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Importance of Human Hookworms Model

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Abstract

Soil transmitted helminthic infections in the community are common and an estimated 4.8 billion people are at risk of getting STH infection. Appropriate animal models, preferably in rodents, are required to perform and conduct experiments in the laboratories. The hookworms of genus *Necator* and *Ancylostoma* parasites can experimentally be maintained in rodent models, and they can effectively be used for evaluating compounds thereby potentially new anthelminthics can be identified. What is the reality in this practical approach and what are the limitations? These points are discussed in this article.

Introduction

Hookworms are soil transmitted helminth parasites and live in the gut of humans. Globally there is an estimated 570 to 750 million people infected with hookworms. Hookworm disease is responsible for more than 4 million disability-adjusted life years (DALY) lost annually with financial impact of over US$100 billion a year [1]. Infection is common in areas where human faecal material is used as a fertilizer or where defaecation onto soil happens thereby environmental faecal- contamination occurs, and people get infected if they walk on barefoot or otherwise on such areas. There is no other way the infection can reach the human body and no vectors and no intermediate hosts are involved. Bare foot and skin are the point of entry into the human body. Infected children are nutritionally and physically impaired. Hookworms are not present in cities they are primarily a rural problem. Hookworms are metazoan parasites and they have therefore not completely culturable; different stages are involved, from eggs to larvae and then to adult stages. In this article, we emphasize relevance of hookworms and their experimental model in rodents and their utility in in *vivo* screening of synthetic compounds.

General Description

Fig 1 describes briefly lifecycle stages of hookworms. Human hookworm eggs isolated from faecal material were suitable to examine for identification (55-76 µm in length and approximately 34-50 µm in width and have a smooth and thin outer shell). Eggs undergo embryonation, hatch and first stage larvae (L-1) emerge. These first stages, mostly free living, and are not suitable for experimental work on drug screening and any objective analysis. Only larvae were partially maintained under in *vitro* conditions provided appropriate conditions were met and suitable culture media were provided. Even if we maintain them *in vitro*, they do not undergo moulting which is a critical condition for initiating subsequent developmental stages. The L-1 stage larvae undergo moulting (development) into L-2 and finally larvae undergo considerable changes and transform into L-3 stage after a moult. The L-3 stage is ensheathed (covered with sheath) and become infective to humans (Fig 2). Under natural conditions infective larvae develop from infected faeces in about 4 to 7 days. Under *in vitro* conditions L-3 larvae are very active and survive for long periods until they come in contact with the human skin. Then L-3 larvae penetrate the skin (takes 10-15 min) and enter the dermis and then reach the lymphatics in the human body and undergo development. Within the body, the developing larvae undergo histotrophic phase, ie., develop certain affinity to specific organs and development continues in lungs (L-4 and L-5) and from lungs they migrate and then swallowed down to pharynx, oesophagus and traverse into the intestine. Then larvae develop to L-5 (pre-adult) and grown into sexually mature - male and female worms. Once in the duodenum, immature 'L-5' hookworms use 'teeth' (*Ancylostoma* spp.) or cutting plates (*Necator* spp.) that line their buccal capsule to lacerate the mucosa and anchor themselves in position to facilitate feeding and avoid being ejected by gut peristalsis. As they begin to feed on blood, juvenile worms mature into sexually dioecious adult parasites. Female hookworms produce as many as 10,000 eggs per day. Eggs are evacuated from the host via the faecal stream. The process from L-3 invasion into patency (egg production) takes approximately 6–8 weeks for *Necator americanus* and possibly a similar period of time for *Ancylostoma duodenale* [2]. Most of the hook worms follow the same trend in the development. In case of *A. caninum*, exceptions are there; the larvae arrested and undergo dormancy in the uterine or mammary gland and then transmitted to pups in the form of prenatal infection. Such infection is recorded very rarely in humans and other hookworms.

Adult hookworms live successfully in the small intestinal region particularly jejunum. They attach to the intestinal wall by drawing a plug of mucosa into their large buccal capsules and began sucking blood, releasing lot of proteases which pass through their intestinal tract. This results with extensive blood loss depending upon the number of worms present. Hookworms are voracious blood suckers. They can live in the intestine for several years, sucking blood and excreting degenerated blood products, and other wastes and with the result the human host suffers from anaemia, blood loss and sometime succumb to infection. This simplistic lifecycle of this parasite has its own detrimental effect on humans depending upon the worm burden and particularly in school-going children and young adults and to some extent working adults suffer from ravages of hookworm infection. Many foundations such as Rockefeller have tried to provide funding and encourage research and hook worms are beyond the scope of projects. There were attempts to develop drugs and vaccines for control of infection.

