

DEVELOPMENT OF AN ANTIBODY-BASED CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTING *ECHINOSTOMA CAPRONI* (TREMATODA) IN EXPERIMENTALLY INFECTED RATS: KINETICS OF COPROANTIGEN EXCRETION

Rafael Toledo, Ana M. Espert, Carla Muñoz-Antoli, Antonio Marcilla, Bernard Fried*, and J. Guillermo Esteban

Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain. e-mail: rafael.toledo@uv.es

ABSTRACT: The present study reports on the development of a coproantigen capture enzyme-linked immunosorbent assay (ELISA) for detecting *Echinostoma caproni* in experimentally infected rats. The capture ELISA was based on polyclonal rabbit antibodies that recognize excretory–secretory (ES) antigens. The detection limit of pure ES was 3 ng/ml in sample buffer and 60 ng/ml in fecal samples. The test was evaluated using a follow-up of 10 rats experimentally infected with 100 metacercariae of *E. caproni*, and the results were compared with those of other diagnostic methods such as parasitological examination and antibody titers determined by indirect ELISA. Coproantigens were detected in all the infected rats from the first day postinfection (DPI). The period of maximal coproantigen excretion was between 7 and 21 DPI. The values remained positive until 49–56 DPI, coinciding with the disappearance of the eggs in the stool samples of the infected rats. The kinetics of coproantigen detection were correlated with those of egg output. The present assay provides an alternative tool for the diagnosis of the echinostome infections. The proposed capture ELISA makes possible an earlier diagnosis than that provided by parasitological examination and indirect ELISA and also allows for the differentiation of past and current infections. Our results show that this assay can also be used to monitor the course of echinostome infections.

Echinostomes are intestinal trematodes with no tissue phase in the final host. Several vertebrates, such as aquatic birds and mammals, including humans (Graczyk and Fried, 1998), have been recorded as definitive hosts of echinostomes. Echinostomiasis in vertebrate hosts has been usually diagnosed by the presence of eggs in stool samples. However, this procedure is tedious, and eggs are not always present in feces because the worms may be preovigerous or mature worms may not be voiding eggs. Moreover, parasite maturation and egg production patterns may vary depending on different factors such as worm crowding or host species (Huffman and Fried, 1990; Toledo et al., 2003). Earlier studies have demonstrated that immunodiagnosis of echinostome infections is feasible by indirect enzyme-linked immunosorbent assay (ELISA) using different antigenic extracts. Agger et al. (1993) showed that anti-*Echinostoma caproni* antibodies are detectable in sera from experimentally infected NMRI mice by the 14th day postinfection (DPI) by using crude adult antigen. Graczyk and Fried (1994, 1995) detected anti-*E. caproni* immunoglobulins by 8 DPI in ICR mice by using glycocalyx membrane crude antigen from adult worms. However, the application of serologic diagnosis has major limitations, particularly in laboratory animals. For example, antibody titers persist after worm loss and also depend on the host species (Simonsen et al., 1991; Toledo et al., 2003). Moreover, animal handling to obtain a sufficient amount of serum samples is often difficult. These factors justify the need for alternative tools for the diagnosis of echinostomiasis both in natural and in experimental infections.

Coproantigen capture ELISA provides an early and specific diagnosis of trematode infections in humans and animals (Espino et al., 1997, 1998; Doumenigo et al., 1999). The main advantages are that coproantigens are detected earlier than antibodies and this assay detects current infection only. In the present article, we describe a polyclonal antibody-based capture ELISA for the immunological detection of excretory–se-

cretory (ES) coproantigens of *E. caproni* in experimentally infected rats. We have also compared the results obtained with those of other diagnostic methods such as parasitological examination of feces and indirect ELISA to evaluate the usefulness of the method described herein for diagnosis and monitoring of echinostome infections.

