IMMUNODIAGNOSIS OF HUMAN FASCIOLIASIS USING AN ANTIGEN OF *FASCIOLA GIGANTICA* ADULT WORM WITH THE MOLECULAR MASS OF 27 KDA BY A DOT-ELISA

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Abstract. Immunodominant antigens of an approximate molecular mass of 27 kDa (FG 27) were obtained from an excretory-secretory product of adult *Fasciola gigantica* by a simple continuous–elution method. A dot–ELISA using the FG 27 antigen was developed for the detection of specific antibodies from patients infected with *F. gigantica*. Control sera were obtained from patients with other parasitic infections and healthy volunteers. The accuracy, sensitivity, specificity, and positive and negative predictive values were 98.2%, 100%, 97.4%, 76.9% and 100%, respectively. This dot-ELISA is a specific, sensitive and easy to perform method for the rapid diagnosis of fascioliasis, particularly when more complex laboratory tests are unavailable.

INTRODUCTION

Liver fluke infection, fascioliasis, is an important public health problem in many parts of the world. The estimated number of these infected is in the order of 2.4 million people (WHO, 1995). The disease is caused by hermaphroditic trematodes of the genus *Fasciola* of which *F. hepatica* and *F. gigantica* are the most common representatives. *F. hepatica* has a worldwide distribution but predominates in temperate zones, while *F. gigantica* is also found on most continents but primarily in tropical regions *ie* Asia, Southeast Asia and Africa (Andrews, 1999). Humans are usually infected by the ingestion of aquatic plants that contain infective metacercariae (Mas-Coma *et al*, 1999).

The parasitological diagnosis of human fascioliasis is often unreliable because the parasite eggs are not found in the stool during the early phase of infection. Even when the worms have matured, the diagnosis may still be difficult since eggs are only intermittently released. Early diagnosis of fascioliasis is necessary for prompt treatment before irreparable damage to the liver occurs. For these reasons, serological tests are the most dependable diagnostic methods (Hillyer, 1999). Attempts have been made to diagnose human fascioliasis by detecting antibodies in the serum of patients suspected of being infected with the fluke. Numerous immunodiagnostic tests have been used with an emphasis on *F. hepatica* (Espino *et al*, 1987; Hillyer and Soler de Galanes, 1988; Hillyer *et al*, 1992; Sampaio Silva *et al*, 1996; O'Neill *et al*, 1998; Cordova *et al*, 1999; Strauss *et al*, 1999; Carnevale *et al*, 2001). Recently, we isolated a 27 kDa component from crude *F. gigantica*-excretory-secretory (ES) products (FG 27) using a continuous elution method and then used this component as an antigen in an indirect ELISA test (Maleewong *et al*, 1999). This assay is simple, sensitive, specific and valuable for the serodiagnosis of human fascioliasis. The standard indirect ELISA format is less practical for field surveys. Another form of the assay, a dot-ELISA has been successfully applied for screening antibodies (Shaheen *et al*, 1989; 1994; Itoh and Sato, 1990). The purpose of the present study was to use the sensitive and specific FG 27 component as the antigen in a dot–ELISA for the diagnosis of

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human fascioliasis.

MATERIALS AND METHODS

Human sera

Human sera were obtained from serum banks kept in different sections of the Faculty of Medicine, Khon Kaen University, Thailand. The collections started several years before the present investigation began. The study protocol was approved by the Scientific-Ethics Committee of Khon Kaen University. Informed consent was obtained from the study subjects using standard guidelines.

Ten fascioliasis sera were obtained from parasitologically confirmed cases of infection with *F. gigantica*. Each of these confirmations was based on the removal of *F. gigantica* adult worms during cholecystectomy, T-tube choledochostomy or other bile duct operations. To assess potential cross-reactivity, 123 serum samples obtained from individuals with parasitic diseases other than fascioliasis were used. These samples were obtained from parasitologically confirmed cases of gnathostomiasis, angiostrongyliasis, paragonimiasis, opisthorchiasis, cysticercosis, trichinosis, strongyloidiasis, and capillariasis. Other parasitosis sera were obtained from cases that were positive for parasites or their products. Negative control sera were obtained from 33 healthy adults. Examinations of their stools at the time of the blood collection using the formalin-ether concentration method (Erdman, 1981), gave no evidence of intestinal parasitic infections.