Besides the two well established species (*Necator americanus* and *Ancylostoma duodenale*) in humans, recent incidence reports and statistics indicate that *A. ceylanicum* is an emerging pathogen in some parts of Asia [3-5]. The climatic and socio-economic condition results with the infection is being reported in several countries regulated with tropical and subtropical environments. Proven drugs such as mebendazole and albendazole are specifically useful against hookworm infection. *Ancylostoma* sucks more blood than *Necator* as few as 25 *N. amercanus* hookworms sufficient to cause 1 ml of blood loss per day [2]. Higher worm burden cause still more blood loss and severe anaemia. The hookworms while sucking blood from humans also introduce several supportive biological materials such as proteases for easy blood flow. This interaction is little understood. Recently Dr Peter Hotez and his collaborators, while trying to develop anti-hookworm vaccine, have identified several molecules in hookworms' infection phase operating against immune function; inhibitors that block the clotting of blood, presence of antioxidants (eg Superoxide dismutase), neutralizing agents, and break- down product of acetylcholine and others. They concluded that these molecular interactions are disruptive for induction of host- protective immune response against hookworms.

For inducing immune-mediated protection conventional approach being injection of parasite derived antigens. This was being performed for a while with no impressive results. Recently some sub-unit antigens were also used as "vaccine- candidates" which resulted with no strong and consistent protection either. There must be a different approach for induction of protective response against this beasty worms. Metazoan (helminthic) parasites have their own way in protecting themselves from hosts and trying themselves to avoid any protective response from hosts. Over the million years they adopted several mechanisms that disrupt host-protective response. With lot of hard work and resources Dr. Miller(1978) [17], was succeeded in inducing protection in dogs by using irradiated larval material. But few irradiated larvae developed into adults, therefore the irradiated larval approach also did not practically found to be useful. One must understand the development of vaccine for such huge and highly active blood suckers is not an easy task. It is an immense problem solving and we need to understand more on the host-parasite interplay and interactions such as parasite tolerance and host intestinal condition and responsiveness. Undoubtedly, we little understood the bionomics of these parasites. Moreover, the way parasite - expulsion occurs under natural condition, without chemotherapy is surprising to note. Molecular mechanisms and other clues are yet to be worked out. This area has become highly intellectual topic with input from molecular biology and immunology. Lots of research has been done by several groups over the years. A flash of intuition is needed to solve the problem of anti-worm protective immune response. Because of the limited knowledge and know-how of parasitism, it is sufficient to say that at the present time the conventional chemotherapeutic approach involving mass drug administration (MDA) and periodic deworming are best available tools to tackle the problems associated with the soil-transmitted helminths (STH).

