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Diagnosis of Cattle Fasciolosis by the Detection of a Circulating Antigen using a Monoclonal Antibody

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SUMMARY A monoclonal antibody (MoAb) 1C12 that reacts with a 66 kDa surface tegumental (ST) antigen of adult worms of *Fasciola gigantica* was used to detect circulating antigen in sera of experimentally and naturally infected cattle. A combination of rabbit anti ST-antigens and MoAb 1C12 were used to capture and detect the circulating antigen in sandwich ELISA. The dilutions of 1:1,000 of rabbit anti ST-antigens and 1:100 for MoAb 1C12 were used to reduce cross-reactivity with other trematodes' antigens. The circulating antigen of *F. gigantica* was demonstrated in sera of all experimentally infected animals as early as the first week after the infection, and it remained detectable until the experiment was terminated at week 32 after the infection. Of the 97 serum samples from naturally infected cattle, the sensitivity of 86.6% was observed when the cut-off point was calculated from 32 serum specimens from uninfected control calves. The sensitivity increased to 100% when the commercial fetal calf and trematode-free baby calves sera were used for calculation of the control cut-off point. Based on these results, the combination of rabbit anti ST-antigens and MoAb 1C12 sandwich ELISA appeared to be sensitive, specific, and applicable in the immunodiagnosis of fasciolosis in cattle for epidemiological study and monitoring of chemotherapeutic efficacy.

Fasciolosis caused by *Fasciola* infection in ruminants produces a great economic loss throughout the world. The current method for diagnosis of infection in cattle is based on the microscopic detection of eggs in feces. Although demonstration of circulating antibodies has been used for epidemiological studies, the presence of antibodies is not the direct indicator of active infection, and cross-reactivity with other parasites is often difficult to differentiate. The detection of circulating antigens rather than antibodies is considered to be a more reliable method for evaluating the status of infection which could be used to monitor the efficacy of treatment and the effectiveness of future candidate vaccines. The method of circulating antigen detection has been used successfully in the diagnosis of many parasitic diseases.[1,2,3] Recently, it has been reported that rabbit antibodies against the 88 kDa antigen of *Fasciola gigantica* can be used for detecting circulating antigens in sera of infected cattle.[4]

In this study, we produced a monoclonal antibody (MoAb) against 66 kDa surface tegumental (ST) antigen of adult *F. gigantica*. By using a combination of this MoAb and rabbit antibodies against the ST-antigens, a sandwich ELISA was developed for detecting directly circulating parasite antigen in sera of experimen-

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tal and naturally infected cattle. The use of monoclonal antibody provides one way of increasing sensitivity and specificity for the immunodiagnostic assay for fasciolosis.

MATERIALS AND METHODS

Parasite antigens

Live, intact adult *F. gigantica* were obtained from bile ducts of naturally infected cattle at local abattoirs. The parasites were washed three times with Hank's balanced salt solution (HBS) containing 100 units/ml penicillin and 100 mg/l streptomycin to remove the host blood, bile and contaminating microorganisms. To obtain the ST-antigens, worms were extracted with non-ionic detergent solution (1% Trition X-100 in Tris buffer pH 8) for 30 minutes at room temperature. The shed pieces of surface tegument in the solution, as monitored under the light microscope as previously reported,[5] were pooled and lyophilyzed. Before using as the antigens, the lyophilized powder was dissolved in double distilled water and dialized against 0.01 M phosphate buffer saline (PBS) pH 7.2 at 4-6°C for 48 hours. The protein content of the tegumental suspension was determined by Lowry's method.[6]

Monoclonal antibodies preparation

Hybridomas were produced by fusion of spleen cells from mice immunized with ST-antigens and P3/x63-Ag8 murine myeloma cells. The hybridoma clones were examined for the production of specific antibody against ST-antigens by ELISA. Ascites containing MoAb produced in pristaine-primed mice were collected and stored at -20°C until use. The monoclonal antibody isotypes and subisotypes were determined by ELISA using MonoAb Screen kit, Zymed Laboratory, Inc., USA.

Characterization of the monoclonal antibodies by enzyme-linked immunoelectrotransfer blot (EITB)

SDS-PAGE was carried out according to the method of Laemmli.[7] The tegumental antigen samples and standard proteins were mixed with the sample buffer and bromophenol blue and heated in a boiling water bath for 3 minutes. The tegumental samples were loaded at the concentration of 50 μg/well into 12.5% SDS-PAGE gel. Electrophoresis was performed with the DC current applied at 100 V, 30 mA from cathode to anode. It was terminated when the bromphenol blue marker reached the bottom of the gel.