MATERIALS AND METHODS

Parasites and experimental infections

The strain of *E. caproni* has been described previously by Fujino and Fried (1993). Encysted metacercariae of *E. caproni* were removed from the kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* and used to infect rats. Ten outbred male albino rats (Wistar) weighing 110–150 g were each infected, via a stomach tube, with 100 metacercariae of *E. caproni*. Five 110- to 150-g rats were left uninfected and used as controls. All the animals were maintained under conventional conditions with food and water ad libitum. At 84 DPI, the rats were necropsied, and the number of worms recovered per rat was recorded.

ES antigens

To obtain ES antigens of *E. caproni*, we followed the methodology described by Toledo et al. (2003). Adult worms were collected from the intestines of rats 4 wk after experimental infection with 100 metacercariae of *E. caproni*. After thorough washings with phosphate-buffered saline (PBS, pH 7.4), the adult worms were maintained in medium at concentrations of 10 worms/ml for 12 hr at 37 C in PBS containing 0.8 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Missouri), 100 U penicillin (Sigma), and 100 µg/ml streptomycin (Sigma). The medium was collected and centrifuged at 15,000 g for 30 min at 4 C, and the supernatant was collected. The protein content was measured by the Bio-Rad (Hercules, California) protein assay and adjusted to 1 mg/ml using an ultrafiltration membrane (YM-3, Millipore, Bellerica, Massachusetts). The antigens were stored at –20 C until use.

Fecal and serum samples

Fecal samples were collected from each rat just before the experimental infection and daily during the first week postinfection (PI) and weekly from 7 to 84 DPI. Fecal samples were examined to determine the number of eggs per gram of feces (EPG), as described in previous studies (Toledo et al., 2003). Briefly, 24-hr fecal productions were collected from each animal and weighed individually. The individual samples were emulsified at 1 g/30 ml in a solution of 0.1 M NaOH. The mixture was shaken at room temperature (RT) for 2 hr and the sediment resuspended in 2 ml. The eggs contained in 200 µl were counted. Five

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* Department of Biology, Lafayette College, Easton, Pennsylvania 18042.

replicated samples were analyzed for each day and animal to determine the EPG, and the average \pm SD was calculated for each week of the experiment.

Fecal eluates for coproantigen detection were prepared as described by Espino et al. (1997), with some modifications. Briefly, 1 g of fresh feces from individual specimens was suspended in 3 ml of PBS containing 0.05% Tween 20 (PBST). These suspensions were centrifuged at 900 g for 30 min. The supernatants were filtered using a 0.20- μ m-pore size membrane (Nalge Nunc, Naperville, Illinois) and collected to measure coproantigens.

Blood was collected weekly from each infected and control rat by cardiac puncture under anesthesia. After clotting of the blood overnight at 4 C, serum was separated from the clot by centrifugation. The supernatant fecal eluates and the serum samples were stored at -20 C until use.

Polyclonal IgG anti-*E. caproni* ES antigens

Polyclonal IgG anti-*E. caproni* ES antigens were obtained by inoculating two 3-kg New Zealand white rabbits with ES trematode products. Three hundred micrograms of ES antigen was emulsified in 0.3 ml of Freund complete adjuvant and used to hyperimmunize each rabbit by giving several inoculations. Rabbits were given similar inoculations on 4 dates, at 2-wk intervals, using Freund incomplete adjuvant. Blood was drawn 21 days after the final inoculation. Serum was pooled, and the anti-*E. caproni* ES antigen globulins containing IgG were obtained by precipitation with 50% ammonium sulfate and then dialyzed extensively against PBS.

Biotinylation of anti-*E. caproni* antibodies

Antibodies against ES products of *E. caproni* were biotinylated as described by Harlow and Lane (1988). Briefly, the antibody solution was adjusted to 3 mg/ml and mixed with a 1% solution of biotin succinimide ester (Sigma) in anhydrous dimethyl sulfoxide (Panreac, Barcelona, Spain) at 80 μ g of biotin per milligram of antibody. The solution was incubated and rotated for 4 hr at RT. The uncoupled biotin was removed by extensive dialysis against PBS.

