DETECTION OF EXCRETORY/SECRETORY COPROANTIGENS IN EXPERIMENTAL HOOKWORM INFECTION

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Abstract. This report describes the detection of hookworm excretory/secretory (ES) antigens in soluble hamster fecal extracts by an enzyme-linked immunosorbent assay (ELISA). A rabbit polyclonal IgG antibody against *Ancylostoma ceylanicum* ES was used to capture hookworm coproantigens that were then detected using pooled, high-titer, infected hamster serum. The ELISA was capable of detecting ES proteins over a range of 10 ng/mL to 10 μg/mL when the antigens were diluted in buffer or uninfected fecal extract, and ES could be detected in infected hamster feces at dilutions up to 1:256. Examination of the kinetics of coproantigen production demonstrated that detectable amounts of ES were produced as early as four days after *A. ceylanicum* infection, whereas fecal eggs were not observed until day 17. Moreover, fecal ES levels correlated well with intestinal worm burden and could be detected in wet or dry stool samples stored for 14 days over a temperature range of −80°C to 37°C. The fecal ELISA was then adapted to analyze the excretion of AceES-2, a novel immunogenic ES protein recently cloned from *A. ceylanicum* cDNA. AceES-2 was found to be excreted in feces with kinetics similar to that of whole ES. Examination of individual hookworm antigens by this method will provide new insights into the molecular host-parasite interaction and may form the basis for future diagnostic methods.

INTRODUCTION

Hookworms are blood-feeding nematodes that infect more than 700 million persons worldwide.1 These intestinal parasites rank among the foremost causes of iron deficiency anemia and malnutrition in the developing world.2 Despite considerable recent progress in the understanding of hookworm molecular biology, pathogenesis, and immunology,3–6 at present hookworm infection is typically diagnosed much as it was at the turn of the last century, namely through the microscopic identification of eggs that are shed by gravid female parasites and passed in the stool of the host. Although fecal examination is a straightforward and well-accepted technique, it is time-intensive, can be affected by variations in egg output,7–9 cannot detect prepatent infections, and would be difficult to automate. Furthermore, a recent study has confirmed that the commonly used Kato-Katz thick fecal smear method has a relatively low sensitivity for hookworm eggs.7 Some of these issues may be addressed by using immunologic methods such as the detection of parasite-specific antibodies in serum.10–13 However, these approaches may in turn be complicated by the need to obtain blood samples, persistence of antibody responses following cure by chemotherapy,14 and immunologic cross-reactivity between co-infecting helminth species.15,16 Consequently, alternative strategies that exploit the utility of immunologic methods to analyze stool samples for parasite material could serve as a foundation for the development of next-generation diagnostic tools.

Here we describe the detection of hookworm excretory/secretory (ES) antigens in feces by an enzyme-linked immunosorbent assay (ELISA). The fecal ELISA was used to analyze samples obtained from hamsters infected with *Ancylostoma ceylanicum*, a model system that has been used extensively by our research group in studies of hookworm pathogenesis and vaccine development.17–23 After first demonstrating the sensitivity of the assay, the kinetics of fecal ES output were evaluated in *A. ceylanicum*-infected hamsters and compared with those of egg excretion. The relationship of fecal ES with intestinal worm burden was examined, and the effects of various sample storage conditions were determined. The fecal ELISA was then adapted to analyze the excretion kinetics of a single ES protein. In addition to forming a conceptual basis for future diagnostic methods, examination of individual hookworm antigens by this method will provide new insights into the molecular interaction between parasite and host.

MATERIALS AND METHODS

Parasites and hosts. The *A. ceylanicum* life cycle was maintained in 3–4-week-old male golden Syrian hamsters of the Lak:LVG(SYR)BR outbred strain as previously described.24 Hamsters were orally infected with 150–200 third-stage hookworm larvae (L₃). Upon development of adult worms (typically 21 days postinfection), the animals were humanely killed and the small intestines were removed. The intestines were dissected longitudinally and repeatedly rinsed with room temperature phosphate-buffered saline (PBS). This treatment caused most of the worms to detach from the mucosa; with the aid of a dissecting microscope, the detached parasites were gently removed from the intestinal debris with fine-tipped forceps and used to prepare ES products as described in this report. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals and were reviewed and approved by the Yale University Animal Care and Use Committee.

