Original Article

Evaluation of Fast-ELISA versus Standard-ELISA to Diagnose Human Fasciolosis

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Abstract

Background: The present study was conducted to evaluate and compare a fast-ELISA (F-ELISA) method versus standard-ELISA (S-ELISA) to diagnose human fasciolosis.

Methods: Serum samples were obtained from 35 individuals infected with fasciolosis, 27 infected with other parasitoses and 22 from healthy people. The samples were examined with S-ELISA (30-minute incubation periods) and F-ELISA (10-minute incubation periods) for total antibody response against fasciolosis.

Results: The optimum conditions for S-ELISA and F-ELISA were respectively as follows: antigen 10 and 5 µg/mL, sera 1:500 dilution for both, peroxidase-conjugated goat anti-human IgG diluted 1:7000 and 1:10000. Data were analyzed using SPSS version 11.0. Cut-off vale for S-ELISA and F-ELISA was determined as 0.56 and 0.42, respectively. The sensitivity, specificity; positive, and negative predictive values were detected as 97.2%, 100%, 94.6%, and 95.6% for both tests.

Conclusion: Cut-off values, sensitivity, specificity, and other important parameters of the two evaluated tests determined that the F-ELISA method could be used with no detectable difference.

Keywords: diagnosis, Enzyme-Linked Immunosorbent Assay, *Fasciola,* validity

Introduction

Fasciolosis, caused by the liver fluke species of the genus *Fasciola*, is a cosmopolitan parasitic disease.[1] The disease is transmitted to human via ingesting metacercaria from contaminated plants and after 3 – 4 months, the parasite is lodged in the biliary ducts of the liver. Afterwards, the final host releases the parasites' eggs through the feces.[2]

Allegedly, locating parasite eggs through stool exam sounds functional as well as effective; moreover, due to its simplicity, unfortunately many routine laboratories use this method. Researchers' findings and the way of excreting eggs by the parasite have shown that this method lacks sufficient sensitivity and oftentimes results in misleading the physician.[3,4] Appraisal of the literature demonstrates that serological and immunological diagnostic methods are at the forefront of investigating fasciolosis. Among all utilized tests to diagnose fasciolosis, ELISA in different forms and with the use of various antigens occupies the top of the scale.[4–8] Studies have reported the sensitivity and specificity of the test as 92 – 100% and 84 – 100%, respectively.

However, considering all factors involving in conducting an ELISA test, such as time, energy, and human power, the necessity for saving time, and reaching a conclusive diagnosis as soon as possible, is of high importance.[9,10] Rapid diagnostic tests have already been utilized on other parasitoses, such as hydatidosis[11–13] of which the results attest to their reliability and functionality.

The present study was conducted based on the aforementioned data and the necessity to establish a fast-ELISA (F-ELISA) method and compare it with the standard-ELISA (S-ELISA) for the diagnosis of human fasciolosis.

Materials and Methods

Antigen preparation

Adult *Fasciola hepatica* worms were obtained from infected sheep livers collected from local abattoirs in Tehran, Iran and were washed six times with PBS (pH 7.2). The parasites were specified according to previous experiences of our laboratory.[14] Somatic antigen was prepared by homogenizing adult worms in 0.045 M PBS (pH 7.2) using an electrical homogenizator (Edmund Buhler Co., model Homo 4/A mit uhr) followed by sonication (Tommy Seiko model UP-200P, Tokyo, Japan) followed by centrifugation at 15000 g at 4°C for 30 minutes (min). The superna-

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tant was filtered and stored at -20°C until further use. The protein content of the antigen was detected according to the Bradford method.[15]

Clinical sera

Blood samples were collected from individuals infected with *Fasciola* spp. (n=35) from Gilan Province, northern Iran, diagnosed based on stool examination and the ELISA test. Informed consent was taken from each patient. Gilan Province has been one of the endemic regions in terms of fasciolosis and thus far, two large outbreaks have been reported.[16–18] Serum samples of patients infected with hydatidosis (n=11), toxoplasmosis (n=3), trichostrongylosis (n=3), and strongyloidosis (n=10) were obtained from the Tehran School of Public Health Serum Blood Bank. The aforementioned parasitic infections were diagnosed based on specific assays such as stool exam, ELISA, and IFA as well as surgical confirmation. Control serum samples were obtained from 22 normal healthy subjects. The human Ethics Committee at the School of Public Health, Tehran University of Medical Sciences approved the study.

