DEVELOPMENT OF RAPID AGGLUTINATION TEST USING *FASCIOLA GIGANTICA* SPECIFIC ANTIGEN FOR SERODIAGNOSIS OF HUMAN FASCIOLIASIS

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Abstract. A latex agglutination test was developed as a simple, fast, convenient, and highly-specific antibody detection tool for diagnosis of human fascioliasis. The assay was performed using immunodominant antigens at molecular mass 27 kDa, obtained from excretory-secretory product of adult *Fasciola gigantica* by continuous-elution method. Four groups of samples from 12 proven fascioliasis, 17 clinically-suspected fascioliasis, 88 other parasitosis (gnathostomiasis, angiostrongyliasis, cysticercosis, paragonimiasis, strongyloidiasis, capillariasis, trichinosis, malaria, hookworm infection, opisthorchiasis, and opisthorchiasis with other parasitic infection) patients, and 31 healthy control sera were used for examination. When the agglutination reaction of 2+ at a serum dilution of 1:4 was used as the cut-off limit to distinguish positive and negative sera, this test gave 75% (9/12) sensitivity, 99.2% (118/119) specificity, 90% (9/10) positive-predictive and 97.5% (118/121) negative-predictive values, and 96.9% (127/131) accuracy. Six of 17 clinically-suspected fascioliasis sera and one serum from a capillariasis patient were reacted. No cross-reaction was demonstrated with the healthy control sera. The advantage of this assay is that all steps are performed at room temperature, and the results can be read by the naked eye; thus, expensive equipment is not required.

INTRODUCTION

The liver fluke infection, fascioliasis, is an important public health problem in many parts of the world. The estimated number of infections 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 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 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 sera 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; Strauss *et al*, 1999; Cordova *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 antigen in an indirect ELISA test (Maleewong *et al*, 1999). This assay is simple, sensitive, specific, and valuable for the serodiagnosis of human fascioliasis. However, the standard indirect ELISA format is less practical for field surveys. Another form of the assay, latex agglutination, has been successfully applied for screening antibodies to *Toxoplasma gondii* (Mazumder *et al*, 1988; Dumas *et al*, 1989; Lin *et al*, 1991; Uga *et al*, 1996) and *Trypanosoma cruzi* (Knecher *et al*, 1994). Evaluation of the latex agglutination test for serodiagnosis of patients infected with *F. gigantica* is still lacking. The purpose of the present study was to use the sensitive and specific FG 27 component as antigen in a latex agglutination test to diagnose human fascioliasis.

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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 Human Research Ethics Committee of Khon Kaen University. Informed consent was obtained from the study subjects, using standard guidelines.

Twelve 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. Seventeen sera were obtained from liver-abscess patients who presented clinical findings compatible with fascioliasis. To assess potential cross-reactivity, 88 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, malaria, and capillariasis. Other parasitosis sera were obtained from cases positive for parasites or their products. Negative control sera were obtained from 31 healthy adults. Examinations of their stools at the time of blood collection, using the formalin-ether concentration method (Erdman, 1981), gave no evidence of intestinal parasitic infection.

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 & Co, Danvers, MA), dialyzed against distilled water containing the same proteinase inhibitors, aliquoted, and stored at -40°C before use.

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 a 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 was operated at 100 ml/minute. The sample was subjected to electrophoresis for 10 hours 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 (OD) 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 conditions 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 component was revealed as previously reported (Maleewong *et al*, 1999) (data not shown).

Preparation of positive and negative reference sera

Positive anti-*F. gigantica* ES antibody serum was obtained by injection of *F. gigantica* ES antigen into New Zealand white rabbit. (Maizels *et al*, 1991). At an appropriate time thereafter, blood was obtained and serum was separated and stored at -20°C until use. Negative reference serum was obtained from a normal healthy rabbit before injection.

Latex sensitization

The suspension of polystyrene latex particles (0.81 μm diameter; Difco Laboratories, Detroit, MI) was

used, unwashed, undiluted and stored at 4°C. Polystyrene latex particle stock solution was diluted 1:5 in glycine-buffered saline, pH 8.2 (GBS; 0.1M glycine, 0.17 M NaCl in distilled water). An equal amount of the latex suspension was mixed with the FG 27 solution (500 µg/ml in GBS pH 8.2). The 2 reagents were mixed thoroughly for 30 minutes at room temperature. After washing twice with GBS pH 8.2, the latex particles were suspended in 1% (v/v) bovine serum albumin (BSA) in GBS pH 8.2 and stored at 4°C. Non-sensitized latex particles (untreated FG 27) were used as control.