Parasite antigens. Live hookworms were rinsed with PBS and used to prepare ES products by incubation in sterile PBS (10 worms/mL) for six hours at 37°C. The worms were removed and the raw ES products were centrifuged at 3,000 × g for 15 minutes to remove particulates. The ES was then concentrated approximately 100-fold using a centrifugal concentrator with a 5-kD molecular mass cutoff (Millipore Corp., Bedford, MA) and protein content determined using the bichinchoninic acid system (Pierce Chemical Co., Rockford, IL) with a bovine serum albumin standard curve. Aliquots of
concentrated ES were stored at -80°C until use. The recombinant *A. ceylanicum* ES protein 2 (rAceES-2) was expressed in *Escherichia coli* using the pET-32 vector (Novagen, Inc., Madison, WI) and purified as previously described.\textsuperscript{20}

**Antibodies.** Rabbit antiserum was prepared by subcutaneously immunizing New Zealand rabbits with 400 μg of ES or 500 μg of rAceES-2 in Freund's complete adjuvant, followed by two booster immunizations at three-week intervals with 100 μg of antigen in Freund's incomplete adjuvant. Rabbit immunization, bleeding, and serum preparation were conducted by the Veterinary Clinical Services, Section of Comparative Medicine, Yale University School of Medicine. Rabbit IgG was purified from hyperimmune serum using HiTrap Protein G HP columns (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions. Immune hamster serum was prepared from animals infected 102 days previously with *A. ceylanicum*.\textsuperscript{17}

**Fecal extracts.** Hamsters were group housed in wire-bottomed cages and fecal pellets were collected onto damp cardboard, then frozen at -20°C until use (unless noted otherwise). Soluble fecal extracts were prepared by homogenizing thawed feces in PBS/0.05% Tween (PBS-T; 2 mL per gram of wet weight). The homogenate was centrifuged for 15 minutes at 3,300 × g to remove particulates and the supernatant was stored at -20°C. Immediately prior to use, thawed extracts were centrifuged for five minutes at 12,000 × g to remove any precipitated material.

**Fecal ELISA.** Immulon-2 microtiter plates (Dynex, Chantilly, VA) were incubated overnight at 4°C with 100 μL/well of rabbit anti-ES or anti-rAceES-2 capture IgG diluted to 10 μg/mL in PBS. The plates were rinsed four times with PBS-T and blocked for one hour at room temperature with 1% non-fat dry milk in PBS. Plates were rinsed four times with PBS-T following blocking and each subsequent step. Fecal extracts or purified ES were diluted in PBS-T to a final volume of 100 μL/well and incubated for three hours at room temperature. Captured antigens were then detected by incubation with a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-hamster IgG (ICN Biochemicals, Irvine, CA) for one hour at room temperature. When assaying for AceES-2, plates were further incubated with a 1:1,000 dilution of HRP-conjugated rabbit anti-goat IgG (ICN Biochemicals) for 30 minutes at RT to enhance sensitivity. Bound HRP was visualized by the addition of 100 μL/well of ABTS substrate solution (1 mg/mL of ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma, St. Louis, MO] in 0.1 M citrate buffer, pH 5.0, 0.03% H₂O₂). After one hour at room temperature, the absorbance at 405 nm was recorded using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). For experiments in which fecal antigen concentrations were determined, extracts from infected animals were diluted 1:2 (for ES) or 1:5 (for AceES-2) in PBS-T, with four parameter standard curves generated by addition of purified ES or rAceES-2 to similarly diluted uninfected fecal extracts. To correct for the difference in molecular mass between native (11.7 kD) and the recombinant AceES-2 fusion protein (29.4 kD), fecal AceES-2 concentrations are presented as molar values.

**Fecal egg counts.** Hookworm eggs were evaluated in hamster feces by a flotation method in which wet fecal pellets were mixed to ensure uniform distribution of eggs and an aliquot was suspended in a saturated NaCl solution (1 mL per 100 mg of feces). The sample was vortexed, strained through gauze into a 15-mL conical tube, and filled to the top with saturated NaCl. A glass coverslip was placed on the meniscus for 30 minutes, then gently removed and placed on a slide for microscopic counting of eggs. A fresh coverslip was placed on the meniscus, incubated, and counted as before; this was repeated until no additional eggs were recovered.

**Statistical analysis.** Data are presented as the mean ± SD. The relationship between fecal ES concentration and intestinal worm burden was evaluated by a Spearman rank correlation test. The effect of various sample storage conditions on fecal ES concentration was evaluated by an analysis of variance, followed by a Tukey-Kramer multiple comparisons post test. In each case, *P* values < 0.05 were considered significant.

