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

Pewpan M Intapan¹, Wanchai Maleewong¹, Saengchai Nateeworanart², Chaisiri Wongkham³, Vichit Pipitgool¹, Varaporn Sukolapong⁴ and Somboon Sangmaneedet⁴

Departments of ¹ Parasitology and ³ Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen; ² Department of Microbiology, Faculty of Medical Technology, Huachiew Chalermprakiet University, Samut Prakan; ⁴ Department of Veterinary Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand

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 (Espinó 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
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 cholecystostomy 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, 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% CO₂. After incubation, the worms were removed and the collected spent medium was clarified by centrifugation at 10,000g 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 cutoff 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-
IMMUNODIAGNOSIS OF HUMAN FASCIOILIASIS

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

**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.

<table>
<thead>
<tr>
<th>Types of serum</th>
<th>No. positive / total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fascioliasis</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>Opisthorchiasis</td>
<td>0/19 (0)</td>
</tr>
<tr>
<td>Paragonimiasis</td>
<td>2/19 (10.5)</td>
</tr>
<tr>
<td>Cysticercosis</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Strongyloidiasis</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>Trichinosis</td>
<td>0/26 (0)</td>
</tr>
<tr>
<td>Gnathostomiasis</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Capillarisis</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Angiostrongyliasis</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>Other parasitosesa</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>0/33 (0)</td>
</tr>
</tbody>
</table>

*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 *Strongyloides 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, 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, avoiding cross-reactions to antibodies induced by other helminthic infections is quite impossible.

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.

**ACKNOWLEDGEMENTS**

This investigation was supported by a grant from Khon Kaen University. The authors wish to thank Dr Nimit Morakote for reviewing the manuscript, and Dr Mark Roselieb for improving the English and the presentation of our research.

**REFERENCES**


Galen RS. Predictive value and efficiency of laboratory
IMMUNODIAGNOSIS OF HUMAN FASCIOLIASIS


