A Simple ELISA for Antigiardial Antibody Using the Membrane Fraction of the Parasite as Antigen*

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SUMMARY Most existing methods for detecting antigiardia lamblia antibody require the use of cultured *Giardia lamblia* trophozoites as antigen in immunofluorescent and enzyme-linked immunosorbent assays. However, the maintenance of trophozoite culture systems limits the large-scale use of these antibody detection systems. An antigen extracted from *Giardia lamblia* by the detergent Triton X-100, when used in an ELISA system, produces specific, objective, quantitative and reproducible results. It is likely that such a test system could be packaged in kit form for large-scale diagnostic and epidemiological application in all parts of the world.

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It is well known that infection with *Giardia lamblia* evokes an antibody response[1,2] which can be demonstrated both by indirect immunofluorescent assay[3] (IFA) and by enzyme-linked immunoadsorbent assay (ELISA).[4] The existing methods for the detection of antigiardial antibody, i.e. IFA and ELISA, have certain disadvantages and are only suitable for highly specialised laboratories since they require expensive equipment and facilities for the axenic cultivation of *Giardia lamblia.* Recently it has been shown[5] that the major surface antigen of *Giardia lamblia* (molecular weight 82,000) is Triton X-100-extractable. This study describes an ELISA for the detection of antigiardia antibody in which filter paper discs were coupled to a Triton X-100-extracted *Giardia lamblia* antigen and compared with an indirect IFA as described by Visvesvara *et al.*[3]

MATERIALS AND METHODS

Giardia antigen

Giardia antigen was prepared from an axenically maintained Portland strain of *Giardia lamblia* obtained from Dr. Louis S. Diamond, N.I.H., U.S.A. The organisms were cultured in a modified Diamond's TYI-S-33 medium supplemented with bile, vitamines and 10% bovine serum.[6] Actively growing 3-4-day-old *Giardia lamblia* organisms were dislodged from the walls of the culture tubes by immersing them in ice-water for five minutes and then centrifuging at 250 x G for 10 minutes. The harvested cells were then suspended in 3-5 ml of 50 mM phosphate buffered saline (PBS) pH 7.2, containing 1% Triton-X-100. The cells were homogenised using a Potter-Elvejhem homogeniser. The homogenates were then centrifuged at 9,000 x G in a Sorvall RC-3B refrigerated centrifuge using an SS34 rotor for 30 minutes. After centrifugation, the supernate containing the antigen was dialysed against 50 mM PBS at 4°C for 48 hours with repeated change of the PBS. On completion of the dialysis, protein was determined by the Lowry method[7] using bovine serum albumin as a standard. Filter paper disc conjugation of this antigen (1 mg/ml Lowry protein equivalent) was carried out with di-vinyl sulfone, as described earlier.[8]

Conjugation of horseradish peroxidase (Sigma type VI, Sigma Chemicals Co., U.S.A.) to rabbit antihuman gammaglobulin (GIBCO) was done according to the fluorodinitrobenzene method of Nakane and Kawaoi;[9] thereafter, it was purified by DEAE cellulose.[10] The optimum working dilution of the

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conjugate was found to be 1 in 1,000.

Detection of antigiardial antibody

Using the *Giardia* antigen-coupled filter paper disc in plastic tubes, ELISA was performed with human sera in serial dilutions from 1/50 to 1/3,200. A minimum dilution of 1/50 was determined by preliminary experiments in which it was noted that some of the serum samples, when used in a lower dilution (<1/50), would give an optical density (OD) value of 2 or above. The sera were diluted in diluent buffer (DB) (phosphate buffered saline containing 0.5% w/v ovalbumin). Diluted sera were distributed in 200-μl volumes (in duplicate) in plastic tubes each containing one antigen disc; they were incubated for three hours at 37°C. The discs were then washed five times with PBS containing 0.05% Tween-20. Finally 200 μl of 1/1,000 diluted peroxidase-conjugated antihuman gammaglobulin in DB were added to each tube. After overnight incubation at 4°C the discs were washed with PBS containing Tween-20. The enzyme activity was then determined by incubation at 37°C with 1 ml of 0.4% orthophenylenediamine (Sigma Chemical Co., U.S.A.) dissolved in 100 ml of 10 mM citrate-phosphate buffer (pH 5.0) contaning 500 μl of 30% hydrogen peroxide. After 30 minutes, the reaction was stopped by the addition of 2 ml of 0.5 N sulphuric acid. (The optimum conditions for incubation had been established by previous experiments.) The colour of the product was then measured at 492 nm in a spectrophotometer.