Indirect ELISA

The presence of antibodies against ES antigens of *E. caproni* was determined by indirect ELISA as described by Toledo et al. (2003), with some modifications. Polystyrene microtiter plates (Nalgene, Naperville, Illinois) were coated overnight at 4 C with 100 μ l/well of a 30- μ g/ml solution of ES antigens of *E. caproni* in 0.1 M carbonate-coating buffer, pH 9.6. The plates were washed 3 times with PBST. Uncoated sites were blocked with 5% nonfat dry milk in PBST. After incubation for 1 hr at 37 C, the plates were emptied, and 100 μ l of diluted serum (1:400 in PBST) was dispensed into each well. The plates were incubated for 2 hr at RT and then washed 5 times with PBST. One hundred microliters of horseradish peroxidase-conjugated goat anti-rat IgG (ICN Biomedicals, Aurora, Ohio) diluted 1:3,000 in PBS was added into each well and incubated for 2 hr at RT. The plates were washed as described above, and 100 μ l of the substrate solution (10 μ l hydrogen peroxide, 30% w/v + 25 ml of 0.1 M citrate buffer, pH 5.0 + 10 mg *o*-phenylenediamine hydrochloride) was added and incubated in the dark at RT. The enzyme reaction was stopped with 50 μ l of 3 N HCl per well. The plates were read at 492 nm in a Bio-Rad 550 microplate ELISA reader.

Polyclonal antibody-based capture ELISA

Polystyrene microtiter plates (Nalgene) were sensitized overnight at 4 C with 100 μ l per well of unlabeled anti-*E. caproni* antibodies at a concentration of 30 μ g/ml in 0.1 M carbonate buffer, pH 9.6. They were then washed 3 times with PBST, and the unbound sites were blocked with 5% nonfat dry milk diluted in PBST. After incubation for 1 hr at 37 C, the plates were emptied by aspiration. One hundred microliters of undiluted fecal eluates were added, and the plates were incubated for 2 hr at RT; thereafter, they were washed 5 times with PBST. One hundred microliters of biotinylated anti-*E. caproni* antibody, diluted 1:2,000 in PBST, was added to each well and incubated for 2 hr at RT, after which it was washed 5 times as described above. One hundred microliters of labeled streptavidin-peroxidase (Sigma), diluted 1:5,000

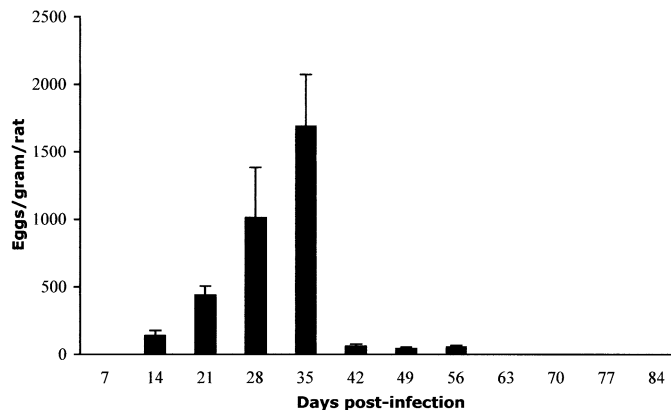


FIGURE 1. Mean EPG in rats experimentally infected with 100 metacercariae of *Echinostoma caproni* each week during the experiment. Vertical bars represent the SD.

in PBS, was added to each well and incubated for 2 hr at RT. The plates were washed as above, and 100 microliters of the substrate solution was added and incubated in the dark at RT. The enzyme reaction was stopped with 50 μ l of 3 N HCl per well. The plates were read spectrophotometrically at 492 nm as above.

Statistical analysis

Each ELISA assay was performed in triplicate, and the absorbance readings from wells with the same sample were expressed as the mean \pm SD. The cutoff for each ELISA was defined as the mean of the samples from the control rats + 3 SD. The difference between the optical density (OD) values for the infection and control specimens at each point in time was calculated and tested using Student's *t*-test. The correlation between the ES concentration in the spiked samples and the capture ELISA values was analyzed by the Pearson product moment correlation test. For correlation of the egg counts and capture ELISA values from 7 to 84 DPI, the Spearman nonparametric correlation test was used; *P* < 0.05 was considered as significant.

