabbit anti-μ chain antibodies. Using a group of 46 positive sera, it was found that the three ELISA's and the IFA were significantly correlated. To discriminate between positive and negative sera we used a cut-off level of average reactivity + 3 standard deviations of 50 negative sera. False negative reactions were not found in any of the ELISA's, while both in the direct and the indirect ELISA one false positive reaction occurred. For further studies or diagnostic use the antibody-capture ELISA is recommended.

Introduction

Infection with *Schistosoma mansoni* leads to a strong and early humoral immune response against gut-associated antigens of the worm [12,14,19,22,24]. SDS-PAGE of adult worm antigen, followed by immunoblotting, as well as ELISA techniques utilizing different worm antigen preparations have been described for determination of antibodies against gut-associated antigens [4,10,13,16,23,24]. Antibodies against the gut-associated circulating anodic

antigen (CAA) and circulating cathodic antigen (CCA) were detected using an immunofluorescence assay (IFA) on Rossman's fixed adult worms [12,19]. Beside the IFA on whole worm sections, ELISA's have been reported in which antibodies against (partly) purified preparations of CAA and CCA [10,13] were detected. CAA and CCA both originate from the schistosome gut and are increasingly used for specific and sensitive immunodiagnosis of schistosomiasis in antigen-detection assays [5,8].

The underlying reasons for the present study were the following. Firstly, a routinely used IFA for immunodiagnosis of schistosomiasis detects IgM antibodies against *Schistosoma* gut-associated antigens in Rossman's fixed sections of adult worms [19]. These IgM antibodies are predominantly directed against CCA and not against CAA, as was shown by inhibition with specific anti-CAA and anti-CCA monoclonal antibodies [14]. This phenomenon needed further investigation and we used immuno-purified CCA preparations in ELISA-techniques to specifically measure the reactivity of human serum IgM antibodies against CCA.

Secondly, using novel immunoaffinity-chromatography techniques and more sensitive methods for antigen-detection, it has now been established that the antigen preparations which were used in the past [10,12], were not entirely pure. As a consequence, antibodies specific for *Schistosoma* CAA or CCA as well as other antibodies could have been detected. This limitation together with improvements in assay-performance and the availability of highly specific and sensitive monoclonal antibodies (McAb), induced us to develop a sensitive assay which detects serum antibodies specific for the *Schistosoma* circulating cathodic antigen.

ELISA methods are well established and convenient assays for rapid screening of large numbers of samples. While for antigen detection absolute concentrations can be read from a standard curve, the most appropriate method for detection of antibody activity (a combination of antibody affinity and concentration) is to express the results as end-point titres [6], as the serum dilution curves of different patients might not be parallel. However, for determination of the end-point titre, absorbance values have to be read in the lowest part of the dilution curve, which is nearly flat and thus most sensitive for errors.

Hancock and Tsang [15] described a modification of the antibody-detection ELISA, the kinetic-based FAST-ELISA, as a truly quantitative assay. Two requirements for quantitative assays are: (1) all reagents except the one being measured are present in excess amounts; (2) the enzyme activity is measured during the initial linear portion of the reaction [15]. Hancock and Tsang described that in their specific case the serum dilution curves for individual sera were parallel, so they could use the standard curve method to estimate the

activity of individual sera (expressed in arbitrary units). In our system we adopted most of the principles described for the kinetic ELISA, namely the excess amounts of reagents and the early measurement of enzyme activity. However, due to limitation of antigen only one serum dilution in duplicate was utilized both for the reference and sample sera and results were expressed as positive–negative ratio.

