Immunodetection of *Fasciola gigantica* Circulating Antigen in Sera of Infected Individuals for Laboratory Diagnosis of Human Fascioliasis

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Running title: A 27-kDa *F. gigantica* antigen in human serum

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ABSTRACT

Currently, the laboratory diagnosis of human fascioliasis is based on the parasitological examination of parasite eggs in stool specimens and serological detection of specific antibodies in serum samples which are often unreliable diagnostic approaches. Ideally, a sensitive and specific diagnostic test for Fasciola infection should be based on the detection of circulating Fasciola antigen which implies active infection. Here, a 27-kDa molecular weight antigen was identified in *Fasciola gigantica* adult worm antigen preparation, excretory-secretory products and in sera from *F. gigantica* infected individuals, and was not detected in antigenic extracts of other parasites and sera from non-infected individuals. The target antigen was isolated and partially characterized as protein. Immunoperoxidase staining located the target epitope within tegument and gut of *F. gigantica* adult worm. The performance characteristics of a newly developed ELISA based on *F. gigantica* circulating antigen detection in serum (FgCA-27 ELISA) were investigated using sera of parasitologically diagnosed 120 *F. gigantica* infected individuals and 80 non-infected individuals. The area under ROC curve (AUC) for ELISA was significantly high (AUC = 0.961, p < 0.0001) for discriminating Fasciola infected and non-infected individuals. The developed assay showed high degrees of sensitivity, specificity and efficiency (> 93 %) and a significant correlation (r = 0.715, p < 0.0001) was shown between antigen level and parasite egg count. In conclusion, a 27-kDa Fasciola antigen was identified in sera of *F. gigantica* infected individuals. A highly sensitive and specific Fasciola antigen detection assay, FgCA-27 ELISA was developed for laboratory diagnosis of human fascioliasis.
INTRODUCTION

Fascioliasis is a worldwide zoonotic infection caused by liver flukes of the genus Fasciola, of which *F. hepatica* and a larger species, *F. gigantica* are the most common representatives. These two food-borne trematodes usually infect domestic ruminants and causing important economic losses to sheep, goats, and cattle, in commercial herds (1). Today, fascioliasis is considered an important human disease and several areas e.g. tropical regions of Africa and Asia have been described as endemic for the disease in humans with estimates of 2.4 to 17 million people infected and 91.1 million are at risk worldwide (2, 3). Egypt, especially Nile Delta is a hot spot and Fasciola infection appeared to be endemic with estimated prevalence varying between 2 to 17% (4-6). Furthermore, fascioliasis has been recognized as an emerging infection in international travelers and migrants, causing significant problems in diagnosis and therapy (7). Owing to its high endemicity in some developing countries and emerging nature, the WHO classified the disease as a neglected parasitic infection and decided to launch a worldwide initiative against this infectious disease (2).

Diagnosis of human fascioliasis is based on clinical findings and laboratory tests. The most reliable means is the finding of parasite eggs in stool of infected individual (8). However, parasitological diagnosis of human fascioliasis is time-consuming and usually lack sensitivity and reproducibility, and often unreliable because parasite eggs are not found during the prepatent period, approximately 3-4 months after infection (9). Moreover, once Fasciola worms have matured diagnosis may still remain difficult since eggs are frequently excreted at irregular intervals and in most cases repeated stool examinations are required to accurate diagnose (10). Anti-Fasciola antibodies can be detected as early as 2 weeks post infection and can thus facilitate early diagnosis and chemotherapeutic intervention (11). Over the past years, several native and recombinant antigens were identified for the detection of serum specific anti-Fasciola antibodies (12-
Many antibody-based immunological techniques have been developed for diagnosis of Fasciola infection in a trial to replace the classical parasitological techniques. However, antibody tests do not distinguish between past, resolving, or current infections, and do not correlate with infection intensity.

In that sense, the direct detection of parasite antigens in stool (coproantigens) or serum (circulating antigens) of Fasciola infected animals or humans has been reported as a new alternative approach with greater diagnostic accuracy. In our previous study, we identified a 26- to 28-kDa circulating antigen in sera of cattle infected with *F. gigantica* using specific rabbit IgG anti-serum. In the present study, we aimed to identify the target *F. gigantica* circulating antigen in sera of infected individuals and described a novel ELISA based on circulating antigen detection in serum for accurate laboratory diagnosis of human *F. gigantica* infection.

