Experimental Hookworm Infection in Laboratory animals:
Parasite behavior, Immune response and Chemotherapeutic Studies

Mahendra K. Bhopale
Department of Microbiology, R.D. Gardi Medical College, Ujjain, MP 456001, India.

Corresponding author: Mahendra K. Bhopale, Ph.D., D. Sc, Department of Microbiology & Director (Research), R.D. Gardi Medical College, Ujjain, MP 456001, India.

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Abstract
Hookworm disease is known to be caused allergic manifestation and severe anemic pathogenicity in man and canine hosts. Attempts have been made to establish laboratory models of Necator americaus, Ancylostoma duodenale, and Ancylostoma ceylanicum, together with canine parasite, Ancylostoma caninum. The studies include pathophysiological aspects of the host-parasite relationship, and develop to establish patent infection. Immunological approach to selecting antigen for diagnosis and protective immunity purpose using larval and adult worm antigens and their secretions became the focus with the subsequent discovery of cloning in vaccine development as main research interest. Chemotherapy of newer drug screening in laboratory models ultimately selected to use for preventive chemotherapy in hookworm endemic areas using recommended drugs.

Keywords: hookworms; laboratory model; hookworm antigens; protective immunity; anti-hookworm drugs; chemotherapy

Introduction
The incidence of intestinal helminthiasis, especially hookworm disease is alarmingly high in tropical and sub-tropical countries [1, 2]. Hookworm disease is known to be caused allergic manifestation and severe anemic pathogenicity commonly occurs in man and canine hosts. Research develops to study in parasite behavior, immunological and chemotherapeutic studies associated with hookworm infection predominantly in suitable models. The first idea for the development of a suitable animal model of human hookworm infection is the ability for the human parasite to develop normally in a laboratory animal. Second, the course of such infection and clinical manifestation produced by these parasites should resemble those seen in infected humans. Attempts have been made earlier to establish hookworm models by infecting a variety of laboratory animals to develop a complete life cycle, namely, Necator americaus, Ancylostoma duodenale, and in canine Ancylostoma ceylanicum. Dog hookworm, Ancylostoma caninum, accidentally infects humans known as zoonosis, wherein they migrate under the skin and remain in the larval stage in the host. Usually these cause an aberrant infection, “creeping eruption” or cutaneous larva migrans.

The established laboratory models of hookworm were used for the study of immunological and pathophysiological aspects of host-parasite relationship and the chemotherapeutical evaluation of newer drugs. All hookworm parasite antigens do not participate in protective immunity. The isolation and identification of antigens which elicit a protective immune response was studied for the full analysis. Research interest in hookworm disease was progressing further using molecular and immunology approach to develop a suitable vaccine [3, 4]. At the same time, search of newer drugs was continued screening in the laboratory animals and finally at the human trials [5].

Parasite behavior
Ancylostoma caninum: The pattern of infection with A. caninum in mice and rats is very similar in both species, but depends on the route of larval entry. Following oral inoculation more than 50% of the larvae penetrate intestinal tissue those which fail to pass out with host faeces. Successful larvae pass through the mucosa, probably through the liver, and by 17 hours can be recovered from the lungs [6]. Within 24 hours, larvae can be observed passing up the trachea, through the larynx and eventually localizing in the laryngeal and pharyngeal muscles. Three to four days post infection the maturity the majority sites only a few migrating further afield and, recovered 30% of the inoculated larvae from the muscles of the head, neck and thorax, in comparison to 3% from the hind limbs. When rodents are exposed to percutaneous (PC) or sub-cutaneous (SC) infection, the larvae migrate directly into the muscles throughout the body [7]. During the distribution phase some
pass through the lungs, but there is no accumulation in the lungs as in orally-infected animals. Muscle larvae in rodents are essentially exsheathed third-stage larvae (L3). They show neither morphological change, nor development over the infective larvae while in the host. Viability is retained for a very long time depending on the intensity of initial exposure. The migration of *A. duodenale* is again very similar. The majorities of larvae localize in the somatic musculature irrespective of the route of inoculation, but survive for a limited period of 66 days.

***Ancylostoma ceylanicum:*** *A. ceylanicum* succeeded in adapting this canine species to regular passage through hamsters, the larvae moulted to L4 by the second or third day and grew rapidly to moult again to pre-adult stage in 7-10 days. Parasite eggs appeared in hamster faeces 14-17 days after infection and peak egg production was usually apparent by 21-28 days [8]. *A. ceylanicum* appears to behave quite differently in mice than *A. caninum* found intestinal worms moulting to the L4 stage by day 3, persisting for longer than a week. However, when cortisone was used worm burdens remained high for about three weeks, and the fertile parasites containing eggs were recovered on day 18, coinciding with the first appearance of eggs in mouse faeces [9].