Materials and Methods

Parasites and antigens

S. mansoni adult worms (Puerto Rico strain) were collected by perfusion of golden hamsters 7 weeks after infection with 1500 cercariae. Adult Worm Antigen (AWA) and a TCA–soluble (7.5% w/v) fraction of AWA (AWA–TCA) were prepared as previously described [9]. CCA was purified using a Protein A–based immunoaffinity column prepared as described by Sisson and Castor [25], using mouse monoclonal antibody 54–5C10–A (IgG3) as capture antibody. The purity of CCA was checked by ELISA as compared with starting–material. Biotin aminocaproylhydrazide (BACH, Pierce, Rockford, USA) was used to biotinylate purified CCA, basically according to O'Shannessy *et al.* [20] and O'Shannessy and Quarles [21]. CCA (2 mg/ml) was oxidized for 15 min at 4°C in acetate buffer (0.1 M, pH 5.5) by adding freshly prepared $NaIO_4$ to a final concentration of 10 mM. The reaction was stopped by sodium sulphite and 0.01 M BACH in acetate buffer was added to a final concentration of 5.4 mM. After stirring the reaction mixture for 2 h at roomtemperature it was dialyzed against PBS (0.035 M phosphate, 0.15 M NaCl, pH 7.6). Glycerine was added (50%) and the preparation was stored in aliquots at −20°C. Final concentration was 0.3 mg CCA–BACH/ml as calculated from the starting amount and assuming that yield was 100%.

Immunofluorescence assay

The IFA was carried out on sections of adult male worms fixed with Rossman's fixative [18,19]. Slides were incubated with two–fold dilution series (10 dilutions, starting at 1/8) of serum of individual patients (45 min), washed, and incubated for 45 min with FITC–labelled swine anti–human IgM (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted 1/40 in PBS containing 0.1 mg/ml Evan's Blue. The slides were observed with a Leitz Dialux 20EB fluorescence microscope with the appropriate filter combination for FITC fluorescence. The reciprocal value of the last serum dilution at which gut–fluorescence was still visible was taken as the titre.

Human serum samples

For optimization of the assays a positive serum pool composed of equal amounts of 5 sera which were moderately positive in the IFA was used. Similarly, 5 sera which showed no reaction in the IFA were pooled in the negative serum pool. This was done to circumvent a possible restriction of antibody-reactivity due to testing with only one serum (in the optimization experiments). A serum showing intermediate reactivity and a low background chosen from a group of 5 positive sera was taken as positive reference serum.

For determination of the assay performance, the same 46 positive sera were used which have been described by Deelder *et al.* [14]. The sera were from patients which had recent (under 6 months), static (between 6 months and 4 years), and chronic (over 5 years) infections. In all patients the infection was parasitologically proven by demonstration of *Schistosoma mansoni* eggs in the stool. None of these patients had been treated with anti-schistosome drugs before serological testing. Fifty sera from non-endemic control persons were used as negative sera.

Enzyme-linked immunosorbent assays

Three ELISA methods were developed for detection of human anti-CCA IgM antibodies. In each ELISA, incubation steps were 15 min at 37°C during which time the plates were shaken on a Cooke AM69 Microshaker [17]. Absorbance readings at 630 nm (A630) were performed within 5-10 min (during this time period the peroxidase reactivity is linear) of substrate incubation with occasional shaking of the plates. Serum samples and reference serum were tested in duplicate in the same dilution (1/200, see Results section) and results were expressed as A630 ratios of samples and reference (S/R ratio), after subtraction of the A630 values of the wells containing diluted sera but without antigen solution. Each plate contained the reference serum and the control without antigen solution. After optimization the following procedures were adopted.

- Direct ELISA

In the direct ELISA purified CCA was directly coated to the plate (Maxisorp, Nunc, Roskilde, Denmark) in a concentration of 2.5 μg/ml PBS (in the first series of optimization experiments the partly purified preparation AWA-TCA was used since the amount of purified CCA was limited). Plates were washed with a 20 times diluted PBS solution and incubated with duplicate serum samples 1/200 diluted in 0.3% Tween-20 in PBS (PT). A positive control serum identically diluted was included on each plate. After washing, plates were incubated with peroxidase conjugated $F(ab')_2$ fragments of rabbit anti-human IgM (Dakopatts, Copenhagen, Denmark) 1/500 in PT. After a thorough final washing, substrate solution (0.42 mM 3,3',5,5'-tetramethylbenzidine (TMB, Polysciences, Warrington, U.S.A.), 1.4 mM H_2O_2 in 0.1 M sodium acetate buffer pH 5.5, [15]) was added and absorbance measured at 630 nm. TMB and H_2O_2 concentrations used were shown to correspond to excess amounts [2].

- Indirect ELISA
In the indirect ELISA anti-CCA McAb 54-5C10-A (IgG3, Protein A purified) was coated in a concentration of 10 μg/ml in PBS, followed, after washing, by incubation of purified CCA (2.5 μg/ml in PBS) (or AWA-TCA in the optimization experiments). The ELISA was further carried out as described for the direct ELISA.