**MATERIALS AND METHODS**

**Stool and blood samples from study subjects.** A total of 200 individuals (101 males and 99 females; age range 6 to 65 yrs; mean 24 yrs) admitted at Tropical Medicine and Parasitology Department, Mansoura University Hospitals, Mansoura, Egypt were included in the present study. Stool specimens were collected from each individual and examined at the day of collection using simple stool sedimentation by centrifugation. The Kato-Katz thick smear technique was used for counting *F. gigantica* eggs in 3 to 5 slides, each containing 41.7 mg stool and the eggs count was expressed as eggs per gram of feces (epg). A total of 120 individuals showing *F. gigantica* eggs in their feces, 57 individuals showing other parasitic infections including 38 individuals infected with *Entamoeba histolytica*, 7 infected with *Schistosoma mansoni*, 5 infected with *Ascaris lumbricoides*, 4 infected with *Hymenolepis nana*, and 3 infected with *Giardia lamblia*, and 23 individuals were diagnosed parasite free-healthy individuals. According to Kato-Katz technique of individuals infected with *F. gigantica*, the intensity of infection was...
classed as light infection (<100 epg) in 76 individuals, moderate infection (100-299 epg)
in 33 individuals and severe infection (≥ 300 epg) in 11 individuals. Blood samples were
collected at the day of stool analysis. Sera were separated from blood, aliquoted and
stored at – 20 °C until used. The Ethical Committee of the Mansoura University
Hospitals, Mansoura, Egypt approved the present study. Informed consents were
obtained from all participants and all individuals were fully informed concerning the
diagnostic procedures involved and nature of the disease.

**F. gigantica and other parasites antigenic preparations.** *F. gigantica* soluble worm
antigen preparation (FWAP) was prepared as described by Attallah et al. (22). The crude
excretory/secretory (E/S) products of adult *F. gigantica* were prepared according to
Santiago de Weil and Hillyer (27). Adult *A. lumbricoides*, from the stools of infected
individuals, were processed in exactly the same way as the *F. gigantica* to prepare *A.
lumbricoides* soluble worm antigenic preparation (AWAP), while *S. mansoni* soluble
worm antigenic preparation (SWAP) was prepared as described by Da Silva and Ferri
(28). The protein content of a sample of each antigenic preparation was determined (29)
before the rest of the preparation was split into aliquots and stored at - 20 °C until used.

**SDS-PAGE and Western blot.** Various samples were subjected to analytical SDS-
PAGE, at 50 µg/lane using vertical slabs of 12% or 16% polyacrylamide (30). Prestained
molecular-weight standards (Sigma) were run on parallel. Samples separated on SDS-
PAGE were electro-transferred onto nitrocellulose (NC) membrane (0.45-µm pore size)
in a protein transfer unit (31). The NC membrane was blocked, using 5% (w/v) non-fat
dry milk dissolved in 0.05 M Tris-buffered saline (TBS) containing 200 mM NaCl (pH
7.4), rinsed in TBS, and incubated with anti-27-kDa Fasciola antigen IgG antibody, see
later diluted (1:150) in the blocking buffer, with constant shaking. The blots were
washed three times (30 minutes each) in TBS, followed by incubation for 2 hrs with goat
anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) diluted 1:350 in TBS. After
washing three more times with TBS (15 minutes each), the blot were soaked in substrate. The color reaction was observed within 10 min and the reaction was then stopped by dipping the blots in distilled water. To assure that the 27-kDa protein purified from serum is a parasite molecule, the developed IgG rabbit anti-serum (diluted 1:150 in blocking buffer) is absorbed with a proper concentration of the purified protein (200-ng/mL) for 2 hrs at 37 °C. Then, the reactivity of rabbit anti-serum was investigated against blots of FWAP and E/S products as described before.

**Gel Electro-elution and purity of the 27-kDa Fasciola antigen.** In preparative slab gel electrophoresis, the SDS-PAGE running condition was adapted to reduce smear of proteins and to enable a considerable long migration distance between bands in the 27-kDa region according to the prestained molecular weight marker (22). In each run, 250-μl of diluted human serum sample of infected individual per preparative gel was electrophoresed and about 35 runs were completed to obtain one mg of the 27-kDa Fasciola antigen. The protein content of a sample of electroeluted antigen was determined before remainder was stored at –20 °C.

**Purity of the electroeluted Fasciola antigen.** The purity of the electroeluted 27-kDa Fasciola antigen was assessed using gel silver staining (32), and capillary zone electrophoresis (CZE) in a modification of the method described by Gordon et al. (33) using an auto sampler (model 1-LIFT; Prince Technologies, Emmen, The Netherlands), a variable ultra-violet-visible detector (Lambda 1010; Metrhom, Herisau, Switzerland) and WinPrince software (version 5; Prince Technologies). The signals were analyzed using Dax software (version 5; Prince Technologies).