**RESULTS**

**Sensitivity of the fecal ELISA.** To detect immunogenic *A. ceylanicum* ES products, a sandwich ELISA protocol was developed using a polyclonal rabbit anti-ES capture IgG antibody coupled with detection antibodies obtained from hookworm-infected hamsters.\textsuperscript{17} As shown in Figure 1, this method allowed ES proteins to be detected over a range of 10 ng/mL to 10 μg/mL when concentrated ES was serially diluted in PBS-T buffer or buffer containing 1:2 diluted uninfected hamster fecal extract. Comparison of optical density (OD) values obtained under the two reaction conditions indicated that fecal material had a modest inhibitory effect on the assay; this was found to be more pronounced at higher ES concentrations.

Once it had been established that known quantities of purified hookworm ES could be detected in fecal extracts by the

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\includegraphics[width=\textwidth]{figure1.png}
\caption{Detection of known quantities of *Ancylostoma ceylanicum* excretory/secretory (ES) products added to phosphate-buffered saline–Tween buffer (PBS-T) or 1:2 uninfected hamster fecal extract (FEX). All points are mean ± SD of background – subtracted optical density (OD) values obtained from duplicate samples.}
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sandwich ELISA, we then evaluated the method using samples from *A. ceylanicum*-infected hamsters. Extracts of pooled feces from infected and uninfected animals were serially diluted in PBS-T from 1:2 to 1:512. Figure 2 shows that mean OD values were higher for the infected samples at all dilutions up to 1:256, confirming the ability of the fecal ELISA to detect *A. ceylanicum* coproantigens shed by worms *in situ*. Since it was found to yield maximal signal with little background, the 1:2 dilution of fecal extract was used in all subsequent assays in which the concentration of fecal ES was determined. To compensate for the moderate inhibitory effect of fecal material (Figure 1), standard curves were prepared in 1:2 uninfected fecal extract.

**Kinetics of *A. ceylanicum* coproantigen excretion.** Having evaluated the sensitivity of the fecal ELISA, we next conducted a study to examine the kinetics of coproantigen production in *A. ceylanicum*-infected hamsters. A group of six hamsters was infected with 175 *A. ceylanicum* L₃, and their feces was collected over a 21-day period. Figure 3 shows that hookworm eggs were first observed in fecal samples on day 17, increasing sharply after day 19, and reaching maximal levels on day 21. In contrast, small but detectable quantities of ES were observed in the first week after infection, starting on day 4. The concentration of ES remained less than 1 µg/mL in fecal extracts until day 10, following which concentrations steadily increased, reaching a maximal value of 4.5 µg/mL by day 17. Fecal ES output remained above 4 µg/ml until day 19, then decreased somewhat over the next two days, coincident with the death of one of the animals on day 20.

**Correlation of ES coproantigens and worm burden.** To assess the relationship between fecal ES and intestinal hookworm burden, individual fecal samples recovered from 27 *A. ceylanicum*-infected hamsters at necropsy were analyzed by the ELISA. Intestinal parasite loads in these hamsters ranged from 3 to 114. Figure 4 shows that ES was detected in all the fecal samples, ranging from 65 ng/mL to 15.7 µg/mL. A positive relationship between fecal ES and worm burden was noted, which was highly significant (rₛ = 0.802, P < 0.0001, by Spearman rank analysis).

**Effect of sample storage conditions on the detection of fecal ES.** To evaluate the ability of the fecal ELISA to detect ES in feces prepared and stored under conditions that might be encountered in field settings, an experiment was conducted to assess the effect of various sample preparation methods and storage temperatures. Pooled wet feces was collected from *A. ceylanicum*-infected hamsters and portions were left untreated, mixed with the antimicrobial preservative sodium azide (3 mg/gram of feces), or air-dried. Aliquots of each preparation were sealed and stored for 14 days at one of five temperatures: 37 °C, room temperature (20–22 °C), 4 °C, −20 °C, and −80 °C. Extracts were then prepared and analyzed...
by the fecal ELISA. Figure 5 shows that although ES was detected in all samples, storage temperature–dependent effects on coproantigen levels were evident for each of the three sample preparation methods. For each method, progressively greater amounts of fecal ES were detected as the sample storage temperature decreased from 37°C to 4°C, although these increases were not statistically significant. However, decreasing the storage temperature to −20°C was associated with a significant two-fold increase compared with the 4°C values for each preparation method. Compared with its −20°C value, an additional significant increase in detectable fecal ES was noted in the untreated wet sample stored at −80°C, whereas values for the wet sample with sodium azide and the dry sample were relatively unchanged. At each of the storage temperatures treatment with sodium azide had no significant effect on fecal ES compared with untreated samples; only at −80°C did the dry sample have significantly lower ES values than the corresponding untreated wet sample.