***Necator americanus:*** The complex life cycle of the hookworm has opportunities for the host-parasite interaction during the larvae invade through the skin and transit through lung tissues to the gut mucosa diverse immune modulation. *Necator americanus* established hamster strain is capable to survive without cortisone [10]. However, it was observed normal development and achieves patent infections when hamsters were exposed to infective larvae within a week old pup [11]. By regular passage, it was possible to improve the adaptation of this strain to the hamster host [12]. Though *N. americanus* is susceptible to the infection in adult hamsters also, but the worms are rejected from the intestine soon after completing their migration from the lungs. *N. americanus* mature in hamsters, but only when 1 to 3-day-old pups infected with larvae. However, older animals acquire resistance quickly and do not develop patent infections. Attempts have been made to use neonatal (two-day-old) rabbits to *N. americanus* with regular passage the pre-patent period was reduced and faecal egg counts rose to 40000 e.p.g. and worms persisted for up to 200 days [13]. Attempts were also made successfully the development of *A. duodenale* in neonatal rabbits when infected larvae orally (buccal pouch) and attained the adult stages to patent infection [14].

**Immune response**

***Immune response to Ancylostoma caninum infection:*** Studies have been shown that repeated doses of the *A. caninum* larva develop immunity in the mice. This study brings to alterations in serum proteins as a single dose or repeated doses following inoculation of *A. caninum* per os in mice with 250, 500, 1000, 2000, and 40000 infective larvae and β-globulin specifically was significantly elevated. However, a significant reduction in the γ-globulin was seen in these animals except in the groups which received 4000 *A. caninum* larvae in mice [15]. Study on *A. caninum* in mice has also provided information on the role of cell-mediated immunity when passively transferred sensitized peritoneal exudate cells [16], small and large intestinal mesenteric lymph node cells [17], and thymus and bone marrow cells separately [18, 19]. The activated immune cells transferred results show diminishing survival of migrating larvae in the tissue and due to the cause of earlier expulsion from the intestines with *A. caninum* infection in mice [20]. Migratory larvae also destroyed in the muscles after allergic immobilized death due to the delayed type hypersensitivity response [21].

***Immune response to Ancylostoma ceylanicum infection:*** Although *A. ceylanicum* has been transmitted experimentally to hamsters [8, 22], but no study on immune response to measure the humoral response to a primary infection until the study made to show positive reaction employing by agar diffusion, counterimmunoelectrophoresis (CIEP) and indirect haemaglutinating (IHA) tests for 20-60 days whereas CIEP until the 150 days of infection using sonicated soluble adult worm antigens [23]. They further measured similar parameters of IHA antibodies and the CIEP reaction in hamsters infected with *A. ceylanicum* treated with mebendazole [24].

***Immune response to Necator americanus infection:*** *N. americanus* surface antigens of adult and L4 recognize serum antibodies and capable of immunoprecipitating on surface antigens recognized by excretory-secretory products during the infection of human and hamster post-infection [25, 26]. Acquired resistance was monitored using the hamster-adapted strain of *N. americanus* infection in BALB/c mice by worm recovery and immunological assays after exposure to primary or secondary infections [27]. Soluble antigens from a third-stage (L3), adult homogenate, and excretory/secretory (ES) were used in various tests to examine humoral responses to *N. americanus* in infant rabbit model. Sera from rabbits infected with *N. americanus* duration the period of of 40-84 days were analyzed positive by CIEP against L3 larval antigen and also showed a significant attenuation in the albumin/globulin ratio in infected animals when compared to controls [13]. Antigen expression during the development of *Necator americanus* L3, L4 and adult is recognized by immunoblotting and immunoprecipitation analysis in natural host man, and the experimental hamster host [28]. There are correlations antibody responses between *Necator americanus* stage-specific antigens and parasite burden in an endemically-infected population in Papua New Guinea. Parasite burden declined significantly with age from positive in younger to significantly negative in older infected hosts. The presence of a positive correlation between eosinophil concentration and the infection intensity in adults indicates that eosinophilia reflects the host’s worm burden, whereas levels of anti-ES antibodies dependent on worm burden. A trend of similar patterns was present for anti-larval-IgG in both pretreatment and after re-infected for anti-ES-IgM and anti-ES-IgE pretreatment [29]. In vitro experiments to assess the skin penetration by exsheathed third-stage infective larvae (L3) of *Necator americanus* visually observed using a novel fluorescein isothiocyanate (FITC)-labeling technique [30]. Studies also emphasized on experimental approaches to develop a recombinant hookworm genetically engineered vaccine in controlling this infection in highly endemic areas. Recombinant polypeptide belonging to the Ancylostoma secreted protein (ASP)-I family has shown promising results of reducing hookworm burdens after larval challenge infection in mice [31].