**Production of specific anti-27-kDa Fasciola antigen IgG antibody.** Specific IgG antibodies were produced in 4 New Zealand white rabbits immunized subcutaneous in three different inoculation sites with the purified 27-kDa Fasciola antigen according to Attallah et al. (22). In brief, equal volumes (500 µl) of the antigen (500-µg/ml) and
complete Freund’s adjuvant (CFA) or incomplete Freund’s adjuvant (IFA) were homogenized together using two Luer-lock syringes connected to a 3-way stainless steel valve. Each rabbit was immunized subcutaneously three times, once with antigen in CFA (on day 0) and twice with antigen in IFA (on days 15 and 28) before being scarified on day 32. Blood samples were collected from all rabbits at 0, 28 and 32 days of immunization. Sera were separated and its immunoreactivity was tested using ELISA (see below) against Fasciola antigenic preparations, and the purified 27-kDa antigen. The specificity of the developed sera was tested using ELISA against adult *S. mansoni* and *A. lumbricoides* antigenic preparations. Sera of non immunized rabbits were tested in parallel. The highly reactive rabbit sera were then aliquoted and stored at –20 °C until used.

**Biochemical treatments.** To determine some of the target Fasciola antigen biochemical characteristics, samples of the purified 27-kDa antigen were treated with protease and sodium meta-periodate being tested in ELISA, to see if these treatments affected the reactive epitope. A periodate oxidation of purified antigen (25 µg/ml) was carried out overnight with 20 mM sodium meta-periodate at RT and the reaction was then inhibited by adding an equal volume of 130 mM glycerol. In the test with protease, purified antigen was incubated at 37 °C with pepsin (1 mg/ml; Sigma) for 5, 10, 15, or 20 min. Serum of Fasciola infected patient and healthy individual were tested in parallel, as positive and negative controls; respectively.

**Immunohistochemical detection of native Fasciola antigen using indirect immunoperoxidase.** A paraffin section (4-µm) of adult worm tissues was deparaffinized and rehydrated through descending grades of alcohols in water. The slides were washed in tris buffered saline (TBS) 3 changes, 5 min each. Excess liquid from around the section was wiped and the slides were layed flat. 4-6 drops of 3 % hydrogen peroxide (H₂O₂) were applied for each slide and incubated for 5 minutes in dark chamber. Normal
goat serum was applied with a dilution 1:5 with 3 % bovine serum albumin (BSA) in TBS for 60 min. Then, the developed rabbit anti-27-kDa Fasciola antigen IgG antibody diluted 1:100 in TBS was applied and incubated for 30 min. After washing, horseradish peroxidase conjugated goat antibody to rabbit immunoglobulins diluted 1: 250 in 1% BSA/TBS was applied and incubated for 60 min. The slides were washed and then tissue reacted with 3-amino-9-ethyl carbazol/H$_2$O$_2$ solution for 30 mins in the dark. The reaction was stopped with distilled water. The sections were counter stained with Mayer’s hematoxyline for 2 min and washed in running tap water for 3 min to the desired intensity of blue color. The slides were dried out and coverslipped using glycerol. All washings and incubations were performed at room temperature.

**Development of F. gigantica circulating antigen detection assay (FgCA-27 ELISA).**

Serial dilutions (1:25 to 1:3200) of selected serum samples from patients with no (n = 4), low (n = 4, < 100 epg), moderate (n = 4, 100-299 epg) and high (n = 4, ≥ 300 epg) parasite egg count were coated in duplicate in carbonate/bicarbonate buffer, pH 9.6 were investigated to get the proper saturation of the polystyrene solid phase with the target antigen. Checkerboard titrations of the specific anti-27-kDa IgG antibody as well as alkaline phosphatase conjugate were also performed. After optimization of assay conditions, polystyrene flat-bottom micro-titer plates (Costar, Acton, MA) were coated with 1:200-diluted human serum samples or serial concentrations (1024 to 0-ng/mL) of the purified 27-kDa Fasciola antigen diluted in normal human serum. In parallel and at 2.5-µg/mL, FWAP and E/S products were tested as positive controls and SWAP and EWAP were tested as negative controls for the reactivity of the developed rabbit antibody. After blocking with 0.3 % BSA, 50 µL/well of 1:400-dilution, in PBS with 0.05% (v/v) Tween 20 (PBS-T20), of rabbit anti-27-kDa antigen IgG antibody was added and the plates were incubated at 37 ºC for 2 hr. After washing, 50 µl/well of anti-rabbit IgG alkaline phosphatase conjugate (The Binding Site, Birmingham, UK) diluted...
1:600 in 0.2% (wt/v) BSA in PBS-T20 was added and the plate was incubated at 37 °C for 1 hr. After washing, the substrate (p-nitrophenyl phosphate in 0.1 M glycine buffer, pH 10.4) was added and the plates incubated for 30 min at 37 °C. Optical densities (OD) were read at 405 nm using a micro-plate auto-reader (Σ960; Metertech Inc., Taipei, Taiwan). The cutoff level of FgCA-27 ELISA above or below which the tested sample is considered positive or negative was calculated as the mean OD for FgCA-27 ELISA (range, 0.093 to 0.227) of a control group of 32 serum samples from healthy individuals with no parasitic infection ± 3 standard deviation (i.e. cut-off level = 0.131 ± [3 × 0.053] = 0.29). The mean OD value of a group of 16 sera from parasitologically diagnosed F. gigantica-infected individuals and investigated in parallel was 0.755 (range, 0.394 to 1.985). The mean OD of another group of 16 serum samples of individuals parasitologically diagnosed Fasciola non-infected but infected with other parasitic infections including schistosomiasis mansoni (n=3), ascariasis (n=3), hymenolepiasis (n=3), giardiasis (n=3), and entamoebiasis (n=4) and investigated in parallel was 0.181 (range, 0.105 to 0.261). To establish the dose response curve, serial concentrations (1 to 1024-ng/mL) of the target 27-kDa antigen were mixed with diluted serum samples showing FgCA-ELISA negative results for antigen detection and with no detected antibody levels (n = 3, OD < 0.25; cut-off level of an in-house ELISA based on specific anti-27-kDa IgG antibodies in human serum) and tested in duplicates as well as selected serum samples with low (n = 3, OD = 0.25-0.5), moderate (n = 3, OD = 0.5-1.5) and high (n = 3, OD > 1.5) levels of specific anti-27-kDa IgG antibodies.