Detection of a single hookworm antigen in fecal extracts. Having established the fecal ELISA as a useful method for the detection of ES coproantigens, we next studied the feasibility of assaying the excretion kinetics of a single hookworm antigen. To evaluate this possibility we selected AceES-2, a highly immunogenic 11.7-kD ES protein previously cloned from A. ceylanicum C DNA.20 The fecal ELISA was modified by substituting capture antibodies against recombinant AceES-2, increasing fecal extract dilutions from 1:2 to 1:5 (to minimize fecal interference observed when rAceES-2 was used to generate standard curves), and adding an additional detection antibody step to the protocol (to enhance sensitivity). The modified fecal ELISA detected rAceES-2 at concentrations as low as 2 ng/mL (approximately 66 nM) and native AceES-2 in hookworm-infected fecal extracts up to a 1:128 dilution. Analysis of hamster fecal extracts obtained from the A. ceylanicum infection study shown in Figure 3 showed that that similar to whole ES, small but detectable quantities of AceES-2 were produced in the first week after infection (Figure 6), well before eggs were observed. As was found for ES, AceES-2 concentrations in fecal extracts began a steady increase coincident with the onset of blood feeding, increasing from 1.2 μM on day 10 to a peak of 53.8 μM on day 19. The latter value corresponds to 630 ng/mL of the native molecule, or approximately 15% of the calculated concentration of total immunoreactive ES at this time point (Figure 3). The AceES-2 values decreased somewhat after day 19; this finding was similar to the ES data (Figure 3) and as previously noted was coincident with the death of one infected hamster.

DISCUSSION

Hookworm infection remains a major global health challenge for which novel diagnostic methods would be expected to improve epidemiologic studies and target control efforts. Accordingly, if simplified and inexpensive protocols are developed, the immunologic detection of hookworm coproantigens may represent an attractive diagnostic alternative to fecal egg assays. Coproantigen ELISAs have previously been reported for nematodes such as Haemonchus contortus25 and Teladorsagia circumcincta26 in sheep, Ostertagia ostertagi in cattle,27 Heligmosomoides polygyrus28 and Trichinella spiralis29 in mice, and Strongyloides ratti in rats.30 However, to our knowledge this is the first report of the technique being applied to the diagnosis and evaluation of experimental hookworm infection. As an initial proof of concept, the fecal ELISA was used to analyze samples obtained from hamsters infected with A. ceylanicum, a model system that has been shown to mimic the major clinical features of human hookworm infection, namely anemia and growth delay.17,24,31 When cultured ex vivo, A. ceylanicum adult worms produce numerous ES proteins,18 many of which are highly immunogenic in infected hamsters.18–20 These observations led us to hypothesize that ES proteins are also produced in significant quantity at the site of attachment in vivo, are shed in the feces, and could therefore be exploited as diagnostic markers.

As described in this report, the fecal ELISA was able to detect as little as 10 ng/mL of A. ceylanicum ES when purified antigens were added to PBS-T or uninfected fecal extract (Figure 1), a sensitivity that is comparable to results for other
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nematode species.\textsuperscript{26–28,30} Although additional studies of the sensitivity and specificity of the fecal ELISA will be necessary if the assay is to be adapted for use as a human diagnostic test, these preliminary results suggest that the fecal ELISA may eventually offer a means to diagnose low intensity infections that might not be detected by conventional microscopy, allowing for more accurate estimates of hookworm prevalence. We noted that OD values were generally lower when ES was diluted in fecal extract as opposed to PBS-T, indicating some inhibition of the assay by fecal material; similar findings have been reported for the previously cited fecal ELISAs for other nematodes. Johnson and others treated fecal extracts from \textit{H. polygyrus}-infected mice with proteolytic inhibitors, detergents, and sonication in an attempt to neutralize the inhibitory activity of feces in their ELISA; however, little improvement in sensitivity was noted.\textsuperscript{28} Our results indicate that despite some loss of signal caused by the fecal material, a suitable level of reactivity was retained in the ES-spiked samples. Furthermore, fecal material did not unacceptably perturb the detection of ES in samples from infected hamsters, which yielded maximal OD values at 1:2, the highest concentration of fecal extract to be evaluated (Figure 2).

In this report, we demonstrate a highly significant relationship between fecal ES and intestinal hookworm burden (Figure 4). This finding is in general agreement with the studies of \textit{H. polygyrus},\textsuperscript{26} \textit{T. circumcincta},\textsuperscript{26} and \textit{O. ostertagi}\textsuperscript{27} and suggests that estimates of hookworm load in humans may ultimately be possible by fecal ELISA. If so, this might represent an attractive alternative to methods that rely on extrapolation from egg counts or recovery of expelled worms following anthelmintic chemotherapy.\textsuperscript{8} It has been demonstrated that intestinal blood loss (as measured by fecal hemoglobin) is highly correlated with egg counts in humans,\textsuperscript{32} and future studies are planned to determine if a similar relationship exists between ES coproantigens and fecal hemoglobin. In addition to the longer-term goal of developing the fecal ELISA for the diagnosis and epidemiologic study of human infections, a more immediate application of the technique will be the study of pathogenesis and vaccination in animal models because it will allow continuous monitoring of infection status.