To determine the degree of nonspecific binding of enzyme-conjugated antibody and to find the significiant cut-off point in each batch of the experiment, several proteins (ovalbumin, bovine serum, bovine serum albumin, bovine gammaglobulin, purified human gammaglobulin from commercial sources having no detectable antigiardial antibody by IFA) were used in dilutions equivalent to 1/20 to 1/40 of human serum. In different sets of experiments, all the above proteins gave ODs within a range of 0.30 to 0.33 and correspondingly, by adding plus 0.02 OD, the significant label of the OD reading was 0.32 to 0.35. Therefore, in different experiments any OD value at or above the range of 0.32 to 0.35 was considered significant for the presence of antigiardial antibody.

Indirect immunofluorescence test

The indirect immunofluorescence was done by the method of Visvesvara *et al*[3] using human sera in serial dilution from 1/5 to 1/2,000. The intensity of fluorescence was graded on a scale of 0 to ++++. This test was considered to be positive for antibody only when 60 per cent of the organisms fluoresced at a value of 1+ or more. The reciprocal of the highest dilution of serum that reacted at 1+ was defined as the titre of the serum. It was noted that all sera up to a dilution of 1/5 invariably gave a high background fluorescence; hence, a minimum serum dilution of 1/10 was used.

Subjects

Sera were obtained from 60 consecutive subjects attending the gastrointestinal clinic of this Centre irrespective of their symptoms. Three stool examinations[11] were carried out on each of the subjects to determine whether parasites were present; the saline-iodine wet-mount and formol ether concentration techniques were used for this purpose. All sera were subjected to antigiardial antibody assay simultaneously by the ELISA and IFA techniques. All test results were read in a blinded fashion.

RESULTS

The specificity of antigiardial antibody was evaluated by absorbing three antibody-positive sera with an equivalent number of *Giardia lamblia* and *Entamoeba histolytica* using the method described by Smith *et al.*[4] It may be noted (Fig. 1) that there was a three- to four-fold fall in the titre of the antigiardial antibody when absorbed with *Giardia lamblia,* but the change was negligible with *Entamoeba histolytica,* suggesting that the antibody measured in these sera by ELISA was specific for *Giardia lamblia.*

Reproducibility

The reproducibility of the ELISA was evaluated by comparing the OD of 21 serum samples tested at different periods of time over three-month period. The coefficient of variation ranged from 5 per cent to 10 per cent for the different dilutions of sera.

Stool findings

Of the 60 subjects whose stool was examined, only 35 (59%) were positive for *Giardia lamblia.* It may be noted that other parasites, viz. *Ascaris lumbricoides, Entamoeba histolytica* and *Ancylostoma duodenale* were also present in the majority of the stool samples.

Antibody response

Thirty-one of the 35 subjects whose stool was positive for *Giardia lamblia* gave a positive titre both by ELISA and IFA. However, 22 subjects whose stool was negative for *Giardia lamblia* also gave a positive antibody titre by ELISA. In contrast, only 16 of the subjects whose stool was negative had a positive antibody response by IFA. It should be noted that four of the subjects whose stool was positive did not have any antibody response either by IFA or ELISA. Thus, irrespective of the stool findings, ELISA appeared to be more sensitive in detecting smaller amounts of antibody than IFA.

Figure 2 shows the OD readings for various serum dilutions in 60 subjects separately for those who were stool-positive and -negative.