- Antibody-capture ELISA
In the antibody-capture ELISA plates were coated with rabbit anti-human IgM (μ-chain, Dakopatts) in a concentration of 5 μg/ml PBS. Serum samples were added in the same distribution as for the direct ELISA and after washing, BACH-conjugated CCA (stock solution (0.3 mg/ml) 1/1000 diluted in PT mixed with peroxidase-conjugated streptavidin (Dakopatts) 1/4000 in PT was added. The final substrate step was performed as described above for the direct ELISA.

Statistics

S/R ratios of the positive sera were normally distributed (Kolmogorov-Smirnov), as well as ^{2}log-values of reciprocal IFA-titres. Therefore the Pearson product-moment correlation coefficient r was calculated to test for association between the different immunoassays. As a threshold for positive/negative discrimination the S/R ratio + 3 standard deviations (SD) of 50 sera from non-endemic control persons was used. Statistical analysis was performed using SPSS/PC+ (SPSS Inc., Chicago, Illinois, USA) on an IBM/XT compatible PC.

Results

Experiments were performed to determine reagent excess. Coating concentrations of McAb 54-5C10-A and CCA were determined using the positive serum pool 1/10 diluted and the peroxidase-conjugate 1/500 diluted (Fig. 1). Concentrations chosen were 10 μg/ml for 54-5C10-A and 2.5 μg/ml for CCA. For the antibody-capture ELISA it was determined that a $F(ab')_2$ rabbit-anti-human IgM coating concentration of 5 μg/ml was already optimal. Using checkerboard titration CCA-BACH stock solution 1/1000 mixed with peroxidase-conjugated streptavidin 1/4000 showed the highest performance.

The influence of post-coating with 0.5% BSA in PBS was tested both in the direct and indirect ELISA. In the former, McAb 54-5C10-A was coated at 5 μg/ml followed by AWA-TCA (50 μg/ml); in the direct ELISA AWA-TCA was coated at 50 μg/ml. Absorbances of the positive serum pool were found to decrease after post-coating, while absorbances of the negative serum pool or the buffer-control were not lowered (Fig. 2).

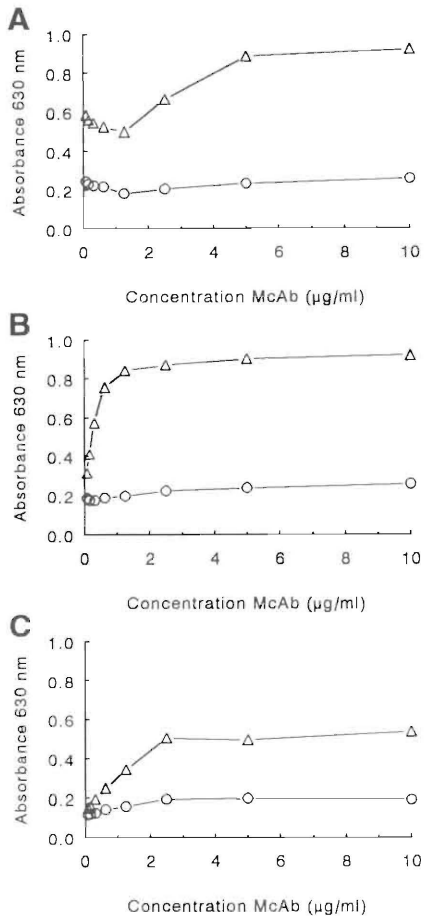

Figure 1. Titration of coating antibody 54-5C10-A in the indirect ELISA (A) **and CCA in the indirect** (B) **and direct** (C) **ELISA.** Serum antibodies, conjugate and substrate were in excess. Δ and o represent respectively positive and negative serum pool 1/10 diluted in PT.

To analyze the influence of buffer-additives, 0.3% BSA, 0.3% Tween-20, or 5% fetal calf serum was added to PBS and the direct and indirect ELISA were performed as described above. Only minor differences were observed, but 0.3% Tween-20 showed a slightly higher performance. On the basis of these results and together with practical considerations, PT was chosen as assay-buffer during serum and conjugate incubation steps.