***Irradiated larval vaccine:*** Earlier, a highly effective vaccine was developed to control *A. caninum* infection in dogs immunized with X-irradiated *A. caninum* L3, protected against subsequent challenge with normal larvae [32]. This immunity could be transferred passively to naive recipients by serum and lymphoid cells [33], but not cleared whether larvae were killed during migration or adults were expelled from the gut due to the effect of vaccination. With UV-irradiated *A. ceylanicum* larvae stimulated resistance in hamsters. A high level of protection afforded by larvae irradiated for 15 min UV-exposure was recorded giving 99.0% and 95.0% worm reduction against the challenge doses of 100 and 1000 normal larvae respectively. There was no marked difference in worm establishment in hamsters vaccinated either orally or...
subcutaneously, followed by oral challenge A. ceylanicum infection in hamsters. In the vaccinated hamsters, the manifestations of resistance at 15 min UV-exposure was shown by marked reduction in worm establishment and highly reduced epg in pellets, and a significantly higher blood hemoglobin levels compared with those given normal larvae as vaccine and challenge controls. [34].

A preliminary study illustrated that active immunization of A. ceylanicum (Ac) whole worm (ww) and larval somatic (som) and excretory-secretory (es) antigen separately in hamsters, gave 93.22% (Ac-ww-som-Ag), 100% (Ac-ww-es-Ag), 92.70% (Ac-larval-som-Ag) and 95.83% (Ac-larval-es-Ag) worm protection respectively against the normal challenge dose. Further, Ac-ww-som-Ag separated through Sephadex G-200 column gave 96.2% worm protection when immunized with fraction-F2 as compared to -F1 (88.62%) and - F3 (72.51%) against the challenge normal dose. Passive immunization of immune serum HHIS (Hyper immunized raise in hamsters) by inoculated UV- irradiated larvae (x3), showed (85% to 90%) worm protection against the normal challenge infection. Immunoglobulin (IgG) separated from the pooled serum samples of HHIS-IgG (Hamster Hyper Immunized Serum-IgG) and RHH-IgG (Rabbit Hyper Immunized Serum-IgG), showed 97.67% and 92.20% worm reduction respectively against the normal challenge dose.

Parasite antigen selection and cloning: Ancylostoma-secreted-protein applied in cloning and characterization of a novel protein, ASP-1 [35]. Vaccination of mice with either third-stage Ancylostoma caninum infective hookworm larva (L3) or alum-precipitated recombinant Ancylostoma secreted protein 1 (Ac-ASP-1) results in protection against hookworm challenge infections. Passive immunization with pooled sera from recombinant Ac-ASP-1-vaccinated mice also resulted in lung hookworm burden reductions in the muscles and also elevated IgG1 and IgG2b in the host [36]. Mice were immunized with recombinant ASPs from different hookworm species which showed different degrees of protection depends on amino acid sequence homology [37]. AceES-2 (Cloning of Ancylostoma ceylanicum-excretory-secretory-protein-2), a novel protein produced by adult worms plays an important role in the host-parasite interaction and represent a useful strategy for controlling hookworm infection [38]. Aspartyl protease inhibitors (API-1) from parasitic nematodes are highly immunogenic, and have been suggested as potential vaccine antigens. API-1 was cloned and characterized from the hookworms, A. caninum and A. ceylanicum. The highly immunogenic properties of nematode asps suggest that Ac-API-1 represents a promising target for a recombinant hookworm vaccine [39].

