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TITLE: Development of a Sensitive and Specific Antigen-Detection System for Strongyloides Stercoralis and Hookworm Infections

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13. ABSTRACT (Maximum 200) ABSTRACT. Prototype antibody and antigen capture DS assays have been developed for the detection of <i>S. stercoralis</i> and human hookworms. Using commercial immunoreagents that were available during the time line of phase I, antibody capture DS manufactured in pilot production were able to differentiate helminth infected patients from uninfected patients, but were not able to differentiate between <i>S. stercoralis</i> and hookworm infected patients; subsequent immunoblot characterizations demonstrated that the antigen extracts used as capture antigen contained a large number of cross-reacting components. Antigen capture DS tests developed during phase I for the analysis of fecal samples have a sensitivity of detection of 5.0 ngs/ml or less. Data developed during phase I has elucidated a number of parasite specific antigens which will be useful in the production of efficacious, species specific antigen and antibody DS tests.				
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FOREWORD

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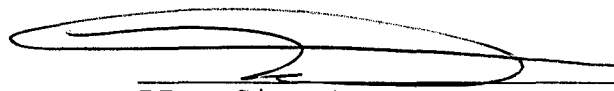
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I. Introduction.

Summary of Phase I. *Integrated Diagnostics, Inc.* ("*INDX*[®]") has completed preliminary work, during the course of the Phase I project reported herein, on development of rapid immunoassays for the soil transmitted helminth diseases hookworm (*Necatur Americanus*; *Ancylostoma duodenale*; *Ancylostoma caninum*) and strongyloidiasis (*Strongyloides stercoralis*). Specifically, prototype dipstick assays for these diseases have been developed in both antibody and antigen capture modes. Demonstrated sensitivity of the prototype dipstick assays is adequate for the development of efficacious diagnostics. Further, while specificity has proven to be a problem while limited to the antigen preparations *INDX* was able to out-source during Phase I, the data in hand is adequate to provide clear guidelines for expeditious development of the highly specific immuno-reagents required to distinguish *S. stercoralis* from hookworms in both antigen and antibody capture formats. Finally, immune compromised patients are especially vulnerable to life threatening complications when infected by these organisms. Thus, there is heightened need for simple, rapid and efficacious diagnostics for these diseases in tropical regions having a high incidence of HIV.

Background. Military personnel deployed to regions highly endemic for soil transmitted helminth diseases -- it is estimated that 1 billion people are infected worldwide -- are at high risk for infection with hookworms and/or *S. stercoralis* (1 - 3). The offending pathogens are often difficult to specifically identify, and failure to implement the correct antibiotic therapy in a timely fashion can result in the onset of acute symptoms and the development of chronic clinical and sub-clinical infections. Consequently, there is a need for logistically simple, rapid diagnostic tests which can confirm diagnoses, specifically identify helminth pathogens and contribute to disease surveillance; these tests must be efficacious for field use and use in underdeveloped countries, as well as in more formal clinical applications. The results of the Phase I work demonstrate that *INDX* dipstick tests can be formatted in either antibody or antigen capture modes to meet this spectrum of needs.

Hookworms are common in most tropical and subtropical regions and some temperate areas. As a family they represent the most common helminth known to infect humans. The two species which most commonly infect humans are *N. Americanus* and *A. duodenale* (3); the hookworm found predominantly in dogs, *A. caninum*, is also relevant. Adult hookworms live for up to thirty years attached to the mucosa of the small intestine, where they feed (mostly on blood) and deposit eggs which are passed with the stool. Blood loss and number of attached hookworms can both be shown to correlate with the number of eggs per gram of stool. The eggs hatch *ex vivo* and the resulting larvae infect new hosts, usually by penetrating the bare skin of the foot ("ground itch") or hand. After penetration the larvae migrate to the lungs, where they bore into the alveoli, are coughed up, and finally swallowed, thus returning to the gut. Larvae successfully completing this cycle find their way to the small intestine, where they attach to the mucosa, mature and begin laying eggs. Large numbers of larvae migrating through the lungs cause a clinical condition known as "Loeffer's syndrome". Common symptoms of active hookworm infections include blood loss with secondary iron deficiency anemia, abdominal pain and intestinal distress.

S. stercoralis is common in many areas of the tropics and subtropics, and is endemic to certain areas in the southeastern United States. Adult females reproduce parthenogenetically and live buried in the mucosa of the duodenum; males do not occur in the vertebrate host phase of the life cycle. Disease presentation is similar to hookworm with irritation at the site of entry of filariform larvae, migration through the lungs (Loefer's syndrome) and intestinal symptoms. Unlike hookworm, however, this parasite has the ability to auto-infect. Auto-infection is probably the major contributing factor in *S. stercoralis*' ability to persist in chronic infections for decades. Efficacious diagnosis of *S. stercoralis* is also important for immuno-compromised, alcoholic and/or malnourished individuals because they are susceptible to life threatening hyper-infections (5).

- **Clinical Manifestations and Treatment.** Hookworm infections are often characterized by severe anemia and iron deficiency. Treatment usually includes blood transfusions and a three day course of mebendazole and iron substitution and/or single dose albendazole outside the U.S. In the U.S. only thiabendazole is approved for treatment and is given for three consecutive days (4,5,6).

Strongyloides infections are usually chronic and may be asymptomatic. Severe infections are characterized by symptoms of peptic ulcer, abdominal pain, and diarrhea. Chronic infection may cause a malabsorption syndrome. The intestinal symptoms are proportionate to the degree of infection and as previously mentioned immuno-compromised patients are particularly at risk. Treatment is similar to hookworms.

Treatment of nematode infection is fairly standard with therapies using either ivermectin and thiabendazole (U.S.) Single dose ivermectin appears most efficient and better tolerated for Strongyloides than thiabendazole. There are reports that ivermectin is not effective against hookworms. Thiabendazole is quite toxic and not tolerated as well ivermectin. The U.S. FDA recently approved ivermectin for use during 1997, (T. Nutman, personal communications, 7,8).

Hookworm diagnosis is made by finding the thin-shelled eggs in **direct smears of feces**. They usually measure 58 - 176 um in length and 36 - 40 um wide and are partially embryonated. The adult rhabditiforms can be differentiated from those of *Strongyloides stercoralis* by differences in mouth parts and inconspicuous genital primordium. Eggs of human hookworms are indistinguishable from other species. The egg count is directly related to the severity of infection where a count of 25 per coverslip denotes a heavy infection which is likely to demonstrate symptoms (9,10,11).

Strongyloides infection is diagnosed by characteristic eggs in feces or on recovery of an adult that has been passed or vomited. 100 eggs/2 mg of feces is considered a heavy infection. The eggs are often embryonated and if stool is left out at warm temperatures, the filariform larvae will be released. These are directly infective and the samples are then hazardous

Other tests are also used to diagnose infection and to enrich for hookworm, Strongyloides, and other nematode infections. These tests include the (i) **Harada-Mori** technique for cultivation and concentration of larvae, the (ii) **Baerman Technique** utilizing charcoal, and an (iii) agar technique which is quite effective but costly. Indirect methods employ an (iv) **IgG ELISA test** for antibodies to Strongyloides and (v) western blot. The sensitivity of the ELISA test is reported to

be 100%. The sensitivity was respectively 92.2% and 13.3% among immune-competent and immune-compromised. When an Avidin-biotinylated ELISA was used, the sensitivity rose to 100% in both groups (12,13). The more sensitive method is recommended for use in hidden and disseminated strongyloidiasis. The antigen was made from freeze-thawed and sonicated preparations of the *Strongyloides*. Other reports suggest use of absorbents to get rid of cross-reactivity (14). References to ELISA tests for hookworm were not found.

Diagnosis of *Strongyloides stercoralis* remains a persistently difficult task and a rapid is of critical importance as eggs are often missed in the patient's stool sample. The current standard diagnostic approach of identification of eosinophilia, use of the CDC ELISA (F. Neva's) for antibody remains the most common approach to diagnosis used. An antigen detection assay would be of great use in monitoring efficacy of treatment as well as diagnosis, (M. Wolfe, personal communications).

The (vi) **dot enzyme-linked immunosorbent assay (dot-ELISA)** proposed for diagnosis *Strongyloides* and hookworm infections currently used in rickettsia diagnosis utilizes serial dilutions of rickettsial antigen spotted on nitrocellulose. Test kits (DS and reagents) for *R. typhi*, *R. rickettsii*, *R. conorii*, *R. tsutsugamushi*, and *C. burnetii* are commercially available (INDX). Although only a single serum concentration is tested, the antigen concentration detected by the test serum is proportional to the serum titer (see below). DS advantages lie in their relative rapidity, good sensitivity and specificity, and the ease with which they can be read. Unlike IFA, the tested DS can be kept as a permanent record. No special equipment is needed, but the INDX DS do require a 50°C water bath. INDX DS can be stored at room temperature although the reagents must be refrigerated. The 6-well DS format is flexible: (i) we can incorporate antigens of different specificities on a single stick (we now make scrub typhus DS with 3 concentrations of Karp strain + 1 screening concentration of Kato and Gilliam strains and we are using a DS with screening concentrations of 3-4 species of rickettsiae and other organisms); (ii) recombinant antigen can be used in some wells to enhance the test specificity (as we are considering in our dengue virus DS development); and (iii) the antigen concentrations can be tailored to specific regions to accommodate local significant threshold titers.