**Statistical analyses.** Descriptive results were expressed as mean ± SD and range or number of patients with a condition. Differences in continuous variables were assessed using Student's t-test or analysis of variance (ANOVA). All tests were two-tailed and statistical significance was assessed at the 0.05 level. The diagnostic accuracy of Fasciola antigen detection test was assessed by the area under the ROC curve (AUC).
The AUC equal to 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no
diagnostic value. The diagnostic accuracy was calculated by sensitivity, specificity,
efficiency and was expressed as percentage. The reproducibility and repeatability of the
developed assay, calculated as intra-assay and inter-assay coefficients of variation
(CV\%) were assessed using 10 serum samples with different concentrations of Fasciola
circulating antigen (17-1200 ng/mL) tested in quadruplicate and undertaken along 4
consecutive weeks. All statistical analyses were done by a statistical software package
(SPSS 15.0 for Microsoft Windows, SPSS Inc., Chicago, Illinois, USA).

RESULTS

Identification of the target Fasciola antigen among \textit{F. gigantic} antigenic extracts
and human sera from infected individuals. FWAP and E/S products of \textit{F. gigantic},
SWAP of \textit{S. mansoni} and AWAP of \textit{A. lumbricoides} were subjected to SDS-PAGE (Fig.
1A) and Western blotting was carried out to detect the target epitope of Fasciola antigen.
It was found that a polypeptide band of 27-kDa in FWAP and E/S product of Fasciola
reacted with anti-27-kDa antigen IgG antibody. This antibody did not recognize any
antigens in the Ascaris and Schistosoma antigenic extracts (Fig. 1B). No reaction was
shown against ESP or FWAP using the saturated rabbit anti-serum with of the 27-kDa
protein (Fig. 1C). The electro-eluted 27-kDa antigen gave a single polypeptide band
when investigated by SDS-PAGE and silver staining, and the purity of the eluted 27-
kDa polypeptide was confirmed using CZE, only a single peak (5.9 minutes) was
observed (Fig. 1D). A sharp band was observed at 27-kDa in sera from individuals
infected with \textit{F. gigantic} but no reaction was observed in sera of uninfected individuals
(Fig. 2). Partial biochemical characterization of the reactive epitope confirmed its
protein moiety. The reactivity of the anti-27-kDa antibody was maintained towards the
purified antigen after Periodate oxidization (i.e. showing positive result using FgCA-27
ELISA). However, antibody reactivity towards the purified 27-kDa antigen decreased by
increasing the incubation time of the antigen with pepsin enzyme and it was completely lost at 20 min (i.e. showing negative result using FgCA-27 ELISA).

**Anatomic localization of the native target antigen in *F. gigantica* adult worm.** The antigenic determinant of the developed IgG antibody was located in the gut and tegumental surface of adult *F. gigantica* worm. A strong immunoperoxidase reaction was found within the tegument (t), the muscularis (ms) and the gut cells (g), Fig. 3A. No peroxidase reaction was shown within tegument, muscularis and gut cells of Fasciola adult worm using normal rabbit serum as a negative control (Fig. 3B).