The separated proteins were electrophoretically transferred onto a nitrocellulose paper and probed with the monoclonal antibody. The reaction was detected by incubation with peroxidase conjugated rabbit anti-mouse Ig and visualized by further incubation in 3, 3 diaminobenzidine (DAB) and H_2O_2.[8]

Immunizing rabbits with ST-antigens

Each rabbits was immunized subcutaneously with 200 μg of ST-antigens in Freund's complete adjuvant. The second and third doses were given on days 14 and 21, followed by the last booster dose of 400 μg of ST-antigens in Freund's incomplete adjuvant on day 28. Serum samples were collected 4 days after the last immunization. Rabbit anti ST-antigens were purified by ammonium sulfate precipitation.

Assay for assessing specificity of MoAbs

Indirect ELISA was used for studying the specificity of MoAbs. The antigens used in this study were crude extracts of *Schistosoma mansoni, S. mekongi, S. spindale* and *Paramphistomum* spp. The parasites' antigens (0.75 μg) were used for coating polystyrene microtiter plates and then allowed to react with 1:10, 1:100, 1:500 and 1:1,000 dilutions of MoAbs. The ELISA technique was performed essentially as described by Srivatanakul *et al.*[9]

Sera from *Fasciola* infected cattle

Four 2-3 months old trematode-free calves were infected orally with 500 *F. gigantica* metacercariae. An uninfected calf was used as the control animal. All of the animals were fed daily with hay and allowed to graze on the pasture near the animal house. The calves were bled once a week during the first 6 weeks of infection and every 2-4 weeks later until the experiment was terminated at 32 weeks after infection. Fecal samples were collected and examined for the parasite eggs by using Beads technique.[10]

Ninety-seven sera from animals naturally infected with *F. gigantica* were obtained from

National Animal Health and Production Institute, Department of Livestock, Ministry of Agriculture and Co-operatives, Bangkok, Thailand. The infection was confirmed by the finding of *F. gigantica* eggs in the feces.

Sandwich ELISA for detection of the 66 kDa circulating antigen

After optimization of the test conditions, the following ELISA procedure was performed; flat-bottom F96 maxisorp (Nunc-immunoplate) was coated with 100 µl of rabbit anti ST-antigens diluted to 1:1000 in coating buffer (0.01 M phosphate buffer, pH 7.2, containing 0.85% NaCl and 0.05% Tween 20) at 4°C overnight. The plate was washed with washing buffer and then blocked by 5% BSA in washing buffer at 37°C for 1 hour. The plate was then washed again before adding 100 µl of infected cow serum diluted to 1:50 with 10% fetal calf serum in washing buffer. The plate was incubated at 4°C overnight and then washed three times with washing buffer. MoAb (diluted to 1:100 with 10% fetal calf serum in washing buffer) was added and incubated for 1 hour at 37°C, and then washed 3 times with washing buffer. Rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase (HRP) enzyme (DAKOPATTS, Denmark) was added, incubated for 1 hour at 37°C, and washed three times with washing buffer. HRP enzyme substrates were added, and the plate was incubated for 30 minutes at 37°C. Color development was read at 490 nm with a Titertek Multiskan spectrophotometer.

Two sets of ELISA control assay were used. The first set of control was performed by using 21 samples of commercially available fetal calf sera (GIBCO BRL, USA.) and four serum samples from trematode-free baby calves obtained before infection with *F. gigantica* metacercariae. The second set of control assay was done by using 32 serum samples obtained from uninfected control calves, whose feces were devoid of the parasites' eggs. The mean optical density (OD) reading from each set of the control assay was calculated and used for the ELISA cut-off from the readings in the experimental assays.

RESULTS

Identification of antigenic molecules and specificity of MoAbs

Three stable clones of hybridomas were used in this study, namely, 1C12, 2C3 and 1H7. The monoclonal antibodies produced by these hybridomas were IgM class, and all reacted with 66 kDa of ST-antigens as shown by EITB analysis (Fig.1). These MoAbs were used for specificity study and ELISA for the detection of circulating 66 kDa antigen. It was found that all three MoAbs cross-reacted to varying degrees with the panel of antigens from four trematode species as already mentioned. However, a significant reduction of their cross-reactivity was observed when the MoAbs were diluted to 1:100, 1:500 and 1:1,000 (Fig. 2). Of the three MoAbs, 1C12 has been found to give the highest specificity and sensitivity; consequently, it was the main MoAb reported in this paper.