II. Phase I Progress and Results.

A. Methods.

NOTE: The methods reported below were developed or optimized during phase I specifically for the detection of *S. stercoralis* and hookworms.

A.1. Dipstick antibody capture assays.

Dipstick antibody capture assays used for the detection of anti- *S. stercoralis* and anti-hookworm antibodies include four steps:

1. Binding of serum antibodies to capture antigens immobilized on dipsticks (serum diluent or "binding buffer"; 5 mins; 50° C);

2. Wash and enhancement (enhancement buffer; 5 mins; 50⁰ C);
3. Binding of alkaline phosphatase conjugated anti-IgG/IgM to captured serum antibodies (labeling buffer; 15 mins; 50⁰ C); and,
4. Color development (color development buffer; 5 mins; 50⁰ C).

Each of the four steps are executed by immersing the dipstick containing immobilized capture antigens in 2.0 mls of the appropriate 50⁰ C buffer. Antibody capture is accomplished by adding 10.0 uls of serum to the 2.0 mls of serum diluent (*i.e.*, a 1:200 dilution) immediately prior to immersing the dipstick. The dipsticks are rinsed in dH₂O between each step.

A.2. Dipstick antigen capture assays.

The optimized method reported in this section is capable of detecting target antigens down to 5.0 nanograms/ml, and represents the end-product of numerous experiments concerned with optimization, including investigation of myriad assay parameters and rigorous evaluation of reagents and methods for labeling detection antibodies. The antigen capture assay is very similar to the antibody capture assay in terms of four step execution:

1. Binding of detection antibody-target antigen complexes to capture antibodies immobilized on dipsticks (binding buffer; 15 - 30 mins as required; 50⁰ C);
2. Wash and enhancement (enhancement buffer; 5 mins; 50⁰ C);
3. Binding of **SIGMA** (St. Louis, MO) *ExtrAvidin*-alkaline phosphatase conjugate (1:20,000) to biotinylated detection antibodies immobilized by successful complex formation (labeling buffer; 15 mins; 50⁰ C); and,
4. Color development (color development buffer; 10 mins; 50⁰ C).

All steps are executed by immersing the dipsticks containing immobilized capture antibodies into 2.0 mls of the appropriate 50⁰ C buffer. Dipsticks are rinsed in dH₂O between each step. Successful execution of antigen capture assays depends on formation of the capture antibody-antigen-detection antibody sandwich during step #1. This is accomplished by adding up to 100.0 uls of the sample to be tested (*i.e.*, potential source of target antigens) to the 2.0 mls of binding buffer in step #1; for antigen capture assays the binding buffer already contains freshly added, biotinylated detection antibody @ 1:500 - 1:2000 as required (see below for biotinylation details): during the 15 - 30 minute incubation used in step #1 complexes form between the immobilized polyclonal capture antibody and the target antigens, and also between the same antigens and the biotinylated polyclonal detection antibody, ultimately resulting in the formation of immobilized [on the dipstick] ternary complexes, or "sandwiches", composed of capture antibody-antigen-detection antibody.

A.3. Biotinylation of detection antibodies used in antigen capture assays: chemical biotinylation of ammonium sulfate precipitated IgG fractions.

The method reported below has been optimized based on the following criteria:

1. Amount of biotinylation;
2. Biotin reagent (and source);
3. Immunogenic reactivity of the resulting biotinylated IgG; and,
4. Nominal non-specific background noise.

The optimized procedure utilizes a biotin disulfide N-hydroxysuccinimide ester available from *SIGMA* (catalog # B 4531) which has been dissolved in dimethylformamide at 25.0 mgs./ml. Prior to biotinylation, the [ammonium sulfate precipitated] IgG fraction to be derivatized is equilibrated with 0.2 micron filter sterilized PBS - pH 7.6 in a *centricon-30* microconcentrator (*AMICON*, Beverly, MA). Subsequent to equilibration the concentration is adjusted such that the resulting OD₂₈₀ is 3.3. Chemical biotinylation is achieved by adding 25.0 uls of the DMF/biotin reagent per ml (biotin:IgG molar ratio is approximately 30), followed by mild reciprocal shaking at ambient room temperature for one hour. At the completion of the biotinylation reaction unreacted biotin is removed by successive dilution/concentration cycles in a *centricon-30* until the theoretical dilution of biotin is at least 5000 fold (recovery of derivatized product is greater using this method than gel filtration; removal of biotin is more effective than dialysis). The resulting biotinylated IgG is highly reactive in standard *INDX* antibody capture assays whether detection is accomplished with avidin-alkaline phosphatase or anti-IgG conjugated alkaline phosphatase. Non-specific background noise is quite minimal at operational dilutions (1:500 - 2:000) used for antigen capture assays.

A.4. Fractionation of antigens by polyacrylamide gel electrophoresis ("PAGE").

PAGE fractionation was accomplished in a *NOVEX* (Dallas, TX) minigel module on 4%-12% continuous gradient polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS). Gel dimensions were 8.0 cm (h) x 10.0 cm (w) x 1.0 mm (d). Immediately prior to electrophoresis samples of antigens to be fractionated were denatured by heat for ten minutes in loading buffer containing SDS and dithiothreitol (DTT); tris/MES run buffers contained the antioxidant sodium bisulfite, in addition to SDS, to maintain proteins in the fully denatured configuration during electrophoresis. Electrophoresis was at $v = 200$ (constant). Precast polyacrylamide gels, loading buffer (20x), run buffer (4x), DTT (10x), antioxidant (400x) and *MultiMark*TM multicolored molecular weight standards were purchased from *NOVEX*. The *MultiMark*TM multicolored molecular weight standards were calibrated in triplicate using native protein molecular weight standards purchased from *BioRad* (Hercules, CA).

A.5. Western blot transfer and immunostaining of PAGE fractionated antigens.

Conditions for semi-dry electroblot transfer, incubation conditions for 1^o and 2^o antibody binding, and color development mediated by alkaline phosphatase conjugated to the 2^o antibody, were optimized during the phase I project. For western blot experiments antigen preparations were denatured and fractionated by PAGE as described above. PAGE fractionated proteins were

transferred onto PVDF membranes by semi-dry electroblot transfer (1.0 hour @ $v = 25$ constant; *NOVEX* transfer buffer), followed by blocking in 13.0% (w/v) dehydrated milk for 30 minutes.

Blocked filters containing PAGE resolved antigens were probed with the appropriate primary antibodies (1:500 - 1:2000 as required) by incubation in binding buffer (step #1 above) for 30 minutes at 42° C. The filters were then washed by incubation for 5 minutes in room temperature enhancement buffer (step #2 above), followed by incubation for 30 minutes at 42° C in labeling buffer (step #3 above) containing an appropriate alkaline phosphatase conjugated anti-IgG @ 1:1000. Color development was achieved by means of *INDX*'s standard alkaline phosphatase developer solution (step #4 above).

B. Antibody Capture Dipstick Assays.

B.1. Pilot production of antibody capture dipsticks for detection of *S. Stercoralis* antibodies in human sera.

S. stercoralis antigen was obtained from *Antibody System, Inc.* (ABS), (Houston, Texas) in the form of extracts prepared from purified third stage larvae ("L3") in phosphate buffered saline ("PBS"). Dipsticks prepared with serial 2x and 4x dilutions up to 1:2048 were tested against confirmed positive and putative negative human sera to determine an appropriate range for pilot production runs. The range selected on the basis of these experiments was 1:16 - 1:1024.

Six spot antibody capture dipsticks for testing human sera were prepared with the *S. stercoralis* L3 extract antigen as follows: (spot #1) positive control (human IgG); (#2) negative control (PBS); (#3) *S.s.-Ag* = 1:16; (#4) *S.s.-Ag* = 1:64; (#5) *S.s.-Ag* = 1:256; (#6) *S.s.-Ag* = 1:1024 (*Figure 1*). This configuration unequivocally distinguished sixteen confirmed positive human sera from a panel of ten putatively negative human sera. Putative negative sera resulted in no colorimetric signal in spot #3 or a very weak signal in spot #3 that scored as +/- . Positive sera were clearly identifiable and amenable to semi-quantitative evaluation based on both the number of *S.s.-Ag* spots displaying signal and the strength of the resulting signals. For example: confirmed sera identified as case numbers 1810, 2391, 3294 and 4695 were provided by Dr. Thomas Nutman of the National Institutes of Health; a 1:200 dilution of 2391 gave a four spot signal (*i.e.*, signals in spots #3, #4, #5 and #6) ranging from 3+ (range = 0 - 4+) in spot #3 to 1 in spot #6; by comparison, a 1:200 dilution of 1810 gave only a two spot signal ranging from 2 in spot #3 to 1 in spot #4, with no detectable signal in spots #5 and #6; 3294 and 4695 were intermediate to 2391 and 1810, with 3294 > 4695 (*Figure 2*). These results were reproducible with different production lots of serum diluent, enhancer, alkaline phosphatase conjugated anti-IgG and color developer solution.

B.2. Specificity of antibody capture dipstick assays using outsourced capture antigens.