Preparation of crude ES antigen

Adult *F. gigantica* were obtained from the livers of infected cattle from an endemic area in Khon Kaen Province, northeastern Thailand. The worms were washed with sterile saline and identified as *F. gigantica* according to criteria previously described (Watanabe, 1965; Sahba *et al*, 1972). The ES antigen was prepared from a spent culture medium (RPMI 1640) containing, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM tosylamide-2-phenylethyl-chloromethyl ketone, 1 μM of L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in which the worms had been maintained for 6 hours at 37°C under 5% CO_2. After incubation, the worms were removed and the collected spent medium was clarified by centrifugation at 10,000*g* for 30 minutes at 4°C. The supernatant was concentrated by ultra-filtration using an Amicon YM 3 membrane filter (Grace and Co, Danvers, MA), dialyzed against distilled water containing the same proteinase inhibitors, aliquoted, and stored at -40°C before being used.

Production of specific antigen by continuous-elution SDS-PAGE

The continuous-elution SDS-PAGE was performed using the Prep-Cell (Model 491; Bio-Rad, Hercules, CA) as recommended by the manufacturer. A discontinuous buffer system was used. A cylindrical column of polyacrylamide gel was prepared using a 28-mm internal diameter casting tube. It consisted of an upper 4% stacking gel and lower 12% gel with heights of 2 cm and 5.5 cm, respectively. The casting tube was assembled with the upper and lower buffer chambers and the latter was connected to an external recirculation pump (Bio-Rad). The crude ES sample containing 5 mg of protein was boiled for 5 minutes in 1 ml of sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 0.001% bromphenol blue) before loading into the gel. The recirculation pump speed was operated at 100 ml/minute. The sample was subjected to electrophoresis for 10 hour at 250 V, 40 mA, and 12 W. Resolved proteins were collected at the base of the gel column using a dialysis membrane with a 6-kDa cut-off. The purified proteins were continuously eluted at a flow rate of 1 ml/minute using an external peristaltic pump (Econo-Pump; Bio-Rad). The absorbance of the eluant at 280 nm was monitored using an UV monitor (Model EM-1; Bio-Rad). The cut-off absorbance was an optical density of 0.05 and fractionated proteins were retrieved using a programable fraction collector (Model 2128; Bio-Rad). Fractions of 2.5 ml provided sufficient separation. The fractions containing the specific component with an approximate molecular mass of 27 kDa were identified by SDS-PAGE. These fractions containing the component of interest were pooled, dialyzed, and lyophilized. The eluants were analyzed by SDS-PAGE using the Mini-Protean II Cell (Bio-Rad), under reducing condi-

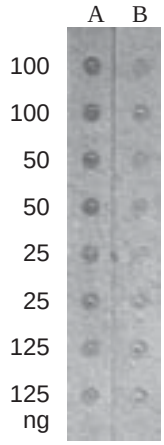

Fig 1–Titration of the FG 27 antigen with human *Fasciola* - positive sera (A) and *Fasciola* - negative sera (B) at a dilution of 1 : 200.

tions on a 10-18% gradient gel prepared by the method of Laemmli (1970). After electrophoresis, the resolved polypeptides were revealed by staining with Coomassie brilliant blue. The fractionated ES antigen containing the prominent 27 kDa (FG 27) component was revealed as previously reported (Maleewong *et al*, 1999) (data not shown).

Dot-ELISA

The optimum amount of the purified FG 27 antigens, 50 ng, was previously determined by titration and used throughout the experiment (Fig 1). Two microliters of the antigens in 0.1 M phosphate-buffered saline solutions (PBS), pH 7.5, were spotted separately on a 6 x 12 mm nitrocellulose paper strip (NC) (Hoefer Pharmacia Biotech, San Francisco, CA) and air-dried for 30 minutes. The unoccupied sites of the NC were then blocked for 30 minutes with 0.1 M PBS, pH 7.5, containing 5% skimmed milk. After blocking, the NC was incubated with 500 µl of human sera, diluted 1: 200 in blocking buffer, for 2 hours at room temperature with gentle shaking. The strips were washed five times with fresh blocking solution and subsequently incubated with 500 µl goat anti-human IgG peroxidase conjugate, diluted 1: 20,000 (Zymed Laboratories Inc, San Francisco, CA) in blocking buffer for 2 hours at room temperature. After washing, the strips were developed in 3, 3′-diaminobenzidine-tetrahydrochloride solution. The reaction was stopped after 5 minutes by washing the strips with distilled water. Results, appearances of brownish dots, were observed with the naked eye and recorded arbitrarily according to colored intensity as +, ++, +++ and ++++, respectively in escalating order. The precision of the dot-ELISA was also investigated by performing the test on different days by using the same pooled positive serum, the same batch of antigens, and the same conditions. Identical results were obtained from all, which indicated that day to day variation was minimal.