Detection of the circulating 66 kDa antigen in experimentally infected cattle

All of the four infected calves exhibited detectable levels of the circulating antigen at the first week after infection. Three levels of absorbance values were observed during the course of 32 week infection. The peak absorbance values were observed during the second and third weeks after infection. The absorbance values declined sharply beginning from the fourth week and remained rather stable until the eighth week after infection. The lowest but relatively stable level at the detectable level was observed beginning from week 12 until week 32. Among the four experimental calves studied, the highest absorbance value observed

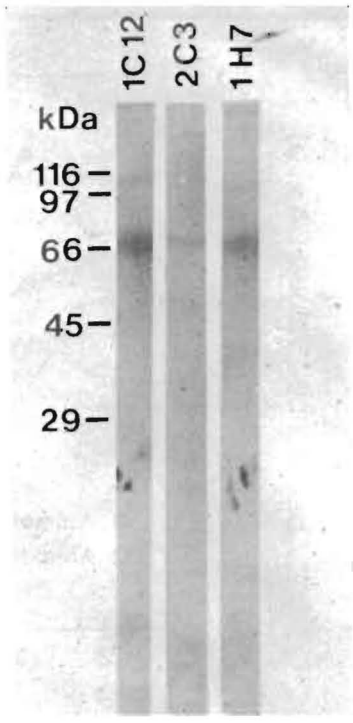

Fig. 1. Immunoelectrotransfer blot of 1C12, 2C3 and 1H7 monoclonal antibodies.

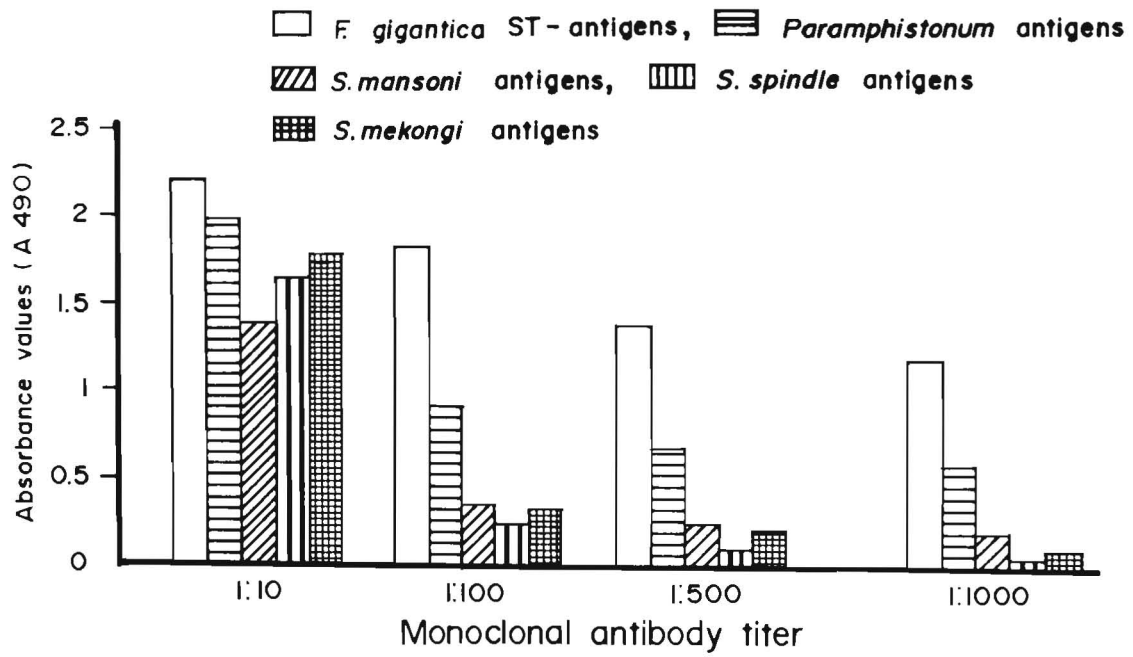

Fig. 2. Mean ELISA values of cross-reactivities studies between MoAb 1C12 and the panel of antigen from various trematode species.