**Detection of the Fasciola circulating antigen in human serum samples using FgCA-27 ELISA.** From the established calibration curve (Fig. 4A), the developed FgCA-27 ELISA successfully detected the 27-kDa Fasciola antigen serially diluted in normal human serum up to 1-ng/mL; the point did not overlap with the concentration corresponding to 3 SD above the mean OD of zero calibrator (n=4). However, the detection limit of the target circulating antigen in human serum samples at 8-ng/mL is corresponding to the cut-off level (OD = 0.29) of the developed assay (n=32). The presence of different levels of specific anti-27-kDa IgG antibodies in serum decreased the antigen levels but did not reach a significant level of difference (p > 0.05) in comparison with normal human serum i.e. negative for specific IgG antibodies to Fasciola (Data not shown). Serum samples from 120 individuals with fascioliasis and 80 non-infected individuals were tested using FgCA-27 ELISA for the detection of the circulating Fasciola antigen. The antigen concentrations (ng/mL) in serum samples are shown in Fig. 4B. The area under the ROC curve (AUC) for Fasciola antigen detection assay was significantly high (AUC = 0.961, p < 0.0001) for discriminating individuals infected with Fasciola and non-infected individuals (Fig. 5A). The developed assay gave a sensitivity of 93.3% and had false-positive results for 4 of 80 non-infected individuals, with a specificity of 95% in detecting human fascioliasis (Table 1). The cross-reactivity
with other parasitic infections was studied based on microscopic stool examinations of 80 non-infected individuals. The FgCA-27 ELISA showed specificity of 100% with S. mansoni (7 cases), 100% with A. lumbricoides (5 cases), 100% with H. nana (4 cases), 100% with G. lamblia (3 cases), 92% with E. histolytica (3 out of 38 cases showing false positive result) and 96% with parasite free-healthy individuals (1 out of 23 cases showing false positive result), Table 1. All serum samples showing false negative and false positive results using FgCA-27 ELISA were investigated using the more sensitive western blot. The 27-kDa antigen was identified in all 8 sera showing false negative ELISA results. Among the four sera showing false positive ELISA result, only one sample of a parasite free-healthy individual showed a sharp band at 27-kDa and the remaining 3 sera of E. histolytica infected individuals showed no bands in their blots. Moreover, antigen detection rates (% positivity) increased by increasing the severity of infection (expressed as parasite egg count), Table 1 and a highly significant correlation (Spearman r = 0.715, p < 0.0001) was shown between circulating Fasciola antigen level in serum (ng/mL) and parasite eggs count (epg), Fig. 5B. The intra-assay and inter-assay coefficients of variation percentage (CV %) in antigen concentrations were 3.3 % and 5.8 %; respectively.

**DISCUSSION**

During the last two decades, a major focus of research has been directed towards the identification of Fasciola antigens during human infection as a step forward toward the development of an efficient diagnostic assay (34). Fasciola antigens are mostly released from the gut and the tegument into the blood circulation and excreted in stool specimens of infected animals and humans (35-39). Several Fasciola antigens have been detected as circulating antigens in serum or as coproantigens in feces and were successfully used in immunodiagnosis of human fascioliasis (8, 11, 16, 34, 40). Of these, one antigenic component with an approximate molecular mass of 27-kDa was found to give a
consistent reaction with human fascioliasis sera (14, 18, 41-43). Of note, all serologic tests based on the 27-kDa antigen was developed and optimized with an emphasis on the detection of antibodies to *F. hepatica* (12, 13, 44, 45). However, the direct detection of Fasciola antigens secreted by the living flukes has apparent advantages over antibody detection tests in that antigenemia implies active infection, has ability to assess efficacy of chemotherapy and determine the effectiveness of future vaccines (24, 46-48).

In our previous study (22), we identified a wide band of 26- to 28-kDa in adult worm and E/S antigenic preparations of *F. gigantica* and also in sera of cattle infected with *F. gigantica* using specific IgG anti-serum developed in rabbit to the target 26- to 28-kDa antigen purified from FWAP. This antibody did not recognize any target epitopes in the Ascaris and Schistosoma antigenic extracts and its antigenic determinant was located in the gut and tegument of adult *F. gigantica* worms. This enhanced our efforts to identify the target antigen of the specific IgG antibody to the 26- to 28-kDa Fasciola antigen in sera of individuals infected with *F. gigantica* and we fortunately observed a sharp narrow band at 27-kDa molecular weight in selected sera from individuals infected with *F. gigantica* and no specific reaction was observed in sera of uninfected individuals using western blot (unpublished data).