A. caninum adult extract prepared in PBS was obtained from *ABS*, and served as a model hookworm antigen in the experiments reported below. Anti-*S. stercoralis* and anti-*A. caninum* hyperimmune rat sera were obtained from *ABS* and were prepared using the *S. stercoralis* L3 (described in B.1.) and *A. caninum* adult extracts, respectively, as immunogens. Hookworm

antibody capture dipsticks were prepared using the *A. caninum* adult extract as capture antigen. The working antigen titration range was calibrated by serial two-fold dilutions as described in B.1., except that the confirmed positive sera were replaced with various titrations of anti-*A. caninum* hyperimmune rat serum (no confirmed hookworm positive human sera were available at this stage of phase I).

The antibody capture dipsticks containing *A. caninum* adult extract as capture antibody were configured as follows: (spot #1) positive control (human IgG); (#2) negative control (PBS); (#3) *A.c.*-Ag = 1:32; (#4) *A.c.*-Ag = 1:128; (#5) *A.c.*-Ag = 1:512; (#6) *A.c.*-Ag = 1:2048 (*Figure 1*). Sticks containing *A. caninum* antigen did not react not at all or only slightly (1/1+ signal in spot #3; no signal in spots #4, #5 or #6) with putative negative human sera, but reacted strongly with anti-*A. caninum* hyperimmune rat serum; these dipsticks also cross-reacted to some degree with anti-*S. stercoralis* hyperimmune rat serum. The reciprocal observation of cross-reactivity is observed with dipsticks containing *S. stercoralis* antigen, suggesting that the outsourced antigens were not providing the specificity requisite to specific helminth identification (note: **ABS** was the only identifiable commercial source with hookworm and *S. stercoralis* immuno-reagents [i.e., those described above] available for timely delivery). The relationship between the antigens and specificity of detection will be given more detailed treatment below (see D.1., D.2., and E.2).

All sixteen human sera confirmed positive for *S. stercoralis* cross-reacted to some degree with the dipsticks containing the *A. caninum* antigen. However, semi-quantitative estimates of reactivity did not uniformly correlate in a comparison of dipsticks containing *S. stercoralis* L3 antigen (as described in B.1.) vs. dipsticks containing *A. caninum* adult extract. Indeed, among twelve *S. stercoralis* positive human sera obtained from Thailand and provided to *INDX* by Dr. John Cross, some were clearly more reactive against the *A. caninum* antigen than against the *S. stercoralis* antigen (*Figure 3*); one case, designated SS-12 scored 4/4+ in spot #3 and 1+ in spot #6 on dipsticks containing the *A. caninum* antigen. We were therefore lead to the conclusion that at least some of these patients were infected with both hookworm and *S. stercoralis*. Dr. Cross subsequently used conventional ELISA assay methods to confirm that all 12 sera were indeed hookworm positive (personal communication).

B.3. Summary.

The prototype dipstick antibody capture assays described above, whether based on *S. stercoralis* L3 or adult hookworm whole animal extracts as capture antigen, are adequate to distinguish helminth infected individuals from uninfected individuals. However, neither variety of dipstick is adequate to specifically identify the helminth parasite. Data presented below will demonstrate that while the two whole animal antigen families have unique and specific immunodominant antigens, they also possess significant overlap in protein content based on western blot comparisons of size and immuno-reactivity. The western blot data will also show that the *A. caninum* adult extract contains a number of proteins found in normal dog feces.

C. Antigen Capture Dipstick Assays.

C.1. Preparation and evaluation of rabbit anti-*S. stercoralis* and Anti-*A. caninum* IgG: new

and more reactive polyclonal antibodies for use in antigen capture assays.

We have reported elsewhere (see: Month #3 Progress report) that the available hyperimmune rat sera and derivatives thereof were of limited value in the development of adequately sensitive antigen capture assays. We therefore investigated the efficacy of rabbit polyclonal antibodies generated *de novo* as part of the phase I project. The anti-*S. stercoralis* and anti-*A. caninum* rabbit polyclonals started becoming available to *INDX* during month #3. They were initially commissioned, as described below, to provide uniform, pilot scale lots of highly reactive anti-helminth polyclonal antibodies.

S. stercoralis L3 and *A. caninum* adult whole animal extracts, originally obtained from *ABS*, were supplied to *American Qualex, Inc.*, San Clemente, CA, (AQ) for inoculation into rabbits. After completion of a standard inoculation regimen, high levels of the desired reactivities were confirmed in ammonium sulfate precipitated IgG fractions and the total IgG fractions were subsequently harvested. All rabbit polyclonals used in experiments reported below were partially purified by ammonium sulfate precipitation before delivery to *INDX*. Two rabbit anti-*S. stercoralis* IgG preparations currently in the *INDX* inventory in large amounts are "SSR11" and "SSR12"; *ibid.* two rabbit anti-*A. caninum* IgG preparations designated "HWR13" and "HWR14". When tested and compared at 1:200 for anti-*S. stercoralis* and anti-*A. caninum* reactivity on the corresponding antibody capture dipsticks, the rabbit polyclonals are at least 16 times more reactive than the corresponding hyperimmune rat sera. However, the rabbit IgG preparations, like the rat sera, display substantial cross-reactivity (see D.2.).

C.2. Successful Development of Antigen Capture Assays Using Biotin/Avidin Detection Systems.

Rabbit anti *S. stercoralis* IgG preparation SSR12 was used as the capture antibody, and chemically biotinylated SSR12 was used as the detection antibody in experiments concerned with optimization of the antigen capture assay; the *S. stercoralis* L3 extract was used as the model antigen. Avidin/alkaline phosphatase color development reagents from *SIGMA* and *AQ*. were evaluated for reactivity and signal:noise and *SIGMA* reagent *ExtraAvidin-alkaline phosphatase* (catalog # E 2636) was ultimately selected and employed in all experiments. These reagents were tested in both the one- and two-step sandwich formation methods (see month #4 progress report) and were found to be effective in both. Only the one-step method will be discussed herein as this is the method of choice.

Optimized dipsticks for *S. stercoralis* antigen capture assays using native SSR12 as the immobilized capture antibody were prepared as follows: (spot #1) positive control (biotinylated IgG); (#2) negative control (PBS); (#3) SSR12 = 1:100; (#4) SSR12 = 1:200; (#5) SSR12 = 1:400; (#6) SSR12 = 1:800.

When biotinylated SSR12 IgG is used as the detection antibody at 1:1000, antigen capture dipsticks prepared with native SSR12 IgG as the capture antibody are capable of detecting *S. stercoralis* L3 antigen at concentrations down to 10.0 ngs/ml (lowest concentration tested). Further, 1.0 ug/ml, 500.0 ng/ml, 100.0 ng/ml and 10.0 ng/ml of antigen are quantitatively differentiable based on the number of reactive spots and signal strength, and 20.0 ngs/ml is differentiable from 10.0 ngs/ml on the basis of signal strength.

The most salient observations with the *S. stercoralis* system were reproduced with the *A. caninum* hookworm system using either HWR13 or HWR14 IgG preparations as the capture antibody and chemically biotinylated HWR13 or HWR14 as the detection antibody. The results in *Figure 4* demonstrate that when HWR14 is used as the capture antibody and chemically biotinylated HWR14 IgG is used as the detection antibody at 1:1000, antigen capture dipsticks configured as described above can detect adult *A. caninum* whole animal extract antigens at concentrations down to 5.0 ngs/ml (lowest concentration tested for either model system).

C.3. Summary.

Pilot scale methods and immuno-reagents have been developed for manufacture of sensitive antigen capture assays in a dipstick dot ELISA format. These dipstick assays employ ammonium sulfate precipitated rabbit IgG as the polyclonal capture antibody and homologous but chemically biotinylated IgG as detection antibody. The reagents and methods for preparation and detection of chemically biotinylated IgG have been fully optimized.

D. Immuno-analysis of western blots containing PAGE fractionated *S. stercoralis* L3 and *A. caninum* adult antigen preparations.

D.1. Results with anti-*S. stercoralis* and anti-*A. caninum* hyperimmune rat serum as primary antibodies.

Figures 5a and *5b* show the results of probing western blots of PAGE fractionated *S. stercoralis* L3 and *A. caninum* adult antigen extracts with anti-*S. stercoralis* L3 or anti-*A. caninum* adult hyperimmune rat sera, respectively, as primary antibodies (all immuno-reagents used in this experiment were sourced from *ABS*). As expected, each rat serum is highly reactive, against a broad range of protein sizes, with lanes containing homologous antigen. However, it is also clear that there is substantial cross-reactivity when anti-*S. stercoralis* serum is reacted with lanes containing *A. caninum* antigen, and *vice versa*.

The most prominent species specific band is seen with anti-*S. stercoralis* L3 serum x *S. stercoralis* L3 antigen, *i.e.*, the prominent band(s) seen at the bottom of the lane. This band migrates to a position corresponding to MW = 24,000 Daltons (*Figure 6a*), and can be isolated from the milieu of antigens by straightforward molecular sieving techniques (*Figure 6b*).

D.2. Results with anti-*S. stercoralis* and anti-*A. caninum* rabbit IgG as primary antibodies.