Both in the direct and indirect ELISA excess antibody was reached at a 1/10 dilution of the positive control serum pool. Therefore, in the subsequent optimization experiments requiring antibody excess, at least a 1/10 dilution of

the (positive or negative) serum pool was used. Serum samples were tested in 1/200 dilution (0.5 μl in 100 μl) which was in the linear part of the serum dilution curve.

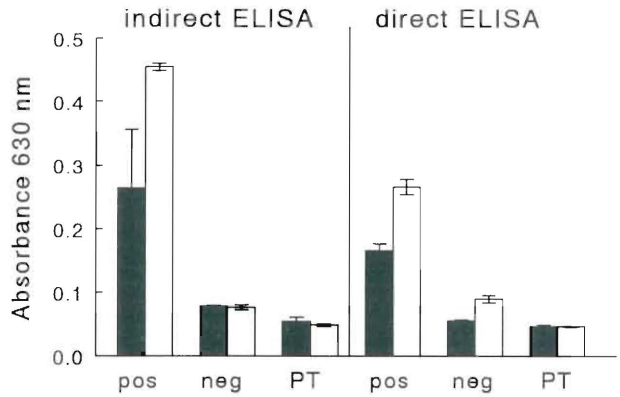

Figure 2. Influence of post-coating with 0.5% BSA in PBS. Absorbances were measured of triplicate samples of the positive serum pool diluted 1/100 in PT (pos), negative serum pool 1/100 in PT (neg) and just PT, in the indirect and direct ELISA, using AWA-TCA as antigen. Closed bars: with postcoating, open bars: without postcoating.

Peroxidase conjugated $F(ab')_2$ rabbit-anti-human IgM was titrated starting from 1/100 dilution with excess antigen and antibody (data not shown). A 1/500 dilution of the conjugate was chosen as optimal for both the direct and indirect ELISA. Substrate reaction was linear using incubation times not exceeding 10 min and absorbance values below 1.0. Outside these ranges S/R ratios were not calculated.

Anti-CCA antibodies of IgM isotype in human sera of 46 *Schistosoma mansoni* infected patients and 50 negative control sera were assayed utilizing the three different enzyme-linked immunoassays. The 46 positive sera were also (previously) tested in an immunofluorescence assay. To determine parallelity of serum dilution curves for all three assays, 3 sera were diluted in a $1/\sqrt{10}$ dilution series starting from 1/20 (Fig. 3).

In table 1 average S/R ratios and standard deviations for the negative control sera are displayed. The average S/R ratio + 3 SD was used to discriminate between positive and negative reaction. Intra-assay and inter-assay reproducibility was analyzed testing 5 repeats of a serum with S/R ratio close to one within the same plate and on 5 different plates (also different coating and conjugate solutions) (table 2). Combined variation due to the spectrophotometer, plates, and multichannel pipette at 0.6 absorbance units was 1%.