RESULTS

Development of the infection

All the rats experimentally exposed to 100 metacercariae of *E. caproni* became infected. The duration of the prepatent period was highly homogeneous. Egg release began at 10–12 (11.11 \pm 0.63) DPI. The kinetics of egg release was monitored during the first 84 DPI (Fig. 1). The egg release progressively increased during the first 5 wk of the infection, to reach a maximum at 35 DPI in all the rats. Thereafter, the EPG values suddenly declined. Eight (80%) of the rats were negative to egg examination at 49 DPI, whereas the other 2 (20%) remained positive until 56 DPI. From 63 DPI until the end of the experiments, all the samples were negative. No adult worm was detected at necropsy of the animals at 84 DPI.

Antibody titers

Antibody titers against ES antigens of *E. caproni* in the experimentally infected rats were investigated by indirect ELISA. The results showed that rats develop only a weak antibody response against *E. caproni* ES antigens (Fig. 2). Moreover, the antibody levels varied widely between individual infections. The cutoff point differentiating negative from positive sera was an OD value of 0.301. Antibodies to *E. caproni* ES antigens were detected in 3 (30%) of the infected rats at 42 DPI. At 49

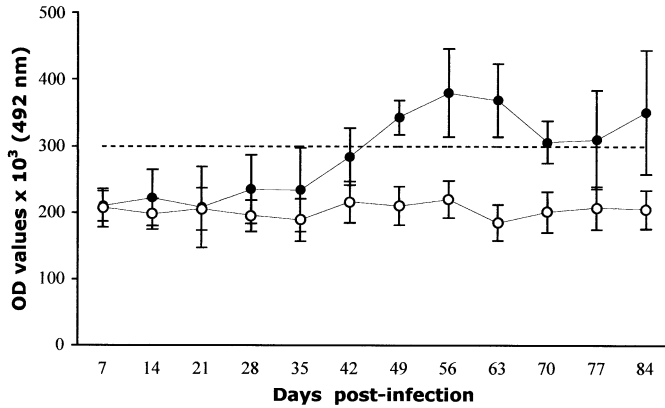


FIGURE 2. Detection of antibodies to *Echinostoma caproni* excretory-secretory antigens by indirect ELISA. Mean OD values of control (○) and infected (●) rats during the course of the experiment. (—) Cutoff point is defined as the mean OD value of the controls + 3 SD. Vertical bars represent the SD.

DPI all the infected animals were positive to indirect ELISA. Antibody levels became stable from 49 to 63 DPI. However, 4 (40%) of the infected rats reverted to negative values at 70 DPI, whereas the other 6 (60%) remained positive until the end of the experiment. The mean OD value during the complete course of the experiment in all the infected rats was 0.285 ± 0.062 , whereas the maximum OD value (0.381 ± 0.066) was observed at 56 DPI. Statistically significant differences ($P < 0.05$) between sera from control and infected rats were observed in each sample analyzed from 49 DPI until the end of the experiment.

Detection limits of the capture ELISA

The detection limits of the capture ELISA were determined by measuring serial dilutions of *E. caproni* ES antigen in sample buffer and fecal extracts from rats naive for helminths. The final concentration of ES antigen in the sample buffer or the spiked fecal extracts ranged from 0.001 to 250 $\mu\text{g}/\text{ml}$. The samples were then tested in triplicate to the capture ELISA. The detection limit of ES antigen was 3 ng/ml in sample buffer and 60 ng/ml in fecal extracts. A significant positive correlation was found between the OD values and the final concentration of ES antigen in the sample buffer ($r_p = 0.93$, $n = 20$, $P < 0.001$) and the fecal extract ($r_p = 0.89$, $n = 20$, $P < 0.001$).