Ad-III-ESA (anti-A. duodenale immunoglobulin (Ig) G4 antibody fraction III of the partially purified excretory secretory antigen of adult worm was studied. The level of serum specific IgG4 was measured by indirect enzyme linked immunosorbent assay and compared with serum specific IgG, IgG1, -2 and -3 subclass antibodies [40]. Two classes of fatty acid and retinol binding proteins produce by nematodes. A partial cDNA was cloned from a polypeptide antigen/ allergen (NPA) to the NPA (AccNPA) which correspond to four subunits of putative A. ceylanicum. The amino acid sequence of AceNPA shares sequence identity with similar proteins from Dicycoaulus viviparos, Ascarsis suum and Ostertagia ostertagi [41]. Another study on Necator americans-Ancylostoma-Secreted-Protein-2 (Na-ASP-2) binds an ascaroide (ascr#3) in its fatty acid binding site has been progressed for the search of small molecules found in E-S products of hookworms include nematode derived metabolites of ascarosides, which are composed of the sugar ascryllose linked to a fatty acid side chain [42].

Chemotherapy
Many reports have attempted screening of anti-hookworm drugs in the laboratory models against the adult stage of hookworm of human and canine origin. Selected drugs were screened where mebendazole was the most effective, with parbendazole, phenylene 1,4-diisothiocyanate, thiabendazole and bpenhium hydroxyphosphate also satisfactory [43]. The compound, 3, 5-dibromo-2-chloro-4'-isothiocyanatosalicylanilide, has been tested against various nematode and cestode parasites in experimental and domestic animals. It showed 100% activity against A. ceylanicum, A. tubaeformis [44]. The anthelmintic activity of Amoscan (C 9333-GO/CGP 4540) has been studied in Necator americans infection in hamsters with single oral doses of 30-60 mg/kg eliminated 94 to 99% parasites in 37-day-old non-parent infection, while single oral doses of 25 mg/kg expelled the entire worm burden in patent infection [45]. Necator americans in infant rabbit system mebendazole appeared to be 100% effective at 100 mg/kg at single dose clear all worms [46]. The efficacy of eight anti-helmintics against Ancylostoma caninum larvae in the skeletal muscles of mice was evaluated using levamisole (5 X 40 mg/kg), thiabendazole (5 X 400 mg/kg), oxendazole (5 X 100 mg/kg), albendazole (5 X 100 mg/kg), flubendazole (5 X 200 mg/kg), benacil (5 X 200 mg/kg) and phenacizole (5 X 200 mg/kg) showed marked larvalcid activity (98 to 99%) whereas Sch 18099 did not show larvicidal activity even at 5 X 400 mg/kg [47]. The benzimidazole derivatives viz. thibendazole, oxibendazole, parbendazole, and fenbendazole showed marked activity only at high dosage rates in A. caninum infection in Mastomys natalensis system [48]. However, none is reported whether the drug is capable of eliminating all the developmental stages of hookworms.

In a preliminary study of A. ceylanicum in golden hamsters, the efficacy of mebendazole, albendazole, parbendazole, flubendazole and ivermectin was evaluated at the stage 3rd stage (day 1), 4th stage, (day 4) 5th stage (day 7) and adult stage (day 12), and necropsies were made on the day 20 for the adult worm recovery prior withhold of food one day before. Among those drugs, albendazole showed 100% efficacy at the dose of 50 mg/kg against the L3 and the L4 stages; 10 mg/kg against the L5 stage; and 5 mg/kg against the adult stage. So also another drug, ivermectin showed 100% efficacy at the dose of 0.005 mg/kg against the L3 stage; 0.003 mg/kg against the L4 stage; 0.002 mg/kg against the L5 stage; and 0.005 mg/kg against the adult stage. This study suggests that albendazole and ivermectin clear all the developmental parasitic stages, including the adults of A. ceylanicum infection in hamsters.
immune cells in *A. caninum* infection in mice. Protective immunity induced by UV-irradiated larvae, somatic and excretory-secretory antigen and passive transfer of IgG in hamsters against the challenge of *A. ceylanicum* infection have been observed. Progress in advance research using molecular and immunological techniques were made in cloning of Ancylostoma ceylanicum excretory-secretory protein 2 (AceES-2), Ac-AP1-1 clone represents a promising target for a recombinant hookworm vaccine. The anti- *A. duodenale* serum derived immunoglobulin (Ig) G4 fraction III of partially purified excretory secretory antigen of adult worm (Ad III ESA), and Nector amercianus-Ancylostoma Secreted Protein-2 (Na-ASP-2) were achieved showing promising results. Among those drugs screened in laboratory animal models of *N. americanus*; *A. ceylanicum* and *A. caninum* against adults or larvae hookworms, but ivermectin and albendazole were found promising drugs to clear all the parasitic developmental stages of *A. ceylanicum* infection in hamsters.

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