Standard ELISA

The immunodiagnostic assay was performed as previously described,[19] with some modifications. To reach optimum conditions, *Fasciola* homogenate antigen with 2.5, 5, 10, and 20 µg/mL concentrations, sera with 1:250 as well as 1:500 dilutions, and peroxidase-conjugated goat anti-human IgG (diluted 1:3000 – 1:10000) were examined in a check board titration process. Eventually the optimum conditions for S-ELISA were obtained as follows: antigen 10 µg/mL, sera 1:500 dilution, and peroxidase-conjugated goat anti-human IgG diluted to 1:7000. Afterwards, 100 µL of antigen was dispensed into the wells of microtiter plates (Nuclon, Kamstrup, Roskilde, Denmark) and incubated overnight at 37°C. Excess binding sites were blocked with 200 µL of bovine serum albumin (2% diluted in PBS/0.1% Tween 20) and incubated for 30 min at 37°C. After the wells were washed three times with PBS/Tween 20, 100 µL of a serum sample was added to each well and incubated for 30 min at 37°C. Following another washing step, 100 µL of peroxidase-conjugated goat anti-human IgG was added to each well and the plates incubated for a further 30 min at 37°C. Following a final washing step, 100 µL of O-phenylenediamine dihydrochloride (OPD) substrate (all from Sigma Chemical Co., Poole, Dorset, United Kingdom) was added to each well and the reaction stopped after 5 min by adding 50 µL of 12.5% H_2SO_4. The optical density (OD) of the samples was measured at 492 nm using a Titerteck (Helsinki, Finland) multiscan ELISA plate reader. All assays were tested in triplicate and repeated twice.

Fast ELISA

The process was conducted according to aforementioned method for S-ELISA but the optimum conditions for F-ELISA were obtained as follows: antigen 5 µg/mL, sera 1:500 dilution, and peroxidase-conjugated goat anti-human IgG diluted 1:10000. All the incubation periods at subsequent steps were reduced from 30 min to 10 min each.

Statistical analysis

The cut-off value was calculated as means plus 3.0 standard deviation OD value of the healthy group sera. Accordingly, higher OD values were considered as positive and vice versa. The sensitivity, specificity, positive and negative predictive values were calculated using the method of Galen.[20] Statistical analysis was carried out using SPSS for Windows, version 11.

Results

Serum samples for this experimental study were obtained from 35 individuals infected with fasciolosis, 27 infected with parasitoses other than fasciolosis and 22 from healthy people as the control group. Samples were examined with S-ELISA (30-min incubation periods) and F-ELISA (10-min incubation periods) for total antibody response against fasciolosis. Cut-off values for S-ELISA and F-ELISA were determined as 0.56 and 0.42, respectively. The cut-off was calculated at 3.0 standard deviations from the mean of the seronegative population, which cuts off 99.0% of the seronegative peak below the cut-off point. According to Figure 1 which shows the analysis of sera from patients infected with fasciolosis and other parasitic infections as well as the control group by S-ELISA and F-ELISA, only one case of false-negative and two cases of false-positive, including one case of hydatidosis and another for strongyloidosis, were detected. This finding was the same for both tests. Hence, the sensitivity, specificity and positive and negative predictive values were detected as 97.2%, 100%, 94.6%, and 95.6% for both tests, respectively.

Figure 2 shows the absorbance readings for S-ELISA and F-ELISA as plotted against each other on scattergram. A compact cluster of negative results with low absorbance readings and another cluster of positive cases with high absorbance readings are shown. Accordingly, 34 out of 35 patients infected with fasciolosis were serpositive by two tests (indicated by open circles), while 49 non-fasciolosis and the control group (indicated by closed circles) were seronegative, with the exception of two cases as previously mentioned.

Absorbance readings for both tests also underwent a boxplot graph (Figure 3). Boxes belong to serpositive absorbance readings for F-ELISA showed smaller measures than S-ELISA.

There was no significant difference between the different non-fasciolosis groups in the ELISA readings recorded; instead, the absorbance readings from all samples from the

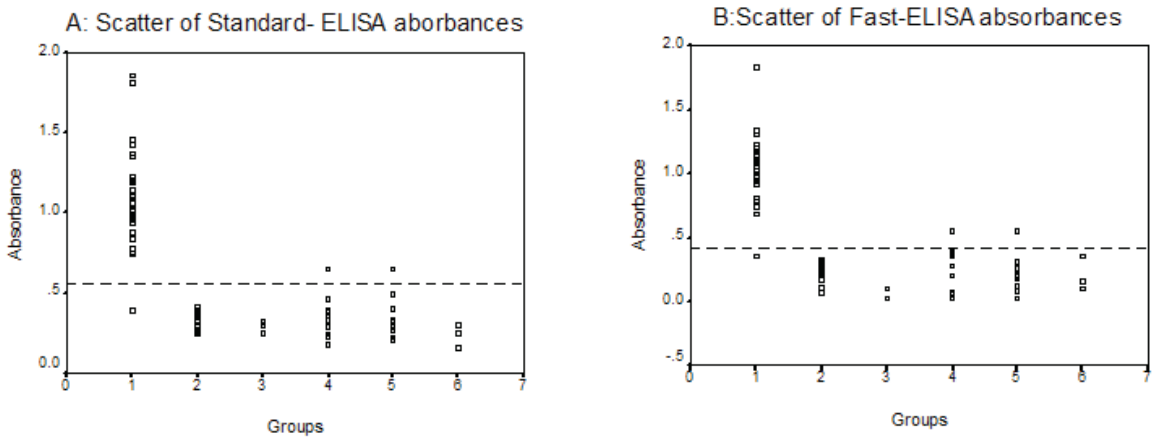

Figure 1. Analysis of sera from patients infected with fasciolosis by standard IgG-ELISA (A) and fast-ELISA (B). Serum samples obtained from patients with fasciolosis (35, lane 1), control human sera (22, lane 2), trichostrongylosis (3, lane 3), hydatidosis (11, lane 4), strongyloidosis (10, lane 5),
and toxoplasmosis (3, lane 6)