Kinetics of *E. caproni* coproantigen excretion

The kinetics of the coproantigen excretion in rats experimentally infected with *E. caproni* was followed daily during the first week of the infection and weekly from 7 to 84 DPI (Fig. 3). The cutoff value for this technique, calculated as described above, was 0.200.

In all the infected rats, the coproantigen levels showed a characteristic and consistent pattern over time. The mean OD value in the infected rats over time was 0.263 ± 0.104 . All the samples analyzed were positive from the first DPI to 49 DPI. The *t*-test showed significant differences ($P < 0.05$) between the OD values from the infected and the control rats in each sample from 1 until 49 DPI. Antigens of *E. caproni* were detectable in the feces of all infected rats from 1 DPI. Infected animals showed time-dependent changes in coproantigen levels

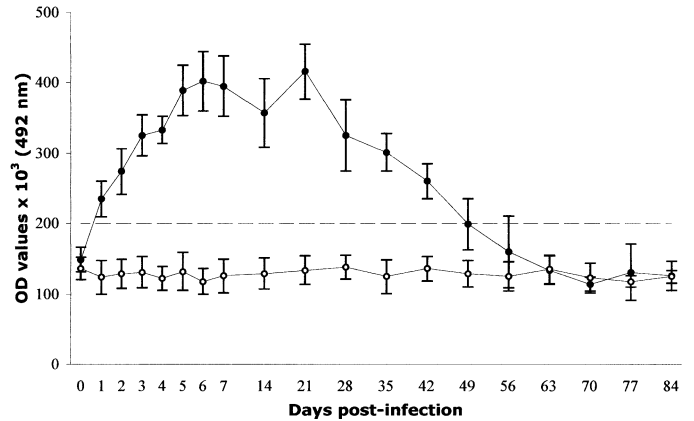


FIGURE 3. Detection of *Echinostoma caproni* excretory-secretory antigens in feces of experimentally infected rats. Mean OD values of control (○) and infected (●) rats during the course of the experiment. (—) Cutoff point is defined as the mean OD value of the controls + 3 SD. Vertical bars represent the SD.

(Fig. 3). The first days of the infection were characterized by a progressive increase in the ELISA values, reaching a period of maximal coproantigen excretion between 7 and 21 DPI. The coproantigen maximal response was observed at 21 DPI (0.421 ± 0.039), just before the period of maximal egg output. Thereafter, the OD values suddenly declined. From this day onward the OD values progressively decreased. In 8 (80%) of the infected rats, the OD values were positive until 49 DPI, whereas in the other 2 (20%), they remained positive until 56 DPI. The latter animals were those that remained positive to egg examination until 56 DPI. From this day onward all the samples analyzed were negative. A significant correlation between the absorbance values and the egg output in the infected rats from 7 DPI until the end of the experiment was found ($r_s = 0.67$, $n = 12$, $P < 0.05$).

DISCUSSION

The present study reports the development of a polyclonal antibody-based capture ELISA for detecting ES antigens of *E. caproni* in fecal samples of experimentally infected rats. The results demonstrate for the first time that the immunological detection of ES antigens of echinostomes in fecal samples is feasible from the first day after infection. In contrast, parasitological diagnosis is likely only by 10–12 DPI, and antibodies against *E. caproni* ES antigens are detectable only from 42–49 DPI by indirect ELISA. The detection limit of the present assay is high, with a detection capacity of 3 ng/ml in sample buffer and 60 ng/ml in fecal samples. The difference in the detection limit when diluting ES antigens in sample buffer or fecal samples indicates that the presence of fecal material interferes with the antigen detection method. However, the differences in the OD values observed between the samples from the infected and the uninfected control rats were of sufficient magnitude to determine the presence of ES antigens.