Figures 6a and *6b* show that the ammonium sulfate precipitated rabbit IgG fractions prepared for *INDX* by *AQ*, are more highly and uniformly reactive, with a broader size range of proteins and peptides, than their hyperimmune rat sera counterparts. Similar to previously described results with the rat sera, however, substantial cross-reactivity is evident with rabbit anti-*S. stercoralis* L3 x *A. caninum* adult antigen, and *vice versa*. These observations are in good agreement with the dipstick results described in section C.1. Finally, it is noteworthy that the rabbit IgG preparations have also elucidated abundant, species specific antigens.

Figure 7a shows that anti-*S. stercoralis* L3 rabbit IgG x L3 antigen resolves the abundant *S. stercoralis* specific band seen at the 24 kd position with the corresponding rat serum. However, the rabbit IgG also resolves an abundant and previously unseen *S. stercoralis* specific band at the 10 kd position. In addition, *Figure 7b* shows that anti-*A. caninum* adult rabbit IgG x *A. caninum* adult antigen resolves an abundant, *A. caninum* specific antigen at the 19 kd position.

D.3. Results with serum from Thai patient #SS06 as primary antibody.

Figure 8 shows the result when serum from Thai patient #SS06 is used as the primary antibody for immuno-detection of reactive antigens on a western blot containing PAGE fractionated *S. stercoralis* L3 and *A. caninum* adult extracts. Serum from patient #SS06 gave a strong response in dipstick antibody capture tests for both *S. stercoralis* and hookworm (*Figures 3a* and *3b*). Serum from patient #SS06 resolves an abundant *S. stercoralis* specific band(s) at the 28 kd position (lane 2). Close scrutiny of the developed filter reveals discrete, parasite specific bands in lanes 2 (*S. stercoralis* antigen) and 3 (*A. caninum* antigen). These results, which must be qualified as a single point determination, nonetheless suggest that a parasite specific serum test can be developed by exploiting unique markers such as the *S. stercoralis* antigen seen at the 28 kd position.

D.4. Results with E/S-1390 and E/S-1393 as primary antibodies.

Dr. Franklin Neva has defined a class of *ex vivo* *S. stercoralis* L3 proteins known as "excreted/secreted" or "E/S" proteins. Vigorous third stage *S. stercoralis* larvae (also known as the filariform life stage) are cultured *in vitro* in fresh RPMI-1640 medium. After 48 hours the larvae are harvested and the E/S proteins are collected from the conditioned culture medium by micro-filtration. Using peptides and proteins isolated in this fashion as immunogens, Dr. Neva generated a panel of polyclonal anti-E/S antibodies (15). Analytical amounts of two of these antibodies, designated "E/S-1390" and "E/S-1393", were made available to *INDX* by project collaborator Dr. Thomas Nutman of NIH.

Figure 9 shows western blots containing PAGE fractionated *S. stercoralis* L3 and *A. caninum* adult extract antigens. The filter shown on top (*Figure 9a*) was developed using E/S-1390 as the primary antibody. Highly abundant antigens reactive with E/S-1390 and common to both preparations can be seen at positions corresponding to 35 kd and 98 kd. Moderately to highly abundant antigens reactive with E/S-1390 but unique to the *S. stercoralis* L3 extract can be seen at positions corresponding to 20 kd, 28 - 33 kd, 70 kd, 90 kd and 140 kd.

The filter shown on the bottom in *Figure 9b* was developed using E/S-1393 as the primary antibody. Comparison to the filter shown in *Figure 9a* indicates that E/S-1393 is much more specific than E/S-1390 by two criteria: first, E/S-1393 reacts with a much smaller population of proteins and peptides than does E/S-1390; second, E/S-1390 reacts almost exclusively with proteins and peptides in the *S. stercoralis* L3 extract. E/S-1393 resolves at least six moderately to highly abundant antigens which are unique to the *S. stercoralis* L3 extract. These antigens are seen at positions corresponding to 11 kd, 20 kd, 22 kd, 34 kd, 95 kd and 125 kd.

D.5. Summary.

Immuno-detection of antigens on western blots of PAGE fractionated extracts of *S. stercoralis* L3 and *A. caninum* adult has been used to compare and characterize these two families of helminth antigens. Rat and rabbit antibody preparations prepared with these extracts as inocula exhibit substantial cross-reactivity. However, abundant, species specific antigens have also been elucidated with these preparations. Discrete antigens contained in these extracts also react with serum from infected human patients; some of these antigens are unique to one extract and suggest that a parasite specific serum test can be devised. Finally, *S. stercoralis* E/S antibodies exhibit highly specific reaction with the *S. stercoralis* L3 extract and serve to confirm the existence of discrete, parasite specific antigens.

E. Detection and analysis of *S. stercoralis* and hookworm antigens in normal, *S. stercoralis* infected and hookworm infected stool samples.

E.1. Antigen capture dipstick assays.

The only salient antibody preparations available to *INDX* for use in pilot production of *S. stercoralis* and hookworm antigen capture dipsticks were the *ABS'* hyperimmune rat sera against *S. stercoralis* L3 and *A. caninum* adult extracts, and the corresponding ammonium sulfate precipitated rabbit polyclonal IgG fractions prepared for *INDX* by *AQ*. Antigen capture dipsticks produced with the rabbit anti-*S. stercoralis* and anti-*A. caninum* IgG preparations SSR12 and HWR-14, respectively, as capture antibodies, are adequately sensitive when samples containing the homologous antigen extract are tested (5.0 - 10.0 ngs antigen/ml; see C.2.), but are ineffective for the analysis of normal and infected stool samples due to lack of specificity and/or lack of reactivity with parasite related antigens typically found in the stool of infected individuals (see below). However, by spiking various stool samples with *S. stercoralis* larvae, *S. stercoralis* larval extract, or *A. caninum* adult extract, we have undertaken preliminary investigations of methods for processing and assaying stool samples.

E.1.a. Example #1.

The first normal and infected samples of dog stool to arrive at *INDX* for the phase I project were supplied by Dr. Shad (project collaborator; University of Pennsylvania) in 10% buffered formalin. Our initial efforts to detect helminth related antigens in these samples by antigen capture assay were unsuccessful, generally due to very low signal strength and/or low signal:noise ratios. We therefore wished to verify the ability to detect antigens spiked into normal stool samples prior to fixation with 10% buffered formalin.

Figure 10 shows the results of spiking fresh normal stool with either the *S. stercoralis* L3 extract or whole third stage *S. stercoralis* larvae. Spiked stool was homogenized in 10% buffered formalin to form a suspension, and was subsequently allowed to stand overnight before processing for antigen capture assays in order to allow the spiked antigens to become "hard fixed" in and with the fecal milieu. The dipsticks shown in *Figure 10* contain anti-*S. stercoralis* rabbit IgG (SSR12) as the capture antibody in the following configuration: (spot #1) positive control (biotinylated IgG);

(spot #2) negative control (PBS); (spot #3) SSR12 = 1:100; (spot #4) SSR12 = 1:200; (spot #5) SSR12 = 1:400; (spot #6) SSR12 = 1:800. The detection antibody used in this experiment was chemically biotinylated SSR12. The dipstick reacted with un-spiked normal dog stool shows only a very weak +/- signal in spot #3. The dipstick reacted with the stool spiked with L3 extract shows clear signals in spots #3 and #4 at a minimum sensitivity of 250 ngs/ml. Finally, the dipstick reacted with stool spiked with third stage larvae shows positive signals in spots #3 and #4. Third stage *S. stercoralis* larvae used in this experiment were supplied by Dr. Shad as snap frozen after washing in PBS; 130 mgs of the frozen larvae/PBS block were added to 500 mgs of stool prior to addition of formalin and subsequent homogenization.

E.1.b. Example #2.

We have investigated whether hidden antigens, *i.e.*, antigens complexed with host antibody, can be uncovered by antibody stripping with acid glycine + 10 minutes at 70^o C. *Figure 11* shows that stripping has little or no effect on the detection of *S. stercoralis* L3 antigen spiked into stool. Spiked stool for this experiment was prepared as described above, except that after overnight incubation in 10% formalin, one aliquot each of spiked, unspiked and buffer control samples was processed through the stripping and tris base neutralization procedures prior to antigen capture assay. Assays of stripped and unstripped normal and spiked samples contained equal amounts of stool in step #1. Minimum sensitivity of detection was 100.0 ngs/ml in the spiked sample.

E.1.c. Example 3.

Figure 12 shows the detection of *A. caninum* adult extract antigens in serially diluted samples utilizing dipsticks containing SSR12 as the capture antibody (as described in E.1.a.) and chemically biotinylated HWR14 as the detection antibody. *Figure 5a* shows that SSR12 substantially cross reacts with antigenic components of the *A. caninum* adult extract; however, as confirmed and clarified by immunoblot experiments reported below, SSR12 dipsticks do not react with antigens in normal feces while HWR14 dipsticks do. For this experiment a sample of normal dog stool was spiked with *A. caninum* adult extract followed by homogenization in 10% buffered formalin. Serial two-fold dilutions were made by diluting the homogenized and spiked sample with a suspension of unspiked normal stool homogenized in 10% formalin. Antigen capture assays were executed after overnight incubation of the homogenized samples in 10% formalin buffer. The unspiked stool sample used as diluent also served as the negative control and produced only a weak +/- signal in spot #3. The spiked samples, however, produced unambiguous positive signals in spot #3 down to 15.6 ngs/ml, the lowest concentration tested, and unambiguous positive signals in spot #2 down to 62.5 ngs/ml.