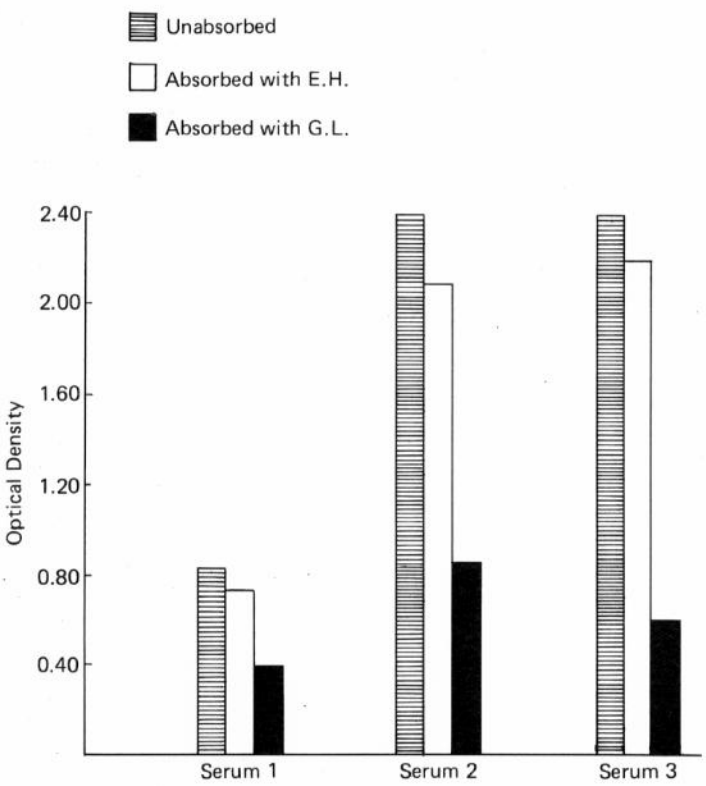

Fig. 1 Optical densities of three antigiardial antibody-positive sera diluted to 1/50 without absorption (unabsorbed) and after absorption with either *E. histolytica* (E.H.) or *Giardia lamblia* (G.L.).

At the 1/50 serum dilution, 89 per cent and 88 per cent of the stool-positive and stool-negative subjects respectively were positive for antigiardial antibody. However, at higher dilutions, the percentage of antibody positivity varied between 51 per cent and 14 per cent for serum dilutions in the range 1/100 to 1/3,000, respectively. In contrast, stool-negative subjects had a lower percentage of antibody positivity (30% to 4%) for the same dilution range of serum samples.

Figure 3 shows a correlation plot between IFA and ELISA titres in 42 subjects who had positive antibody response by the above tests. There was a close correlation between these two methods.

DISCUSSION

Serum antibody to *Giardia lamblia* could serve as useful clinical or epidemiological indicators of infection or disease. However, the major constraint to the widespread application of presently available