Other technique

The protein concentration was determined as described by Lowry *et al* (1951) with bovine serum albumin used as the standard.

Data analysis

The diagnostic accuracy, sensitivity, specificity, and predictive values were calculated using the method of Galen (1980).

RESULTS

The reaction of ++ was used as the cut-off limit to distinguish positive and negative sera.

Table 1
Sensitivity and specificity of the dot-ELISA using the FG27 component for the serodiagnosis of fascioliasis.

Types of serum	No. positive / total (%)
Fascioliasis	10/10 (100)
Opisthorchiasis	0/19 (0)
Paragonimiasis	2/19 (10.5)
Cysticercosis	1/5 (20)
Strongyloidiasis	0/12 (0)
Trichinosis	0/26 (0)
Gnathostomiasis	0/8 (0)
Capillariasis	0/8 (0)
Angiostrongyliasis	0/9 (0)
Other parasitoses[a]	0/17 (0)
Healthy controls	0/33 (0)

[a]Of the total 17 cases, 4 were infected with *Opisthorchis viverrini*, hookworm and minute intestinal flukes, another 4 with *O. viverrini* and hookworm, 2 with *O. viverrini* and *Strongyliodes stercoralis*, 3 with *Echinostoma* spp, 2 with hookworms, 1 with *Thelazia* spp and the last 1 with *Entamoeba histolytica* infections.

The results of the dot-ELISA are summarized in Table 1. The overall accuracy of the test was 98.2%, while the sensitivity was 100% *ie* all fascioliasis sera were positive, the specificity was found 97.4% as a result of false positive results associated with two paragonimiasis and one cysticercosis sera. The positive and negative predictive values of the test were 76.9% and 100%, respectively.

DISCUSSION

Several groups reported the use of dot-ELISA for screening anti-*Fasciola* antibodies in cattle (Ibarra *et al*, 1998; Maisonnave, 1999; Castro *et al*, 2000), in llamas (Rickard, 1995), in sheep (Zimmerman *et al*, 1985; Arriaga de Morilla *et al*, 1989) and in rabbits (Rivera Marrero *et al*, 1988). This serodiagnostic test has also been applied to diagnose of human fascioliasis with an emphasis on crude helminthic antigens (Itoh and Sato, 1990; Shaheen *et al*, 1994) or partially purified antigen of somatic adult worms by conventional G-200 Sephadex column (Shaheen *et al*, 1989). Although higher sensitivity was reported in antibody detection from partially purified fractions of *Fasciola* spp antigens, avoiding cross-reactions to antibodies induced by other helminthic infections is quite impossible.

In the present report, a dot-ELISA using antigens with an approximate molecular mass of 27 kDa from *F. gigantica* adult worms demonstrated high accuracy, sensitivity and specificity. The production of specific antigen by continuous SDS-PAGE is simple and practical for use. Cross-reactivity occurred with this dot-ELISA particularly with paragonimiasis and cysticercosis, however, no cross reaction was shown by the indirect ELISA (Maleewong *et al*, 1999). This cross-reaction is difficult to explain. Sera from paragonimiasis and cysticercosis showed positive with *Fasciola* spp. antigens (Maleewong *et al*, 1996; Ikeda, 1998; Intapan *et al*, 1998). The dot-ELISA should be carefully interpreted in an endemic area of paragonimiasis and cysticercosis.

The dot-ELISA has several advantages over the indirect ELISA. Nitrocellulose papers spotted with antigen are stable for at least three months at -20°C (data not shown), all incubation steps are performed at room temperature, and the results can be read with the naked eye, thus an expensive spectrophotometer is not required. The test is applicable to diagnose in the field setting as well as in laboratories that are not well equipped. The dot–ELISA is simpler and allows testing of multiple samples at the same time.

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