E.2. SSR12 and HWR14 immuno-detection of reactive antigens on western blots containing PAGE fractionated fecal samples from normal and infected dogs.

Data reported above indicate that the dipstick antigen capture assays utilizing SSR12 (rabbit IgG against *S. stercoralis* L3 extract) and HWR14 (rabbit IgG against *A. caninum* adult extract) as the capture antibodies can detect reactive helminth antigens *if* they are present in stool. Therefore, the failure to unambiguously detect such antigens in the unspiked stool of experimentally infected dogs suggested that the antibodies generated with commercially available antigen preparations, *i.e.*, *S. stercoralis* L3 extract and *A. caninum* adult extract, were poorly

matched to helminth antigens assumed to be present in stool. Support for this notion derives from consideration of the reproduction and life cycles of the parasites.

The third larval stage of *S. stercoralis* represents the infectious filariform stage of the organism's life cycle. Filariform larvae are typically found in the small intestine. However, feces of *S. stercoralis* infected individuals can be expected to contain recently hatched rhabidiform larvae and E/S proteins from filariform larvae, but few if any filariform larvae. Similarly, hookworm adults are found primarily in the small intestine, while feces from infected individuals can be expected to contain embryogenic eggs and E/S proteins from adults, but few if any adults.

We therefore developed methods to prepare fecal samples for effective PAGE fractionation, and used western blots of PAGE fractionated samples to characterize helminth related antigens.

Fresh feces from normal dogs, *S. stercoralis* infected dogs + one experimentally infected human patient, and hookworm (*A. caninum* and *A. duodenale*) infected dogs were homogenized in liquid nitrogen, with a mortar and pestle, until reduced to fine powder. The still frozen powder from each sample was uniformly suspended 1:10 (w/v) in deionized water which contained 0.5% (v/v) methanol and 1.0 mM phenylmethylsulfonyl fluoride (PMSF). An aliquot of each suspension was diluted with an equal volume of 4x *NOVEX* PAGE loading buffer, resulting in a final sodium dodecyl sulfate (SDS) concentration of 4.0% (w/v), followed by heating at 90° C for 10 minutes. The samples were stored overnight at 4° C, and then heated a second time for 10 minutes at 90° C prior to removing an aliquot for PAGE fractionation. For PAGE fractionation an aliquot of each sample was diluted with an equal volume of deionized water containing the prescribed amount of *NOVEX* dithiothreitol (DTT) protein reducing reagent, followed by heating a third and final time at 90° C for 10 min. The double heat denaturation employed prior to preparing an aliquot for PAGE fractionation significantly improved the resolution of discrete bands. *A. caninum* eggs harvested by floatation in ZnSO₄ solution were similarly processed for PAGE fractionation except that eggs were not homogenized.

Figures 13a and 13b show western blots which were probed with SSR12 and HWR14, respectively. Consistent with results discussed above, SSR12 displays negligible reactivity with antigens in normal dog feces. Further, SSR12 is reactive with a cluster of antigens (55 - 60 kd) in *S. stercoralis* positive stool, but not in a consistent fashion: stool from one infected dog has a relatively strong signal; stool from a second infected dog has no signal; stool from an experimentally infected human has a weak signal.

The filter shown in *Figure 13b* illustrates that HWR14 reacts with abundant antigens which are common to stool samples from normal dogs and dogs infected with both parasites. The results also suggest, however, that HWR14 reacts with helminth specific antigens seen primarily in lanes containing the *S. stercoralis* positive samples.

E.3. Identification of *S. stercoralis* specific E/S proteins in stool samples from infected dogs.

Western blots containing PAGE fractionated stool samples were probed with E/S-1390 and E/S-1393 as primary antibodies. *Figure 13* shows the filter probed with the more specific E/S-1393 (see D.4. and *Figures 9a and 9b*). The results shown in *Figure 14* confirm the presence of *S. stercoralis* specific E/S proteins in the stool of infected individuals. However, they also indicate that the pattern and distribution of E/S proteins is not uniform: one positive sample has

moderate to highly abundant *S. stercoralis* specific E/S proteins at positions corresponding to 42 kd, 50 kd and 110 kd; a second positive sample has very highly abundant *S. stercoralis* specific E/S proteins at positions corresponding to 30 kd and 40 kd, and moderately abundant *S. stercoralis* specific E/S proteins at positions corresponding to 80 kd and 110 kd. The E/S protein seen at 110 kd is common to both *S. stercoralis* positive samples, but is not seen in the normal or hookworm positive samples.

E.4. Summary.

The available antigen capture dipsticks are capable of sensitive detection of helminth antigens spiked into fecal samples, even after hard fixing in 10% buffered formalin. However, various immunoblot data shown above indicate that the anti-helminth capture antibodies available for manufacture of these dipsticks are poorly matched to helminth antigens actually contained in feces from infected individuals. However, the results with E/S-1393 indicate that moderately to highly abundant, species specific E/S proteins are manifest in feces and can be exploited (used to capture antigens, purify antibodies, etc.) for the development clinically efficacious antigen capture dipstick tests using polyclonal or monoclonal antibodies generated.

III. SUMMARY OF RESULTS.

Phase I of this project was focused on four areas of effort:

1. Development of prototype antibody and antigen capture assays, in dipstick dot ELISA formats, for the detection of *Strongyloides stercoralis* and hookworms with clinical relevance to humans;
2. Evaluation of commercially available and other outsourced immuno-reagents for *S. stercoralis* and hookworm dipstick assays;
3. Generation of an empirical data base and other information requisite to the development of antigen and antibody preparations necessary for clinically efficacious and parasite specific detection of *S. stercoralis* vs. hookworms, including;
4. Identification of relevant antigens for use in fecal antigen capture assays specific for *S. stercoralis* vs. hookworms.

We have reported the development of antibody capture dipstick assays capable of clinically efficacious detection of anti-helminth antibodies in human sera. These assays can qualitatively and semi-quantitatively differentiate uninfected patients from those infected with hookworms or *S. stercoralis*, but cannot differentiate hookworm infected patients *from* those infected with *S. stercoralis* (see *Figures 2* and *3*) at this time. Pilot production lots of dipsticks were prepared with the only commercially available antigens germane to the phase I project, *i.e.*, *S. stercoralis* third stage larval extract ("SsL3") and *Ancylostoma caninum* adult (canine hookworm)

extract ("AcA"). Both of these extract type antigens were obtained from *ABS* at the beginning of the phase I project, and a portion of each were in turn supplied to *AQ* for the purpose of generating pilot production amounts of anti-*SsL3* and anti-*AcA* rabbit IgG. No other materials were readily available for the preparation of antibodies and the phase I timeline did not provide for the de novo development of adequate antigen preparations.

Subsequent investigations, based primarily on PAGE fractionation followed by immunoblot characterizations, demonstrated that the *ABS* extract antigen preparations share a wide range of cross-reacting proteins and peptides. In addition, the *AcA* preparation was shown to contain a number of antigens found in normal dog feces (*SsL3* does not): the *A. caninum* adults used in preparation of this extract were harvested and immediately washed in PBS and homogenized; no steps were taken to clear the mouth, bucal cavity or gut of these organisms prior to homogenization, resulting in contamination with fecal antigens. Consequently, neither of these antigen preparations, nor antibodies generated by means of these antigens, are adequate for parasite specific assay development. However, we have confirmed the existence of abundant, parasite specific antigens in both the *SsL3* and *AcA* extracts. Using microanalytical detection and recovery techniques (to be described in the phase II proposal), such antigens can be rapidly exploited, as required, for the expedited development of parasite specific assays.

We have also reported the development of reagent synthesis and pilot scale manufacturing methods for the production of sensitive dipstick antigen capture assays. The prototype dipsticks for *S. stercoralis* and hookworm antigen capture assays contained the above mentioned rabbit IgG preparations as the immobilized capture antibodies, and utilized homologous but chemically biotinylated rabbit IgG as detection antibody. These immuno-reagents provided an antigen capture sensitivity of 10.0 ngs/ml (lowest concentration tested) for the *SsL3* based model system, and 5.0 ngs/ml (lowest concentration tested: see *Figure 4*) for the *AcA* based model system. In addition, these dipsticks were useful in developing techniques for antigen capture assays performed on fresh and formalin fixed stool samples spiked with *SsL3* or *AcA* antigen. However, in addition to lack of parasite specificity, antibodies generated with these antigens are not largely reactive with parasite related antigens found in the stool of infected dogs.

Infected stool from *S. stercoralis* patients (or dogs) can be expected to contain newly hatched rhabidiform larvae, and excreted/secreted ("E/S") proteins from filariform larvae (*i.e.*, *SsL3*) attached to the mucosa of the duodenum. We have confirmed the presence of *S. stercoralis* specific E/S proteins in infected dog stool by probing western blots of PAGE fractionated feces with two anti-*S. stercoralis* E/S polyclonal antibody preparations. Analytical quantities of these antibodies, designated "E/S-1390" and "E/S-1393", were available to *INDX* via project collaborator Dr. Thomas Nutman (NIH). E/S-1393 revealed the presence of highly specific (not seen in normal or hookworm infected stool), abundant antigens (see *Figure 14*). By comparison, the anti-*SsL3* rabbit IgG was largely unreactive with similar western blots; however, a potentially unique and specific *S. stercoralis* antigen was seen in one of three infected stool samples tested (see *Figure 13*). As above, micro-analytical detection and recovery techniques will be used during phase II to harness E/S and other antigens, as required, in the rapid development of efficacious, *S. stercoralis* specific antigen capture tests for stool.