In the present study, the target 27-kDa circulating antigen was isolated and purified from serum sample of *F. gigantica* infected human and a highly reactive IgG antibody was developed in rabbit to the purified 27-kDa antigen. The newly developed IgG antibody identified a polypeptide band of 27-kDa molecular weight in adult worm and E/S antigenic preparations of *F. gigantica* but did not recognize any target epitopes in the Ascaris and Schistosoma antigenic extracts and its antigenic determinant was located in the gut and tegument of adult *F. gigantica* worms. Moreover, the target circulating antigen was identified at 27-kDa molecular weight in sera from individuals infected with *F. gigantica* and no specific reaction was observed in sera of uninfected individuals.
using western blot and also detected by using ELISA. An inhibition-western blot assay was performed to confirm that the 27-kDa protein isolated from serum is a parasite molecule. Our antigen is similar in molecular weight to a *F. gigantica* antigen designated FG27 (42,49). The partial biochemical characterization of our purified antigen confirmed its protein moiety. However, further molecular characterization of our 27-kDa target antigen including peptide mapping followed by MS amino acid sequence will be performed in collaboration with a well-equipped laboratory to clarify its identity. The identification of the Fasciola antigens in different FWAP, E/S products and in human serum is of crucial importance for reliable diagnosis of active Fasciola infection. Several sensitive and specific methods based on Fasciola antigen detection in serum and stool using specific antibodies were applied to *F. hepatica* and *F. gigantica* infections in animals (8, 25, 26, 47). The antigen tests can detect experimental infection a few days after inoculation (23). However, few antigen detection assays have been developed for diagnosis of *F. gigantica* in human fluids with varied ranges of sensitivities and specificities (21). In the present study, we have developed FgCA-27 ELISA successfully detected the 27-kDa Fasciola antigen with a detection limit of 8-ng/mL in human serum samples. The presence of Fasciola antigens in free form in the circulation of infected individuals may be attributed to continuous shedding and release of circulating antigens as a possible escape mechanism by the parasites, reinfections and exposure to maternal antigens that may lead to developing tolerance rather than immunity to free antigens of natural infections (50). Moreover, the presence of high titers of specific IgG antibodies in the sera of infected individuals does not indicate a complete elimination of the pathogen; such high titers of antibodies may not neutralize all of the circulating antigens i.e. via immune complex formation and, also, the specific IgM antibodies’ response will not be stimulated for new infections. The presence of different levels of specific anti-27 kDa IgG antibodies in serum samples did not affect the detection of different
concentrations of the target Fasciola antigen using the optimized FgCA-ELISA. A non-significant decrease (p > 0.05) in the antigen concentrations was shown with the increase of anti-27-kDa IgG antibodies levels in serum (Data not shown). This indicates that the 27-kDa antigen could attach the solid phase in presence of specific IgG antibodies or other serum proteins.

The performance characteristics of the developed assay were investigated using sera of parasitologically diagnosed 200 individuals. The developed assay gave high degrees of sensitivity, specificity, and efficiency (> 93%). The accuracy of the developed ELISA, calculated by the area under ROC curve (AUC), yielded a 0.961 value, indicating the high performance of the assay that could significantly (p < 0.0001) discriminate individuals infected with *F. gigantica* and non-infected individuals. Moreover, the precision of the test, calculated as the intra-assay and inter-assay coefficients of variation were 3.3 % and 5.8 % respectively, indicating also high performance in reproducibility and repeatability of the assay. Interestingly, our developed assay show superior or at least similar results to the previously reported antigen tests for diagnosis of human fascioliasis (37, 51, 52) and the differences may be due to the different nature of antigens used. A recent study evaluated an antigen capture ELISA using a pair of monoclonal antibodies raised against *F. gigantica* E/S antigens in human serum and stools at a lower detection limit of 3-ng/mL (25). The sensitivity (94%) and specificity (94.6%) of the capture ELISA in serum were similar to those shown in our study. Another more recent study showed similar degree of sensitivity (94.5%) but lower specificity (84.6%) using sandwich ELISA based on monospecific rabbit IgG antibody to 14.5 kDa FABP antigen obtained from *F. gigantica* adult worms (26). Protein-based (e.g. protein microarrays) as well as DNA-based molecular tests (including PCR) can be used for clinical detections as well as field screening (53). Further investigations
comparing the immunodiagnostic performances of our FgCA-27 ELISA with such tests will be performed in collaboration with a well-equipped laboratory.

In the present study, all serum samples showing false negative ELISA results were of light infection (i.e. egg count < 100 epg) except one was of moderated parasite burden (i.e. egg count 100-299 epg), however, the target 27-kDa antigen was identified in all these samples using the more sensitive western blot. This result may support the idea of immune complexes formation in the samples showing false negative ELISA results.

Therefore, the absence of the target 27-kDa antigen in 8 sera of our infected individuals using ELISA may be due to undetectable levels of circulating antigen in these serum samples as a result of immune complex formation with host antibodies that tend to decrease the potential rate of circulating antigen (47, 54). Consequently, the developed assay conditions will be optimized to detect 100% of positive sample by using sample pretreatment with a dissociating buffer for immune complexes. The use of specific capturing of the 27-kDa antigen by using specific rabbit IgG polyclonal antibody coating of the solid phase will be also investigated. Moreover, the identification and detection of the target 27-kDa antigen in stool samples will be investigated to overcome the drawback of immune complex formation.