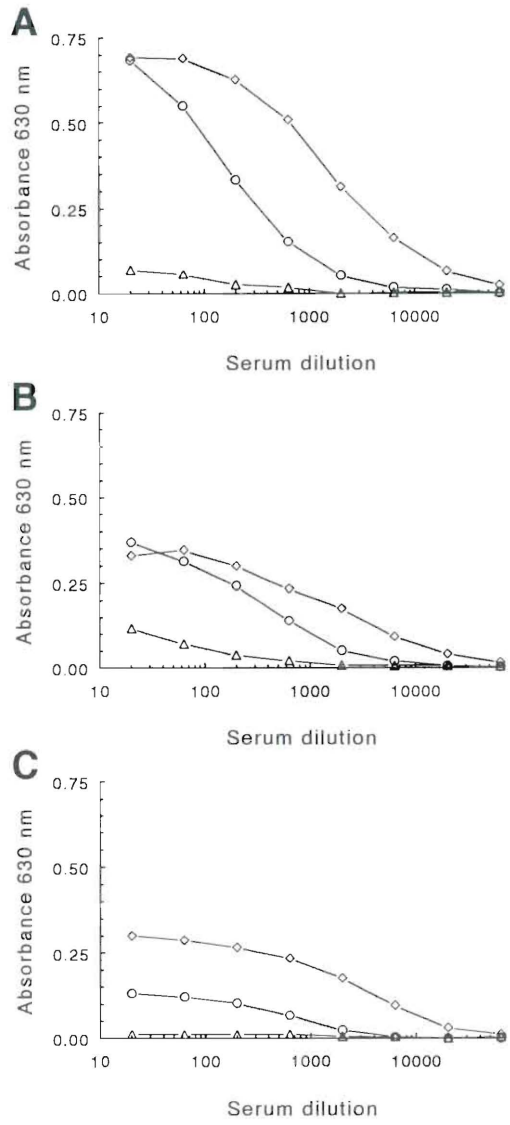

Figure 3. Serum dilution curves of 3 individual sera, including the positive control serum; (A) in indirect ELISA; (B) in direct ELISA; (C) in Ab-capture ELISA. Symbols represent: ◇ highly positive serum; o positive control serum; △ weakly positive serum. Absorbances are corrected for nonspecific binding.

Correlations between the assays were expressed using the Pearson product-moment correlation coefficient *r* (table 3). Some of the results are visualized in Fig. 4 using scattergrams.

Table 1. Values of average S/R ratio's and standard deviations for 50 negative control sera in indirect, direct and Ab-capture ELISA.

assay	average S/R ratio (sd)	average S/R ratio + 3 * sd
indirect ELISA	−0.002 (0.012)	0.033
direct ELISA	0.045 (0.024)	0.118
Ab-capture ELISA	0.005 (0.034)	0.107

Table 2. Intra-assay and inter-assay variability of S/R ratio's for a serum of moderate activity in indirect, direct and Ab-capture ELISA.

assay	intra-assay C.V.[a]	inter-assay C.V.
indirect ELISA	1.2	1.3
direct ELISA	2.4	3.0
Ab-capture ELISA	3.3	5.2

[a] C.V. = coefficient of variation

Table 3. Pearson product-moment correlation coefficient r for the association between the three ELISA's and for the IFA (expressed as ^{2}log(titre)), utilizing 46 positive sera.

assay	direct ELISA	Ab-capture ELISA	^{2}log(IFA-titre)
indirect ELISA	0.9734 [a]	0.8888	0.5738
direct ELISA		0.8508	0.5225
Ab-capture ELISA			0.5971

[a] r value (all p-values <0.0005)

Discussion

In contrast to previous findings in this laboratory [10] it was established that purified CCA bound very well to the ELISA-plates used in the present study, with an excess coating concentration already reached at 2.5 μg/ml. This discrepancy between the two studies might be explained (1) by the use of a different and highly activated type of ELISA-plates (Nunc Maxisorp), which

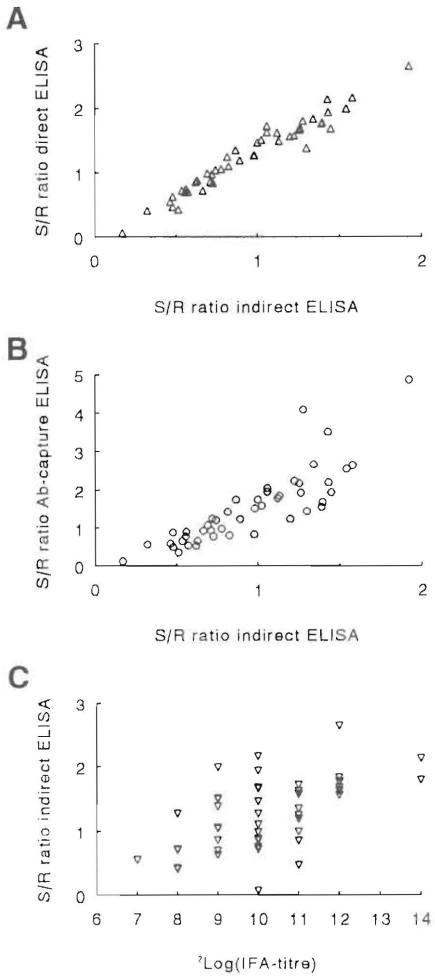

Figure 4. Correlation scattergrams of sample/reference ratios of human IgM anti-CCA antibodies obtained by three variant ELISA procedures and by the IFA. (A) direct ELISA vs. indirect ELISA; (B) Ab-capture ELISA vs. indirect ELISA; (C) indirect ELISA vs. ^{2}log(IFA-titre).

might show different surface characteristics towards binding of CCA, a highly glycosylated antigen, and (2) by competition of other antigens possibly present in the antigen preparation used in the past, which was only partly purified with respect to CCA.