Infected stool from hookworm patients (or dogs) can be expected to contain embryogenic eggs, and E/S proteins from adults attached to the mucosa of the small intestine. During the last month of the phase I project *INDX* was able to obtain fresh and formalin fixed dog feces from hookworm (*A. caninum* or *A. duodenale*) infected dogs. When western blots of PAGE fractionated

normal, hookworm and *S. stercoralis* infected dogs were probed with anti-AcA rabbit IgG, a number of antigens were consistently resolved in normal feces. However, it is noteworthy that a number of helminth specific bands not seen in normal dog feces were resolved in three of three *S. stercoralis* feces samples (2 dog; 1 human) (see *Figure 13*).

I.V. Conclusions and discussion.

The data presented above demonstrate the feasibility of utilizing antigen and antibody capture DS assays for the detection of helminth infections. DS for antibody capture were able to differentiate infected from uninfected patients, but, due to limitations in the specificity of the available antigens, were not able to differentiate *S. stercoralis* from hookworm infections. However, it has been shown that among the components of the extracts exist antigens which are parasite specific, thus providing the potential to develop specific DS antibody capture assays.

Highly sensitive fecal antigen capture DS assays for *S. stercoralis* and hookworms have been developed. However, while the immuno-reagents available to *INDX* for this segment of the phase I study proved invaluable as model systems, they did not correlate well with helminth antigens actually present in feces from infected individuals. Thus, we were forced to rely on spiking experiments for method and technique development. Nonetheless, an extremely important observation which emerged from the analysis of fecal antigens was detection of abundant *S. stercoralis* specific E/S proteins. Antigens of this type, many of which are probably represented as clones in *S. stercoralis* cDNA libraries recently made available to *INDX* by Dr. Thomas Nutman (NIH project collaborator), will provide the means to construct efficacious, parasite specific antigen capture DS tests for rapid fecal analysis.

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FIGURE 1

Typical Dipstick Configuration

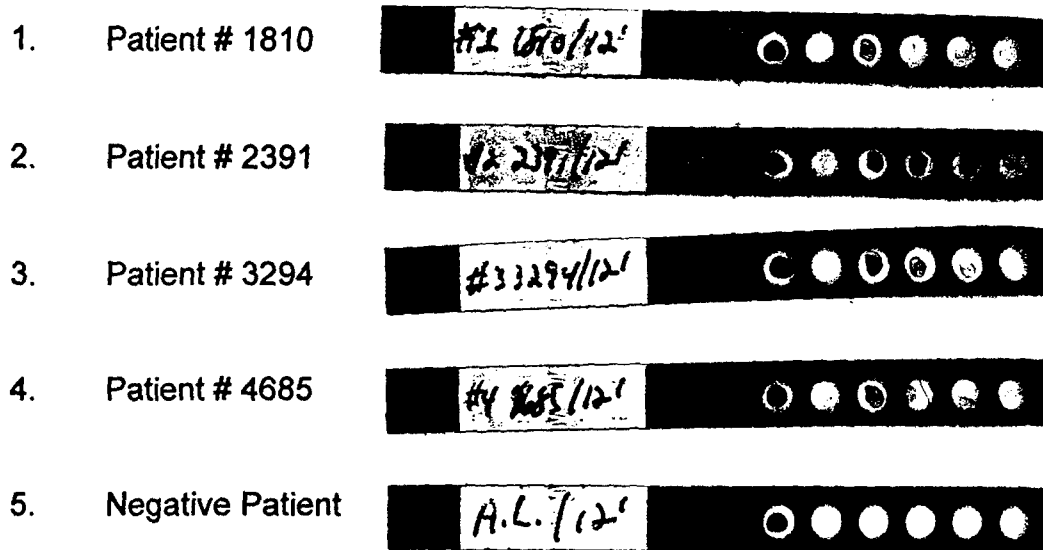


Shown above is a typical six (6) spot dipstick . Dipsticks for capture of *S. stercoralis* antibodies contain *S. stercoralis* L3 extract as capture antigen and have the following configuration:

<u>Spot #</u>	<u>Spot contents</u>
1.	IgG (positive control)
2.	PBS (negative control)
3.	SsL3 capture antigen @ 1:16
4.	SsL3 capture antigen @ 1:64
5.	SsL3 capture antigen @ 1:256
6.	SsL3 capture antigen @ 1:1024

FIGURE 2

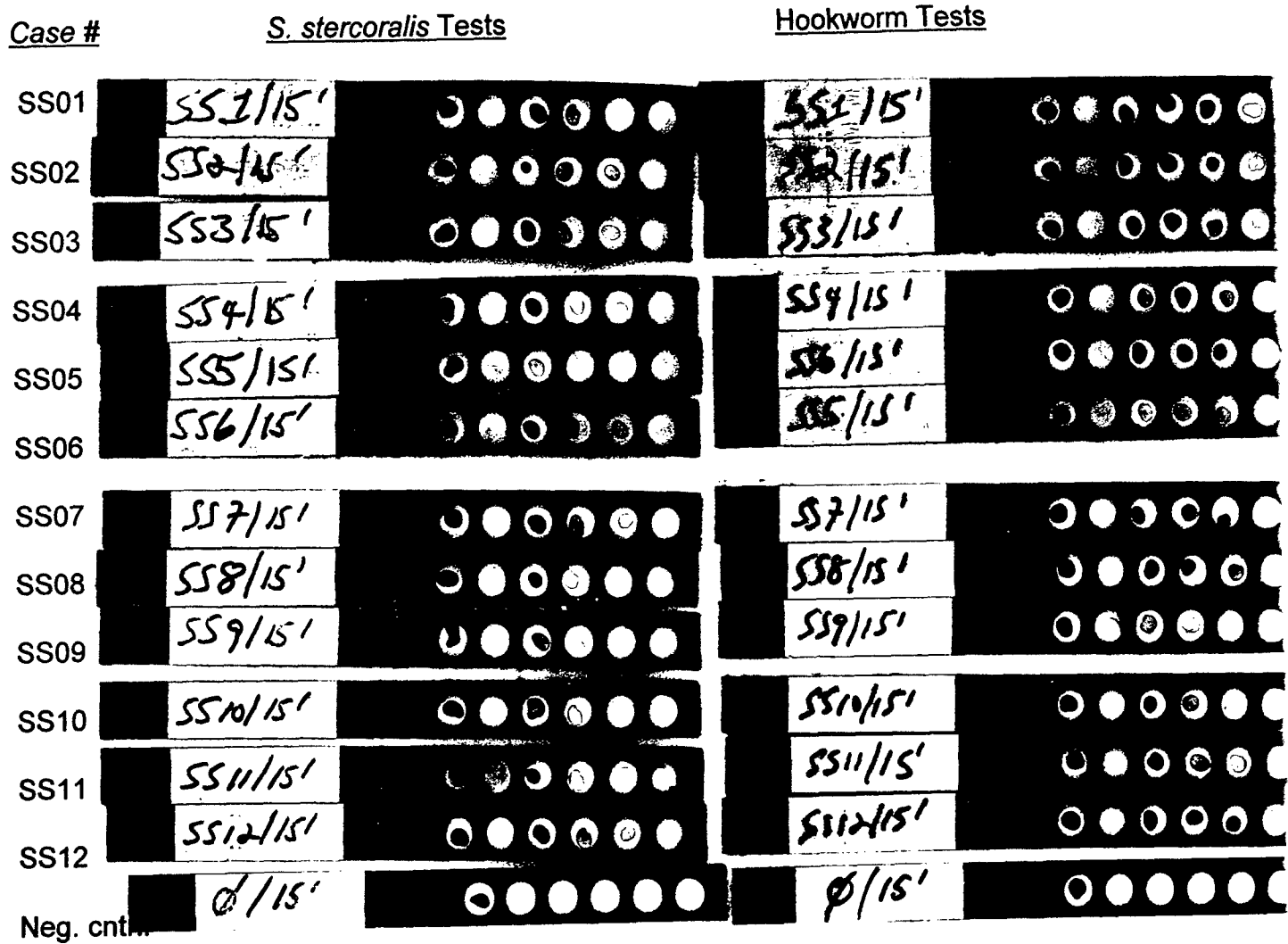
Detection of Anti-*S. stercoralis* Antibodies in Sera from Infected Human Patients



Serum from four confirmed *S. stercoralis* patients manifesting varying levels of infection were provided by Dr. Thomas Nutman, NIH. Putatively negative human serum was drawn from *INDX* archives.