Diagnosis and monitoring over time of echinostome infections have been typically done by finding eggs in fecal samples, although the difficulties entailed in the parasitological procedures gave rise to the need for development of immunological methods of diagnosis (Graczyk and Fried, 1994). However, only

a limited number of studies have considered this topic, and only indirect ELISA methods have been developed (Simonsen et al., 1991; Agger et al., 1993; Graczyk and Fried, 1994, 1995; Toledo et al., 2003). These studies have demonstrated that anti-*E. caproni* immunoglobulins can be detected in experimentally infected mice by 14 and 8 DPI depending on the antigen used (Agger et al., 1993; Graczyk and Fried, 1994, 1995). Moreover, antibody detection methods have major limitations, i.e., the presence of antibodies indicates previous exposures rather than active infections, and considerably different patterns of anti-*E. caproni* antibody titers have been observed depending on the host species (Simonsen et al., 1991; Toledo et al., 2003). In the current study, detectable levels of antibodies were obtained only from 42–49 DPI, when the animals were free of parasites by parasitological criteria. Moreover, according to our results, rats seem to develop a weaker antibody response to *E. caproni* than mice. All these facts show that the utility of serologic diagnosis in echinostome infections is limited and alternative tools may be needed.

The coproantigen capture ELISA developed herein provides a rapid and sensitive diagnosis of an echinostome infection. The main advantages of the present assay over other diagnostic methods are that coproantigens are detected earlier than eggs (1 DPI vs. 10–12 DPI) and antibodies (1 DPI vs. 42–49 DPI) and that the present assay does not depend on the capacity of the host to develop antibody response. Other advantages of the present assay are that fecal samples are collected and hence avoid animal handling and possible damage for serum collection and a large number of animals can be screened without time-consuming parasitological examination procedures.

The use of coproantigen detection has been already successfully demonstrated for other trematodes such as *Fasciola hepatica* (Espino et al., 1997; Abdel-Rahman et al., 1998; Doumenigo et al., 1999; Almazan et al., 2001). However, the kinetics of coproantigen excretion is clearly different from that observed for *E. caproni* in relation to the respective life cycles. In *F. hepatica* infections, coproantigens are first detected approximately 4–6 wk PI, coinciding with the arrival of most of the flukes in the bile ducts and the subsequent release of the ES products to the bile. In contrast, echinostomes are intestinal digenans with no tissue phase. Consequently, ES products are released in the feces immediately after metacercarial excystment, and coproantigens are detected from the first DPI. The first days of infection are characterized by a progressive increase of coproantigen excretion. This is probably related to a period of high activity of the newly excysted metacercaria and juvenile adult worms. Further analysis of the products released in the first days of the infection may be of great interest because they could be involved in host–parasite interactions such as habitat location and establishment within the intestine and even in chemical communication and mate attraction. The presence of enzymes associated with excretory concretions in the metacercariae of *E. caproni* may be involved in some of these events as previously noted (Ursone and Fried, 1995).

We have found a significant degree of correlation between the capture ELISA values and the egg output from 7 DPI until the end of the experiment. The detection of coproantigen is maximal from 7 to 21 DPI, immediately preceding the period of maximal egg output. The high degree of coproantigen excretion during this period may be attributed to progressive

growth and maturation of the adult worms associated with a higher metabolic rate and increased shedding of surface antigens (Andresen et al., 1989). A parallel decrease of coproantigen release and egg output was observed, probably in relation to the decrease of the worm burden. Considering all the infected animals, coproantigen mean values were negative from 56 DPI until the end of the experiment. However, it is worth mentioning that at 56 DPI, 2 of the rats were still positive to capture ELISA and egg examination. These animals simultaneously reverted to negative values for both analyses at 63 DPI. Considering all these facts, the present assay may be a useful method for monitoring the course of the echinostome infections over time.

In summary, we have developed a polyclonal antibody-based capture ELISA to detect coproantigen in rats experimentally infected with *E. caproni*, providing a sensitive and rapid diagnostic tool. The present assay has allowed us to describe for the first time the kinetics of coproantigen excretion in an echinostome infection. Coproantigens are detected from the first DPI, and their excretion is correlated with the egg output. This makes this method a suitable tool to follow and analyze the course of echinostome infections.

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