The cross reactivity with other parasites represents a major problem in specificity of immunodiagnostic assays like ELISA, especially in endemic areas (13). Moreover, Fasciola shares cross-reactive antigens with some parasites such as Schistosoma and Echinococcus (12, 54). Regarding the specificity of the developed FgCA-27 ELISA, there were no major problems related to cross-reactivity when common protozoans or helminthes were present. The developed assay had false-positive results for only 4 of 80 non-infected individuals, with a high degree specificity of 95% in detecting human fascioliasis. This is not surprising because the specificity of the FgCA-27 ELISA mainly depends on the recognition of a single Fasciola antigen (27-kDa) using monospecific
IgG antibody. However, the ELISA positivity of 4 individuals showing false positive ELISA result was investigated using western blot. Only one sample of a parasite free-healthy individual showed a sharp band at 27-kDa and consequently this individual may be infected with Fasciola but not identified by stool analysis due to the variable sensitivity of that technique and the eggs might be present in a small number at irregular intervals (55) and in most cases repeated stool examinations are required to accurate diagnose (10). Regarding the remaining 3 sera of *E. histolytica* infected individuals; no bands were identified in their blots. The ELISA positivity of these sera may attributed to a cross reactivity with some epitopes on antigens of other parasites that not identified in our study (26, 55) or may due to further purification steps of the developed anti-27 IgG polyclonal antibody are required to improve the assay specificity (41). However, further study using a large number of individuals with other common parasitic infections is required to draw the final conclusion. Also, it will be necessary to carry out new study in countries where other types of human trematodosis occur to confirm the usefulness of the FgCA-27 ELISA assay in countries where these parasitic diseases are endemic.

Of note, a highly significant correlation (Spearman $r = 0.715$, $p < 0.0001$) was shown between circulating Fasciola antigen level in serum (ng/mL) and parasite egg count (epg). Significant correlations have been demonstrated between Fasciola antigens and the parasite burden in animals (23, 56) and also with egg counts in human fascioliasis (25, 51). In contrast, Ubeira et al. (24) reported that there was no correlation between egg counts and antigens levels in stool measured by ELISA as the egg excretion is probably more erratic in patients with chronic infections. However, the presence of a highly significant correlation between circulating Fasciola antigen levels and parasite egg count which is presumably dependent on the number of flukes in the host is of crucial importance to the use developed assay to monitor the efficiency of flukicide treatment in Fasciola infected individuals and to assess potential new vaccine efficacy.
In conclusion, a 27-kDa Fasciola antigen was identified in sera of *F. gigantica* infected individuals. A highly sensitive and specific Fasciola circulating antigen detection assay, FgCA-27 ELISA was developed for reliable laboratory diagnosis of human fascioliasis. Further characterization of the target antigen will be performed and further optimization and field studies are necessary to draw a final conclusion regarding the use of FgCA-27 ELISA for diagnosis and follow up of Fasciola infected individuals.

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REFERENCES


**26. Allam G, Bauomy IR, Hemyeda ZM, Sakran TF.** 2012. Evaluation of a 14.5 kDa-


FIG 1 Identification and purification of the target Fasciola antigen. (A) Silver stained SDS-PAGE. (B) Western blot for different antigenic sources of *F. gigantica*, *S. mansoni* and *A. lumbricoides* using rabbit anti-27-kDa Fasciola IgG antibody. Lane 1: *F. gigantica* adult worm antigen preparation (FWAP); lane 2: Excretory/Secretory (E/S) products from *F. gigantica*; lane 3: *S. mansoni* adult worm antigenic preparation (SWAP); lane 4: *A. lumbricoides* adult worm antigenic preparation (AWAP); lane 5: The purified 27-kDa antigen. The developed anti-Fasciola antibody identified its target epitope at 27-kDa in FWAP, E/S products and the purified antigen (lanes 1, 2, 5) but not in SWAP and AWAP (lanes 3, 4). (C) Inhibition western blot for different antigenic sources of *F. gigantica* using rabbit anti-27-kDa Fasciola IgG antibody saturated with purified 27-kDa antigen. Lane 1: FWAP immunostained with the anti-Fasciola antibody; Lane 2: FWAP immunostained with the saturated antibody; lane 3: E/S products immunostained with the anti-Fasciola antibody; lane 4: E/S products immunostained with saturated antibody. The saturated anti-Fasciola antibody did not identify its target epitope at 27-kDa in FWAP and E/S products (lanes 2, 4). Standard molecular weights (Mr.) was included Phosphorylase B (97.4 kDa), Bovine serum albumin (66.2 kDa), Glutamate dehydrogenase (55 kDa), ovalbumin (42.7 kDa), aldolase (40 kDa), Carbonic anhydrase (31 kDa) and Soybean trypsin inhibitor (21.5 kDa). (D) The 27-kDa antigen purified from serum of *F. gigantica* infected individual showing a single peak, in absorbance of 200 nm, at 5.9 min using CZE.