Figure 3

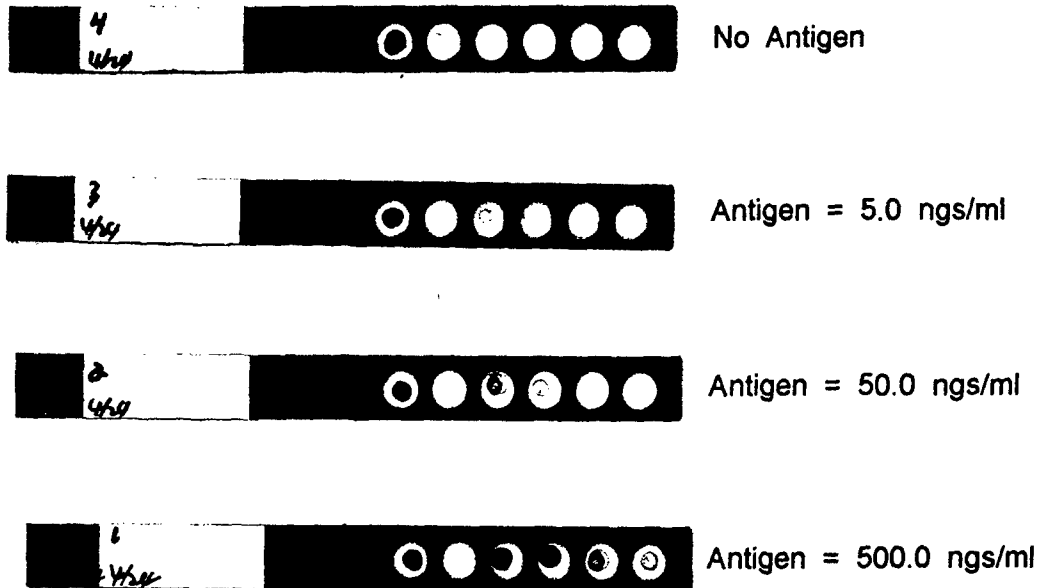
Analysis of 12 Helminth Infected Thai Patients with Antibody Capture Dipstick Tests for *S. stercoralis* and Hookworm.



Serum samples from 12 Thai patients confirmed positive for *S. stercoralis* were provided to *INDX* by Dr. John Cross (NIH) and were tested for anti-*S. stercoralis* and anti-hookworm antibodies with DS tests. Due to the results of the experiment shown above, all 12 were subsequently confirmed positive for hookworm by conventional ELISA assay (personal communication; J. Cross).

FIGURE 4

DIPSTICK ANTIGEN CAPTURE ASSAY



Capture antibody = Anti-*A. caninum* adult

Detection antibody = Biotinylated anti-*A. caninum* adult

Color development = **SIGMA** "Extravidin"-alkaline phosphatase

Antigen = *A. caninum* adult extract

Spot #1 = Positive control (Biotinylated antibody)

Spot #2 = Negative control (PBS)

Spot #3 = Capture Ab @ 1:100

Spot #4 = Capture Ab @ 1:200

Spot #5 = Capture Ab @ 1:400

Spot #6 = Capture Ab @ 1:800

FIGURE 5
Immunoblot Characterization of *S. stercoralis* L3 and *A. caninum* Adult Extract Antigens with homologous Anti-*S. stercoralis* and Anti- *A. caninum* Hyperimmune Rat Sera.

Figure 5a: Primary Antibody = Anti- *S. stercoralis* L3

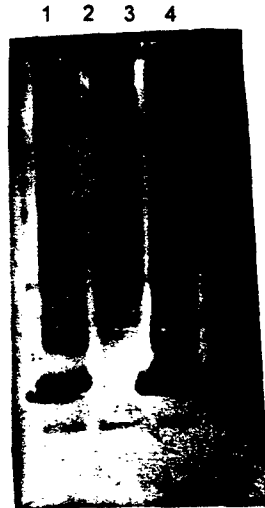


Figure 5b. Primary Antibody = Anti-*A. caninum* adult



For Figures 5a and 5b:

- Lane 1. *S. stercoralis* L3 extract (lot #1)
- Lane 2. *A. caninum* adult extract (lot #1)
- Lane 3. *S. stercoralis* L3 extract (lot #2)
- Lane 4. *A. caninum* adult extract (lot #1)

FIGURE 6

Characterization of the 24,000 MW Antigen Specific to *S. stercoralis* L3 Extract

Figure 6a. Lane 1 = Molecular weight markers; Lanes 2 and 3 = Lot #1 and lot #2 of *S. stercoralis* L3 extract.

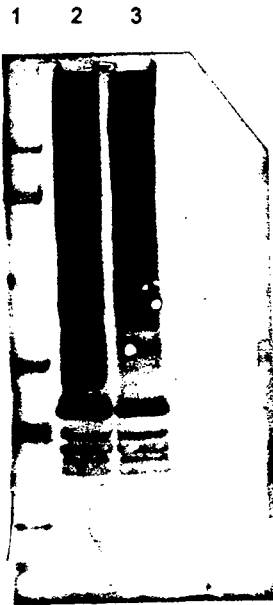


Figure 6b. Purification of the 24,000 MW antigen. Lane 1 = native extract; Lanes 2 - 4 = intermediate purification fractions; Lane 5 = 24,000 MW antigen purified by molecular sieving,



FIGURE 7
Immunoblot Characterization of *S. stercoralis* L3 and *A. caninum* Adult Extract Antigens with homologous Anti-*S. stercoralis* and Anti-*A. caninum* Rabbit IgG.

Figure 7a: Primary Antibody = Anti- *S. stercoralis* L3

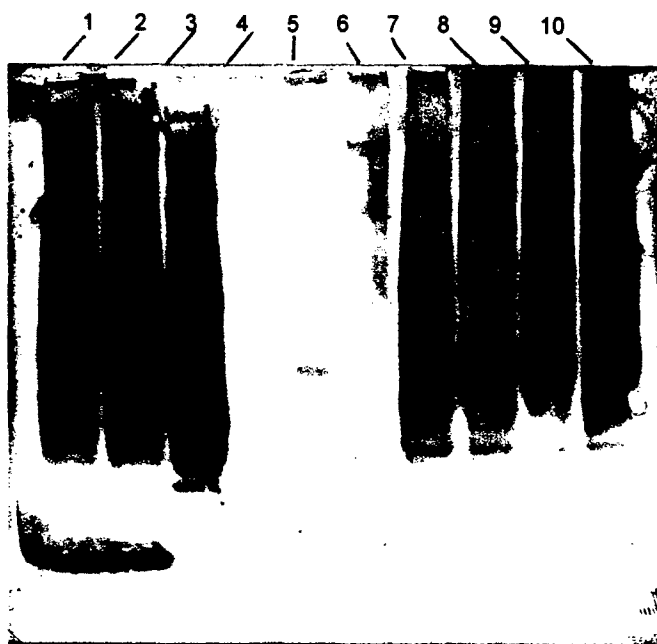


Figure 7b. Primary Antibody = Anti-*A. caninum* adult



FOR 7a and 7b: Lanes 1 and 2 = *S. stercoralis* L3 extract; Lane 3 = 30,000 - 100,000 native MW fraction of L3 extract ; Lanes 4 and 6 = blank; Lane 5 = MW markers; Lane 7 = 30,000 - 100,000 native MW fraction of *A. caninum* extract; Lanes 8, 9 and 10 = *A. caninum* adult extract.

FIGURE 8

Immunoblot Characterization of *S. stercoralis* L3 and *A. caninum* Adult Extracts Using Serum from an Infected Human Patient as Primary Antibody.



Lane 1. Molecular weight markers

Lane 2. *S. stercoralis* L3 extract

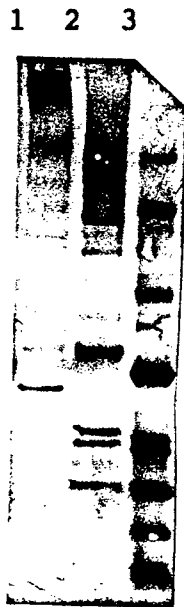
Lane 3. *A. caninum* adult extract

The above western blot was developed with serum from Thai patient # SS06 as primary antibody. Patient SS06 gave a strong positive result in antibody capture DS tests for both *S. stercoralis* and hookworm (see Figure 3).



FIGURE 9

Primary = E/S 1390



Primary = E/S 1393

- 1. *A. caninum* adult extract
- 2. *Strongyloides* L3 extract
- 3. Markers

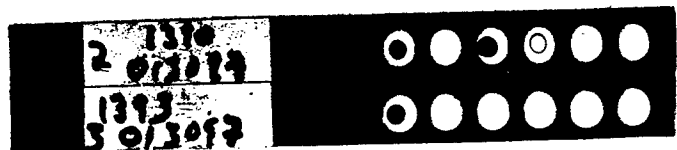


FIGURE 10

**Antigen Capture Assays: Detection of Antigen in Spiked,
Formalin Fixed Fecal Samples.**

1. Normal Dog Feces



2. Normal Dog Feces Spiked with *S. stercoralis* L3 Extract



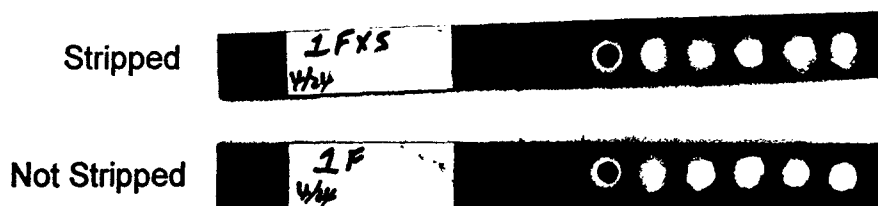
3. Normal Dog Feces Spiked with Intact *S. stercoralis* Larvae.