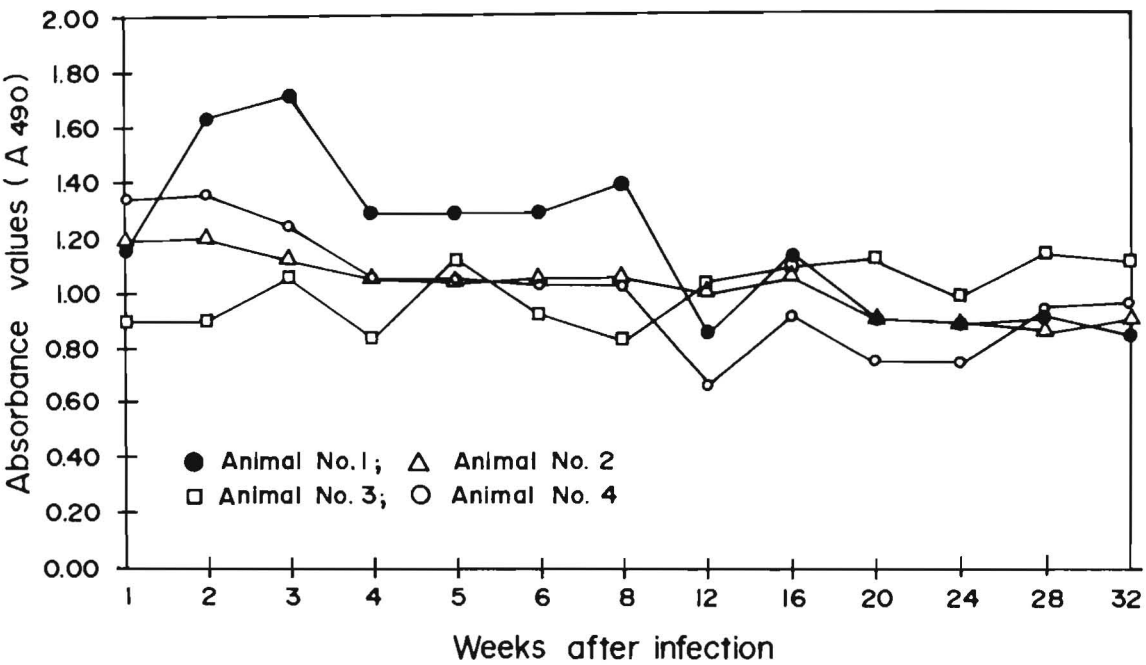

Fig. 3. Detection of circulating 66 kDa antigen in cattle experimentally infected with *Fasciola gigantica*.

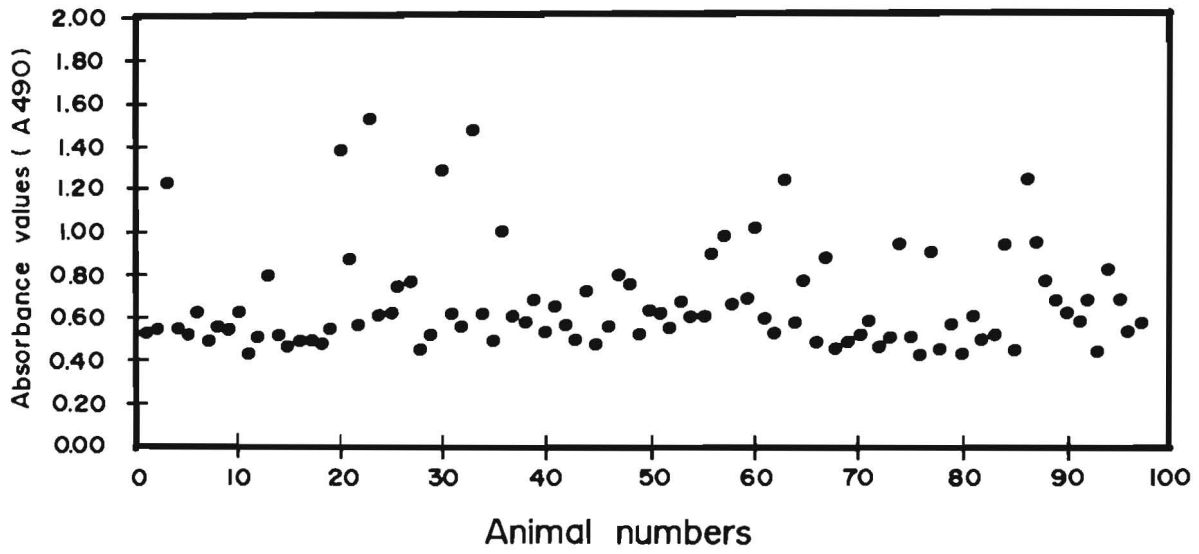

Fig. 4. Detection of circulating 66 kDa antigen in cattle naturally infected with *Fasciola gigantica*.

was 1.716, and the lowest value was 0.680 (Fig. 3). The mean cut-off value calculated from control fetal calf sera and sera from trematode-free baby calves was 0.3 ± 0.08, and that of the uninfected control calves used in the experiment was 0.5 ± 0.05.