FIG 2 Western blot of serum samples from Fasciola infected and non-infected individuals for the detection of circulating Fasciola antigen. Lane 1: FWAP, lanes 2-4: serum samples from 3 non-infected individuals, and lanes 5-9: serum samples from 5 individuals infected with *F. gigantica*. Fifty μg/lane of each sample were separated on 12 % acrylamide gels then transferred to NC sheet and reacted with 100 μg/mL Rabbit anti-27-kDa IgG antibody. Anti-Rabbit IgG alkaline phosphatase and BCIP/NBT
substrate were used to visualize the reaction products. The developed rabbit antibody identified a 27-kDa antigen in all sera of infected individuals (lanes 5-9) but not in sera of non-infected individuals (lanes 2-4). Standard molecular weights (Mr) was included Phosphorylase B (97.4 kDa), Bovine serum albumin (66.2 kDa), Glutamate dehydrogenase (55 kDa), ovalbumin (42.7 kDa), aldolase (40 kDa), Carbonic anhydrase (31 kDa) and Soybean trypsin inhibitor (21.5 kDa).

**FIG 3** Localization of the target 27-kDa Fasciola antigen within *F. gigantica* adult worm using specific rabbit antisera and indirect immunoperoxidase staining. (A) Section through *F. gigantica* adult worm showing positive immunoperoxidase reaction; red color within tegument (t), muscularis (ms) and gut cells (g) (100x). (B) Section through *F. gigantica* adult worm showing no immunoperoxidase reaction, (100x).

**FIG 4** Quantification of the 27-kDa *Fasciola gigantica* antigen in human serum using the developed FgCA-ELISA. (A) Calibration curve of the 27-kDa Fasciola antigen. The purified 27-kDa antigen (ng/mL) was serially diluted in normal human serum and tested using the developed ELISA. The optical densities (OD) were measured at 405 nm and the cut-off level was set at OD = 0.29. (B) Scattergram showing Fasciola antigen levels (expressed as ng/mL) in sera of 120 parasitologically diagnosed infected individuals classed as light infection (<100 epg, n = 76), moderate infection (100-299 epg, n = 33) and severe infection (≥300 epg, n = 11) in comparison with antigen levels in sera of 80 non-infected individuals. The cut-off level of the developed ELISA was set at 8-ng/mL.

**FIG 5** Diagnostic accuracy of the developed FgCA-ELISA. (A) Receiver operating characteristic (ROC) curve of circulating Fasciola antigen detection using ELISA for discriminating *F. gigantica* infected individuals and non-infected individuals. The true positive rate (sensitivity) is plotted as a function of the false positive rate (1 - specificity). Each point on the ROC curve represents a sensitivity/specificity pair.
corresponding to a particular decision threshold. Area under curve (AUC) value represents the combined effects of both sensitivity and specificity of circulating Fasciola antigen in diagnosing individuals infected with *F. gigantica* (AUC = 0.961, p < 0.0001).

(B) Correlation between the levels of Fasciola circulating antigen measured in human sera by ELISA (expressed in Log_{10} ng/mL) and the egg count measured by Kato-Katz technique (expressed in EPG). The results of both assays for 120 serum samples were statistically analyzed and showing highly significant correlation (Spearman r = 0.715, p < 0.0001).
**TABLE 1** Performance characteristics of a newly developed ELISA based on Fasciola circulating antigen detection in sera of parasitologically diagnosed 120 *F. gigantica* infected individuals and 80 non-infected individuals.

<table>
<thead>
<tr>
<th>Parasitological diagnosis</th>
<th>No.</th>
<th>FgCA-27 ELISA*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td><em>F. gigantica</em> infected individuals:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light infection (&lt; 100 epg)</td>
<td>76</td>
<td>69</td>
</tr>
<tr>
<td>Moderate infection (100-299 epg)</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>Severe infection (≥ 300 epg)</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Non-infected individuals:</td>
<td>80</td>
<td>4 (FP)</td>
</tr>
<tr>
<td>Other parasitic infection</td>
<td>57</td>
<td>3</td>
</tr>
<tr>
<td>No parasitic infection</td>
<td>23</td>
<td>1</td>
</tr>
</tbody>
</table>

Abbreviations: TP, true positive; FP, false positive; TN, true negative; FN, false negative. TP: a serum sample from individual with confirmed Fasciola infection showing positive result by the ELISA; FN: a serum sample from patient with confirmed Fasciola infection showing negative result by the ELISA; TN: a serum sample from individual with confirmed absence of Fasciola infection showing negative result by the ELISA; FP: a serum sample from individual with confirmed absence of Fasciola infection showing positive result by the ELISA.

* Sensitivity (%) = TP / (TP + FN) = 112 / (112 + 8) × 100 = 93.3 %,
  Specificity (%) = TN / (TN + FP) = 76 / (76 + 4) × 100 = 95.0 %,
  Efficiency of the test (%) = (TP + TN) / Total = (112 + 76) / 200 × 100 = 94.0 %.
Antigen concentration (Log10 ng/mL) vs. Eggs per gram (EPG)

- Correlation coefficient: $r = 0.715$
- Significance: $p < 0.0001$

The data points show a positive correlation between antigen concentration and eggs per gram, with the regression line indicating a significant linear relationship.