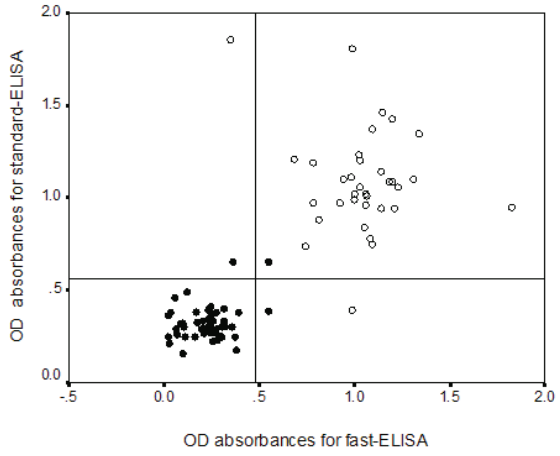

Figure 2. Scatter graph of the combined data for 35 *Fasciola*-infected and 49 non-*Fasciola* infected as well as control groups obtained by standard-ELISA and fast-ELISA. The K-means cluster analysis using the combined data separated the population into seronegative (indicated by closed circles) and serpositive (indicated by open circles) subpopulations. The vertical and horizontal lines in the figure indicate the calculated cut-off points for each tests

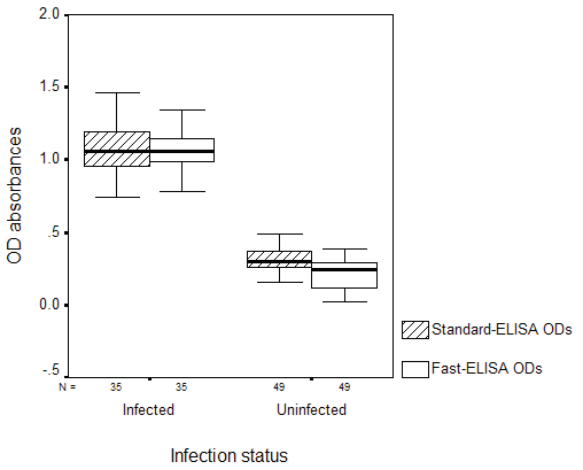

Figure 3. Boxplots of standard-ELISA and fast-ELISA absorbances obtained from *Fasciola*-infected and non-*Fasciola* infected as well as control groups

Fasciola serpositive individuals were significantly higher than those obtained from patients that were seronegative or were infected with other parasites ($P<0.001$)

Discussion

Many factors impose the idea of evaluating and establishing serological methods for diagnosing of fasciolosis instead of parasitological tests. These factors mostly get their source from parasite's life cycle. Parasitological diagnosis suffers from the weak points as follows: releasing the parasites' eggs is started 3 – 4 months post infection, which accordingly results in the unsuitability of a stool exam during the acute phase, intermittent egg output dynamics, very low or even absence of egg shedding in cases of only one or a few adult flukes, chronic and ectopic infections, transit infection due to eating of *Fasciola* infected livers, or flukes unable to attain maturity in human subjects in non-human endemic areas.[1, 21,22]

Researchers have focused mostly on improving the method of ELISA due to its simplicity and functionality, and a broad spectrum of antigens have been used so far.[3,4,19] Iran is one of the endemic countries for human fasciolosis,[1,16,17] thus evaluating and establishing a screening test for

the diagnosis of fasciolosis is of great importance in Iran as well as all endemic countries. Saving time and maintenance of the validity of the test are undoubtedly imperative criteria. Accordingly, the idea of conducting F-ELISA (10-min incubation periods) versus S-ELISA (30-min incubation periods) was challenged in this study and has shown that the F-ELISA with minor modifications can be used instead of the latter test. Our team has succeeded to evaluate and conduct the F-ELISA test for the diagnosis of hydatidosis, which is being implemented with success.[11] It is worth mentioning that a commercial F-ELISA test has been evaluated and implemented[9,10] with more or less acceptable outcomes, but regarding the expenses and difficulties of providing such kits, many endemic countries for fasciolosis prefer to take advantage of a cheaper and reliable test with the same qualifications.

In conclusion, the obtained results testify that F-ELISA can be used with some minor modifications versus S-ELISA. Cut-off values, sensitivity, specificity, and other important parameters of the two evaluated tests determine that both methods can be used with no sensed difference.

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