Post-coating with 0.5% BSA resulted in equal (direct ELISA) or decreased (indirect ELISA) positive/negative ratios while a decrease in non-specific binding was not observed. The differences between various buffer-additives, in terms of positive/negative ratio and background level, was only marginal. However, even

PBS without any additive on unblocked plates did not give high background levels, a phenomenon which might be explained by the high purity of the antigen preparation and the specific epitopes on CCA (supposed to be identical to antigen M [3]). A CCA-specific McAb has been used for immunopurification of CCA and this McAb has *e.g.* been applied by De Water *et al.* [7] for studies of ultrastructural localization of CCA in the digestive tract of various life-cycle stages of *Schistosoma mansoni*. A more detailed description of this and other CCA-specific McAbs from our laboratory is in preparation (Deelder, *et al.*, manuscript in preparation). The specificity of the antibody-peroxidase conjugate is also of importance, as higher absorbances for negative sera or buffer controls were observed with conjugates from other suppliers.

For both the direct and the indirect assay the serum dilution curves were parallel at a 1/200 serum dilution (Fig. 3) as could be expected since except for antigen presentation both assays are the same. In these assays, competition might occur by CCA-specific antibodies from other isotypes, while in the Ab-capture ELISA IgM antibodies of other specificities might compete with the IgM anti-CCA antibodies. Taking into consideration maximal protein binding capacity per well together with average IgM serum concentrations (1-2 mg/ml, [26]), limiting amounts of IgM antibodies are only reached at dilutions exceeding about 1/1000. Therefore, at the dilution used (1/200) only the ratio of CCA-specific vs. nonspecific IgM antibodies could be determined in the Ab-capture ELISA. However, measuring the samples at dilutions greater than 1/1000 would decrease the sensitivity of the assay. Despite these differences in assay limitations correlations between the three ELISA's were high, which indicates a high dependency of the parameter measured. This suggests only a limited influence of the different assay conditions.

Background absorbances were generally lowest in the Ab-capture ELISA, followed by those in the direct ELISA. In the indirect ELISA some sera showed a considerable binding to the mouse McAb-coated plate. Boerman *et al.* [1] indeed described that a substantial portion of human sera contains anti-mouse antibodies (mostly of the IgM isotype). In our assays, the presence of these antibodies could result in non-specific binding only in the indirect ELISA.

If a cut-off level of average sample/reference ratio of negative control sera + 3 standard deviations was used, no false negatives were found in any of the three ELISA's. In both the direct and indirect assay one false positive was found (a different serum). It can be concluded therefore, that all three assays correctly discriminate between positives and negatives. However, limited inference can be made regarding assay sensitivity, since the positive sera were (previously) selected for showing positive reactivity in the IFA. Nevertheless, observations on assay performance are valid and reliable and the optimal assay and assay-conditions could be established.

The good correlation of the three ELISA's with the IFA confirms the previous observations that in the IFA predominantly CCA is recognized [14]. Regarding only the technical aspects of the assays, the ELISA is preferred above the IFA because of practical and economical considerations. Many samples can easily be screened and the results are expressed in a more quantitative form. However, from the results presented in this study it could not be concluded that for immunodiagnostic purposes the IFA can be replaced by the ELISA, because the sensitivity and specificity of the assays could not properly be compared with this group of patient sera.

Comparing only the ELISA's, the antibody-capture ELISA is the assay of choice for further studies in immunodiagnosis and epidemiology for the following reasons: 1) background absorbance is close to buffer-control, even with samples which show significant non-specific reaction in the other ELISA's; 2) false negatives or false positives were not found; 3) intra-and inter-assay variation is low; 4) in terms of labor and materials it is the most economic assay. The limitation that only the ratio of CCA-specific vs. non CCA-specific IgM antibodies is determined would argue against the Ab-capture assay. However, competition by CCA-specific antibodies of other than IgM isotypes is a constraint of both the direct or indirect ELISA. Principally speaking, this should be verified in every ELISA system which is used to specifically detect antibodies of different isotypes and specificities.

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