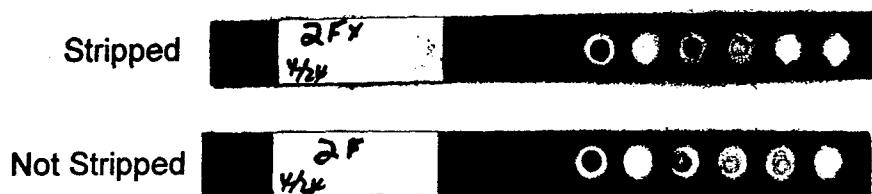
FIGURE 11

Effect of Acid Glycine Stripping on Detection of Spiked Antigens in Formalin Fixed Feces.

Normal Feces: Not spiked



Normal Feces: Spiked with *S. stercoralis* L3 extract.



Normal Feces: Spiked with *S. stercoralis* L3 larvae

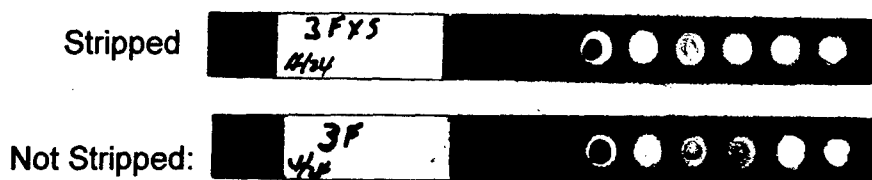
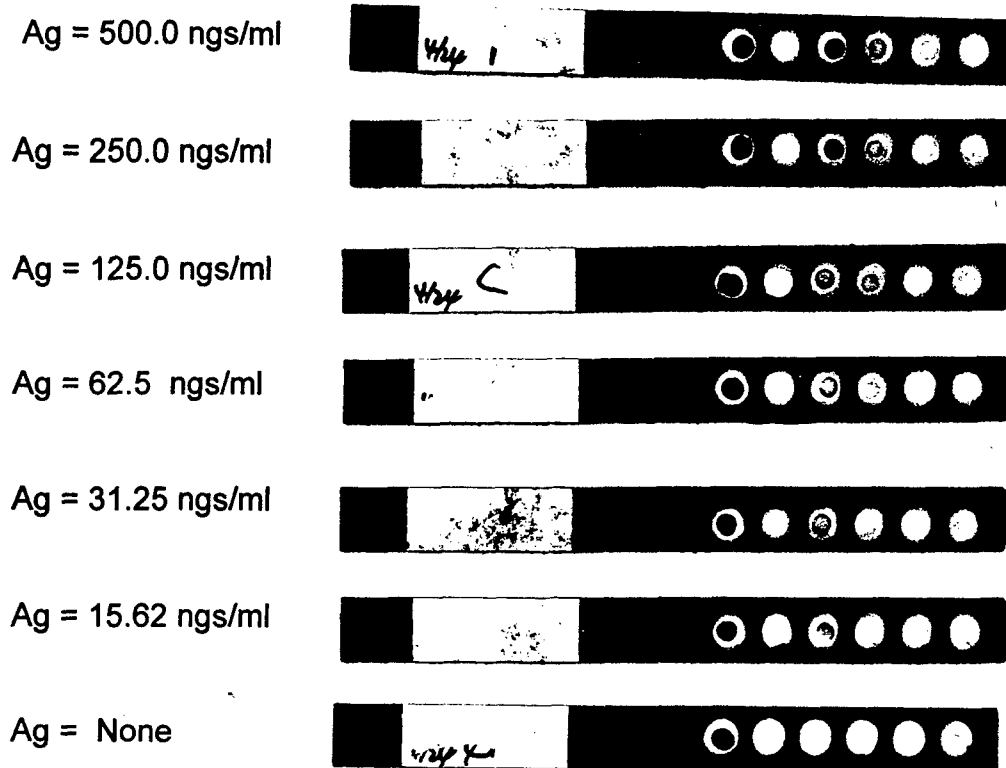


FIGURE 12

Sensitivity of Fecal Antigen Capture Assays: Detection of Spiked Antigen in Serially Diluted Fecal Samples.



A sample of normal dog feces was spiked with *S. stercoralis* L3 antigen prior to homogenization in 10% buffered formalin. Serial two-fold dilutions were made by dilution of spiked feces homogenate with homogenate of unspiked feces.

1 2 3 4 5 6 7 8 9 10

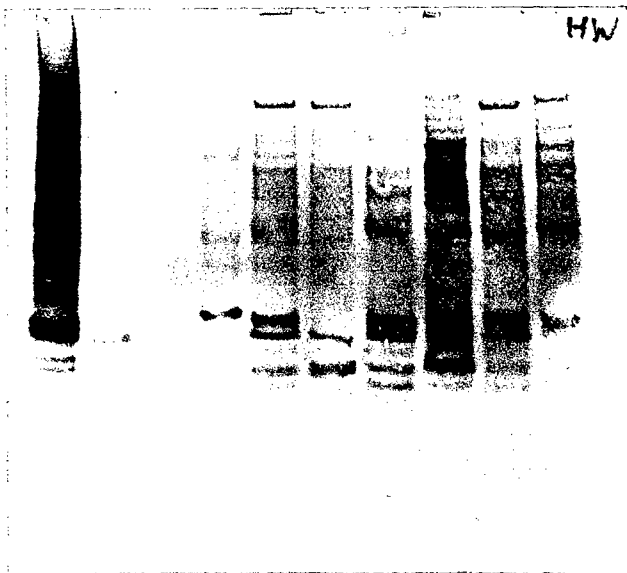
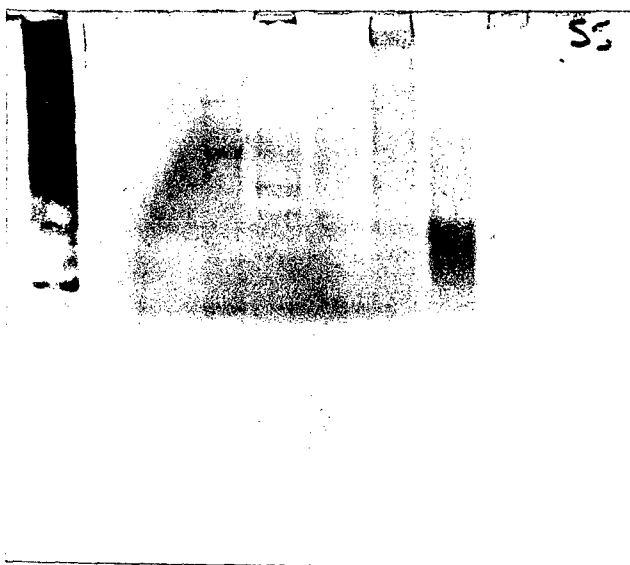


FIGURE 13

Primary = Anti-*A. caninum* adult

1 2 3 4 5 6 7 8 9 10



Primary = Anti-*Strongyloides* L3

1. Adult *A. caninum* extract (top)
L3 *S. stercoralis* extract (bottom)
2. Floated material (hookworm dog feces)
3. Floated material (hookworm dog feces)
4. Markers
5. Normal dog feces (sample #1)
6. Normal dog feces (sample #2)
7. Hookworm dog feces
8. *Strongyloides* dog feces (sample #1)
9. *Strongyloides* dog feces (sample #2)
10. *Strongyloides* human feces (Nutman)

1 2 3 4 5

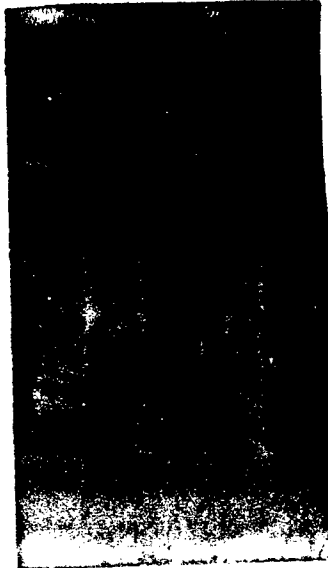
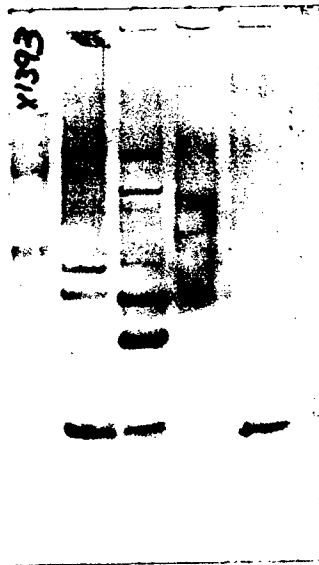


FIGURE 14

Primary = E/S 1390

1 2 3 4 5



Primary = E/S 1393

1. Markers
2. *Strongyloides* dog feces (sample #1)
3. *Strongyloides* dog feces (sample #2)
4. Normal dog feces (sample #1)
5. Hookworm dog feces

Contract Number: DAMD17-97-C-7025

Title: Development of a Sensitive and Specific Antigen-Detection System for Strongyloides Stercoralis and Hookworm Infections

Personnel receiving pay from this effort:

Helene Paxton, M.S., MT (ASCP), P.I.

Santo Grillo, Ph.D

Bruce Goodwin

Weimin Liu



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REPLY TO
ATTENTION OF:

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4 Dec 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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