Detection of the circulating antigen in naturally infected cattle

Sandwich ELISA was used for the detection of the circulating antigen in naturally infected cattle. A total of 97 serum samples together with corresponding fecal samples from sick animals were collected and examined by National Animal Health and Production Institute as a part of service provided for cattle farmers throughout the country. *Fasciola gigantica* eggs were detected in all of these fecal samples. Twenty three of these animals were also found to be positive for *Paramphistomum* eggs, and one was positive for *Paramphistomum* and *Schistosoma* eggs. Among the 24 animals positive for *Fasciola, Paramphistomum*, and *Schistosoma* eggs, the highest OD reading was 1.478 and the lowest was 0.449. Statistical analysis revealed the mean OD of 0.683 ± 0.253. Of the 73 animals which only *Fasciola* eggs were detected, the highest OD was 1.525 and the lowest was 0.426. The mean calculated OD of this group of animals was 0.666 ± 0.230. Using the cut-off point calculated from fetal calf sera and trematode-free baby calves (OD 0.3 ± 0.08), the sensitivity of the test was 100%. However, when the cut-off point from uninfected control calves (OD 0.5 ± 0.05) was used, the sensitivity was reduced to about 86.6 % (Fig.4).

DISCUSSION

Immunodiagnosis of cattle fasciolosis by the detection of circulating antigen has not yet been thoroughly investigated. Recently, Fagbemi *et al.*[4] successfully used rabbit antibodies against the 88 kDa antigen of adult *F. gigantica* for the detection of circulating antigens in experimentally infected cattle sera. The authors reported that circulating antigens can be detected as early as the second and third weeks after infection. In the present study a monoclonal antibody specific to a 66 kDa antigen of *F. gigantica* was used for detection of the circulating antigen in experimental and naturally infected cattle. This antigen was associated with the surface of the parasites' tegument[5] and the assay reported herein utilized the binding abilities of rabbit antibodies and the specific MoAb against this

antigen in sandwich ELISA method.

In the experimentally infected animals, high levels of circulating antigens were detected during the first, second and third weeks after infection in three of the four animals, while a relatively low but detectable level of circulating antigens was detected in the fourth calf. Detectable antigens in serum apparently decreased by the fourth week after infection and remained stable at the detectable level throughout the course of this study (Fig. 3). Apparently the levels of the circulating antigen in infected sera were correlated with the pattern of the worms' migration in the host. During the first few weeks young parasites penetrated the intestinal wall and migrated through the peritoneal cavity where they might release copious amounts of their antigens into the hosts' circulation. Once they reach and become established in the biliary system of the liver, they might release a smaller amount of antigens or these antigens might simply not gain access to the hosts' circulation as during the migrating stage. However, the result did indicate that the parasite antigen was still continuously released into the host circulation, even after the adult worms had already established themselves in the bile ducts.

Among the 97 serum samples obtained from naturally infected cattle the sensitivities of 100% and 86.6% were observed depending on the levels of the cut-off points being used. The high cut-off point observed in the uninfected control calf might be due to the possibility of the animal actually being infected with a few worms which failed to be detected by fecal examination. It is also possible that the animal may be infected with other parasites with cross reacting antigens, since it was reared under natural condition. Sensitivity of the assay as obtained in our experiment is similar to that reported by Fagbemi *et al.*[4] where about 87.5% of the infected animals were found to be positive. The sensitivity, however, was improved to 100% when the cut-off point was calculated from fetal calf sera and the trematode-free baby calf control set. Our study also indicated that the presence of *Paramphistomum* and *Schistosoma* parasites does not appear to interfere with the assay, since the mean OD (0.683 ± 0.257) of the group of animals infected with *Paramphistomum*, *Schistosoma* and *Fasciola* is only slightly higher than that of the animals infected with *Fasciola* alone (0.666 ± 0.230).

In the present study, we have shown that the circulating antigen was detectable as early as the first week after infection, with the peak levels occurred during the second and third weeks. Our result correlated with the report of Langley and Hillyer[11] who used mice as the experimental animals. On the other hand, based on the detection of parasite eggs in the hosts' feces, it was reported that the prepatent period of *F. gigantica* in cattle is between 114-116 days[12] and the circulating antibody levels usually reach the peak at about 16 weeks after infection.[13] Thus circulating antigen detection assay reported in the present study rendered much higher sensitivity, sufficient specificity and earlier detection than the parasitological examination or the circulating-antibody assay. This illustrates the potential improvement of immunodiagnosis for fasciolosis, especially during the acute infection.

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