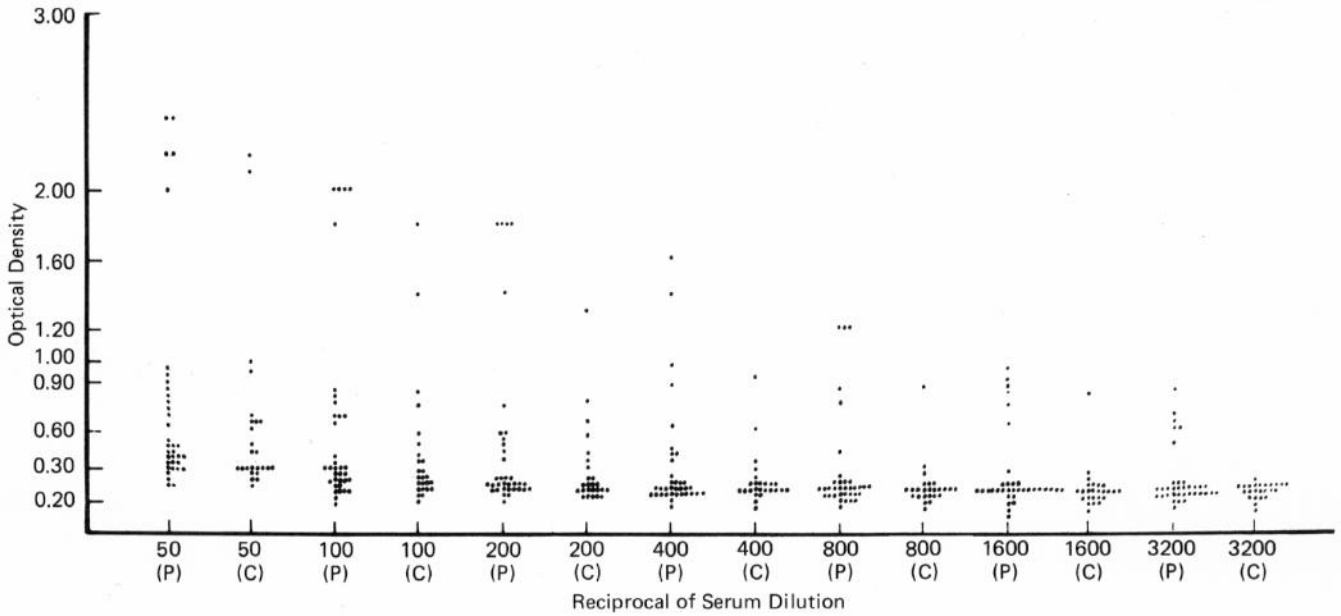

Fig. 2 Distribution of serum optical density by ELISA of 35 stool-positive subjects (P) and 25 stool-negative subjects (C) at dilutions 1/50 to 1/3,200. (The findings represent the readings of one batch in the experiment).

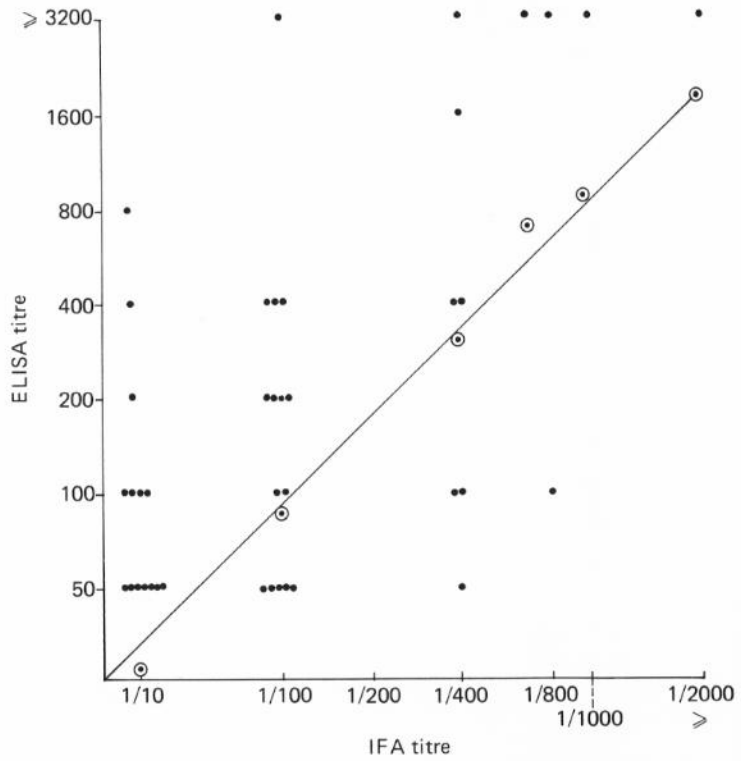

Fig. 3 Antigiardia lamblia antibody titres by indirect immunofluorescent (IFA) and enzyme-linked immunosorbent assays (ELISA) in 42 sera.

antigiardia antibody detection tests, i.e. IFA[3] and ELISA,[4] is the limited availability of facilities for the axenic cultivation of *Giardia lamblia;* both of these tests require live trophozoites of *Giardia lamblia.* It is well known that such facilities are not available even in many of the well-equipped laboratories of developing countries. It would also be useful if the ELISA could be packaged in a kit form for use by laboratories.

In this study we have tried to develop a simple ELISA using a detergent-extractable membrane antigen of *Giardia lamblia.* Einfeld and Stibbs[5] have recently shown that the major surface antigen (M.W. 82,000) of *Giardia lamblia* is Triton X-100-extractable; it is expected that a test system using this antigen will be more sensitive than currently available ones. The ELISA described herein is specific, quantitative, objective and reproducible; also it could be packaged in kit form. Furthermore, it also appears to be more sensitive than IFA in detecting smaller amounts of antigiardial antibody.

It has been shown that a very high percentage of subjects from our endemic area have antibody responses to *Giardia lamblia* irrespective of the stool findings. Since there is poor concordance between positive stool findings and positive serum antibody titres, it would appear that in areas with high rates of infection, a single serum antibody titre cannot be used as a marker for *Giardia lamblia* infection. However, such an antibody detection test may be a useful epidemiological tool for identifying a hyperendemic focus and for determining the age of initial infection.

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