Latex agglutination test

The slide latex agglutination test was performed according to a modification of Hopwood *et al* (1985). Five µl diluted serum (1:4) in 1% BSA in GBS pH 8.2 and 5 µl sensitized latex particles were added to the ring slide. The 2 reagents were mixed thoroughly for 5 minutes with an electric rotating apparatus (Sarstede, Germany) and the agglutination was read with the naked eye. Hyperimmune and healthy control rabbit sera were used as positive and negative reference sera, respectively.

The results, the appearance of agglutination, were observed with the naked eye and recorded arbitrarily according to agglutination intensity as negative (0), 1+, 2+, 3 + and 4+, respectively, in escalating order. Typically, a 4+ reaction was observed as large floccules formed instantly with clear background fluid, a 3+ reaction was observed as medium floccules formed instantly with clear background fluid, a 2+ reaction was observed as small floccules formed instantly with clear background fluid, and a 1+ reaction was observed as faint floccules formed with milky background fluid. A negative reaction was characterized by a uniform milky fluid with no visible floccules. The precision of the latex agglutination test was also investigated by performing the test on different days using the same positive sera, the same batch of antigens, and the same conditions. Identical results were obtained from all, which indicated that day to day variation was minimal.

Protein concentration was determined as described by Lowry *et al* (1951), with BSA used as the standard.

Data analysis

The diagnostic accuracy, sensitivity, specificity, and predictive values were calculated using the method of Galen (1980). These values were calculated and expressed as follows : accuracy = [(no. of true positives + no. of true negatives) / (no. of true positives + no. of false negatives + no. of true negatives + no. of false positives)] x 100; sensitivity = [no. of true positives/ (no. of true positives + no. of false negatives)] x 100; specificity = [no. of true negatives/(no. of false positives + no. of true negatives)] x 100; positive predictive value = [no. of true positives/(no. of true positives + no. of false positives)] x 100; negative predictive value = [no. of true negatives/(no. of true negatives + no. of false negatives)] x 100; true negative = number of control samples (other parasitosis and healthy controls) that were negative by latex agglutination test; true positive = number of proven fascioliasis samples that were positive by latex agglutination test; false positive = number of control samples that were positive by latex agglutination test; false negative = number of proven fascioliasis samples that were negative by latex agglutination test.

RESULTS

The reaction of 2+ was used as the cut-off limit to distinguish positive and negative sera. The results of the latex agglutination test are summarized in Table 1. The overall accuracy of the test was 96.9%, while the sensitivity was 75% *ie* 9 of 12 fascioliasis sera were positive. Specificity was 99.2% as a result of false-positive results associated with one capillariasis serum. The positive and negative predictive values of the test were 90 and 97.5%, respectively.

DISCUSSION

In the present report, a latex agglutination test using antigens with a 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. Cross-reactivity occurred with this latex agglutination test, particularly with capillariasis, however, no cross reaction was shown by indirect ELISA (Maleewong *et al*, 1999). This cross-reaction is difficult to explain. However, it was noted that sera from paragonimiasis and cysticercosis showed positive with *Fasciola* spp. antigens (Maleewong *et al*, 1996; Ikeda, 1998; Intapan *et al*, 1998). Nevertheless, the latex agglutination test should be carefully interpreted in an endemic area of capillariasis.

The latex agglutination test has several advantages over the indirect ELISA. The results can be demonstrated within 5 minutes, 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 for diagnosis in the field setting, as well as in laboratories that are not well-equipped. The latex agglutination test is simpler, rapid, and allows testing of multiple samples at the same time.

Table 1
Sensitivity and specificity of the latex agglutination test using the FG27 component for the serodiagnosis of fascioliasis.

Types of serum	No. positive / total (%)
Fascioliasis	9/12 (75)
Suspected fascioliasis	6/17 (35.2)
Opisthorchiasis	0/9 (0)
Paragonimiasis	0/10 (0)
Cysticercosis	0/2 (0)
Strongyloidiasis	0/10 (0)
Trichinosis	0/10 (0)
Gnathostomiasis	0/10 (0)
Capillariasis	1/8 (12.5)
Angiostrongyliasis	0/9 (0)
Malaria	0/10 (0)
Other parasitosis [a]	0/10 (0)
Healthy controls	0/31 (0)

[a] Of the total 10 cases, 6 were infected with *Opisthorchis viverrini* and hookworm, 2 with *O. viverrini* and minute intestinal flukes, 1 with *O. viverrini* and *Strongyliodes stercoralis*, and 1 with hookworm.

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