For an assay such as the fecal ELISA to be adopted for practical use in clinical or epidemiologic applications, it will be necessary to determine the conditions under which fecal samples may be stored prior to analysis. We show here that ES could be detected in samples subjected to a wide range of storage temperatures over a 14-day period (Figure 5). It was noted, however, that a statistically significant loss of signal occurred when samples were stored at temperatures above the freezing point. Compared with untreated wet feces, air-drying of samples prior to storage had relatively little effect on ES signal at temperatures other than \textdegree{-80°C}. Likewise, inclusion of sodium azide, an antimicrobial agent that has been previously used to preserve nematode eggs in fecal samples,\textsuperscript{33} had no significant effect on fecal ES concentration at any storage temperature. The reduced signal occurring in unfrozen samples is thus probably due to spontaneous breakdown of the ES proteins and/or the proteolytic action of gut-derived digestive enzymes, as opposed to microbial contamination. For the conditions evaluated here, these results suggest that initial sample treatment has less effect on the level of detectable ES than does subsequent storage temperature. Further study will be necessary to determine if other fecal treatments such as chemical fixatives\textsuperscript{34} will improve the stability of ES at higher storage temperatures to maximize assay sensitivity.

Among the more noteworthy findings in this study is the ability of the fecal ELISA to detect hookworm coproantigens early in the prepatent period. Although the chronic nature of most human hookworm infections means that in hookworm-endemic areas there would be few opportunities to detect prepatent infections, the ability to evaluate prepatent infections in experimental animals by fecal ELISA is of considerable interest because it provides a novel method that can characterize early events in the molecular pathogenesis of hookworm disease by examining the excretion kinetics of individual coproantigens. The onset of patency in other hamster strains infected with \textit{A. ceylanicum} has been reported to range between 12 and 21 days postinfection,\textsuperscript{24} and we have occasionally observed low levels of eggs as early as day 14 in the outbred strain used here (Bungiro RD, Jr. and Cappello M, unpublished data). It is therefore plausible that in this study small amounts of eggs were produced by adult females between day 14 and when they were first detected on day 17. In any case, analysis of total ES (Figure 3) and its constituent antigen AceES-2 (Figure 6) demonstrated that detectable quantities of coproantigens were produced by day 4, which is much earlier than egg production could have began and coincident with previously published estimates of the \textit{L}_2 to \textit{L}_4 molt in \textit{A. ceylanicum}.\textsuperscript{24} Moreover, the steady increases in ES and AceES-2 excretion that began on day 10 were concurrent with the \textit{L}_4 to pre-adult molt and subsequent onset of blood feeding in this species.\textsuperscript{24} The biologic function of AceES-2 is currently unknown but given its high degree of immunogenicity\textsuperscript{20} and expression kinetics as reported here, we hypothesize that it plays an important early role in hookworm pathogenesis, perhaps as an immunomodulator or decoy antigen. Additional evidence for a role of AceES-2 in pathogenesis includes our prior observation that mucosal immunization with the recombinant molecule confers partial protection against anemia following challenge infection.\textsuperscript{20} Further studies of AceES-2 by the fecal ELISA, as well as other secreted proteins that are proposed to function as virulence factors,\textsuperscript{3,6} will help to further clarify their role in the disease process. Moreover, adaptation of the fecal ELISA to the study of various single antigens should make it possible to develop species-specific diagnostic assays, perhaps ultimately using monoclonal antibodies and dipstick technology.\textsuperscript{35} Work is currently underway to evaluate the fecal ELISA as a diagnostic tool in communities endemic for the major anthropophilic hookworm species \textit{Ancylostoma duodenale} and/or \textit{Necator americanus}.

In conclusion, we have demonstrated a sensitive ELISA-based method for the detection of hookworm ES antigens in the feces of experimentally infected animals. The fecal ELISA detected prepatent infections, demonstrated a significant positive relationship between fecal ES and worm burdens, and provided a means to study the excretion kinetics of a single antigen. In addition to serving as a starting point for the development of future diagnostic methods, examination of hookworm antigens by the fecal ELISA is expected to provide new insights into the molecular pathogenesis of hookworm disease.
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