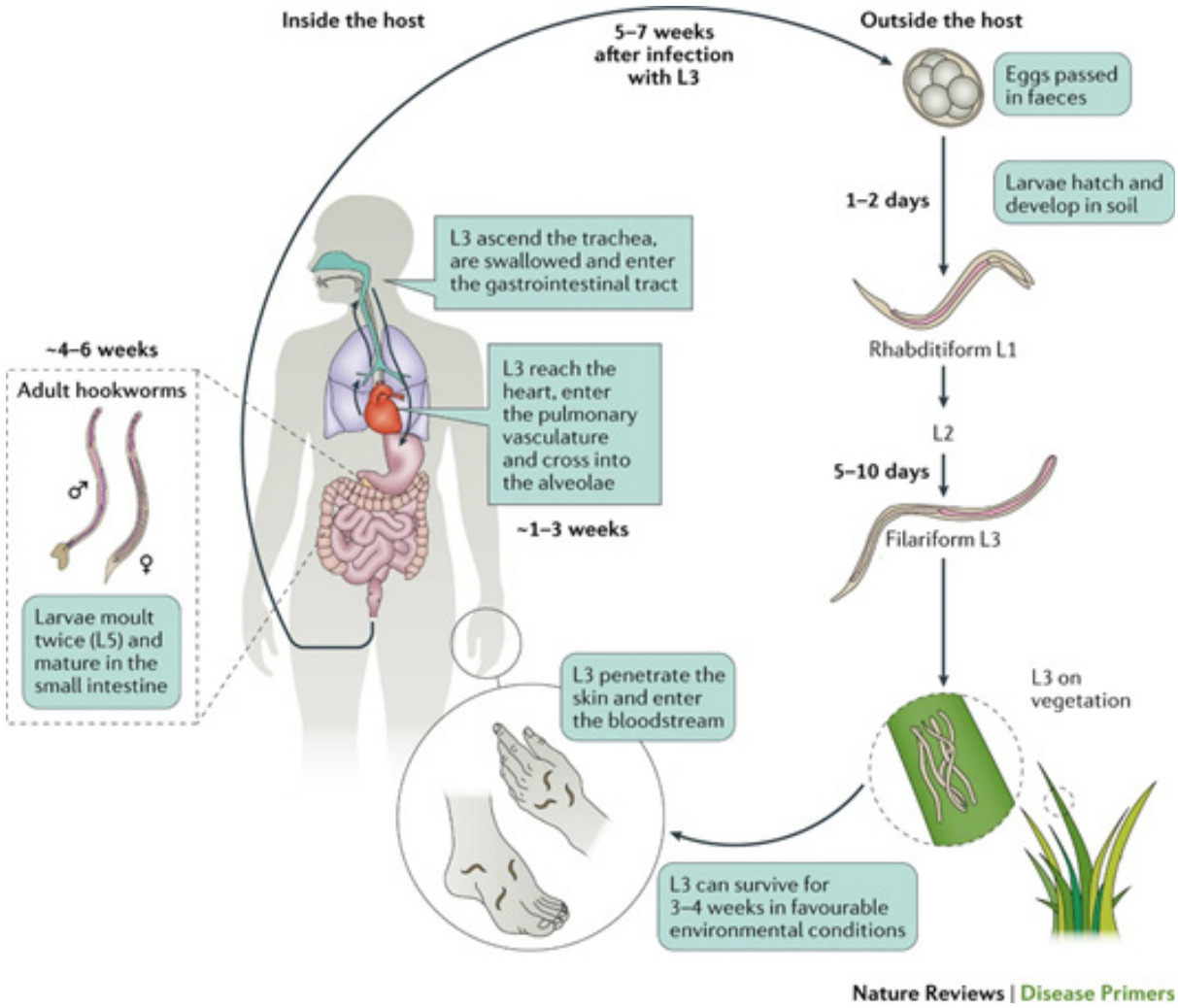

Figure 1. Life cycle stages of hook worms> (From Loukas A et al. 2016 Hookworm infection Nature Reviews Disease primers 2, 1-18).

Figure 2. Infective larva L3 stage (From CDC Website downloaded from Internet).

Experimental

Different stage of hookworms can be used for screening the synthetic compounds and for conducting any immunological experiments.

Infective larvae through skin barrier

Infective L-3 stages of hookworm larvae are very active, culturable and get them in sufficient quantity and could they be used for evaluation? Infective L-3 stages usually ensheathed and are highly protective and resilient against any compounds to be screened. Unless they made them exsheathed by some other method, the exsheathed larvae could be used for screening purpose. Ability of NaL3 passing through skin, the first barrier of infection, is important criterion for establishment of infection. Attempts were made to develop a model for this aspect of study. Experiments were designed to perform the degree of skin penetration by ensheathed NaL3 through the perfused hamster skin. NaL3 were washed three times by centrifugation (3x) in tissue culture medium (RPMI 1640 GIBCO) with Hepes and bicarbonate buffer (pH 7.2) supplemented with serum 5%, glucose (1%) and then transferred into a glass tube (diameter 1.5 cm and length 10 cm), one end of which was closed by tying the freshly dissected skin of 3-week-old hamster on its outer rim. Care was taken to avoid any leakage. The tube containing a known number (a total of 2000 EnNaL-3) were deposited in 2 ml medium on the external (dorsal) surface of the skin. The tube was then suspended into an external container (25 ml glass container with 10 ml of medium with the closed end of the tube covered with skin fully immersed in the medium. The whole system was suspended with a clamp and incubated at 37C for a duration of 24 h under sterile condition (Fig 3). The EnNaL3 after skin penetration shed its external membrane and transformed into exsheathed larvae (Ex NaL-3). After the perfused skin penetration, larvae were collected into a beaker with medium under sterile condition. The larval penetration is quantified periodically. The penetration results successfully into absolute number of exsheathed (ExNaL3) larvae. These larvae will be useful for screening the synthetic compounds. This will mimic exactly skin penetration under natural condition. As shown in Fig 3 and monitored periodically to study the ability of larval passage through the skin. Periodically the samples were taken for microscopic examination. The penetrated larvae were counted after 1, 4, 8, 20 h while hamster skin was perfused (immersed) in sterile RPMI medium supplemented with 1% glucose. During the incubation period EnNaL-3 penetrated the skin, exsheathed (ExNaL-3) and were liberated on the other side of skin and ultimately dropped into fluid medium covering the skin as perfusion and ultimately reaching the beaker as the external container (Fig 3). Periodically medium in the beaker was replenished and the ExNaL-3 larvae were collected in the beaker for inverted microscopic examination. The content of the beaker was checked thoroughly for the studying the larval condition. Most of the larvae were exsheathed and were active. The larvae were en-sheathed before penetration and after the penetration underwent into ex-sheathed (Ex-NaL-3) larvae. In this way, we developed exheathing larvae successfully under in *vitro* condition. Figs 4 and 5 show how many larvae were exsheathed during 1-24 h incubation. A total of 10 experiments were conducted and exsheathed ExNaL3 were enumerated in Fig 5. None were seen during the 1 h period. But they penetrated after 4 h onwards.

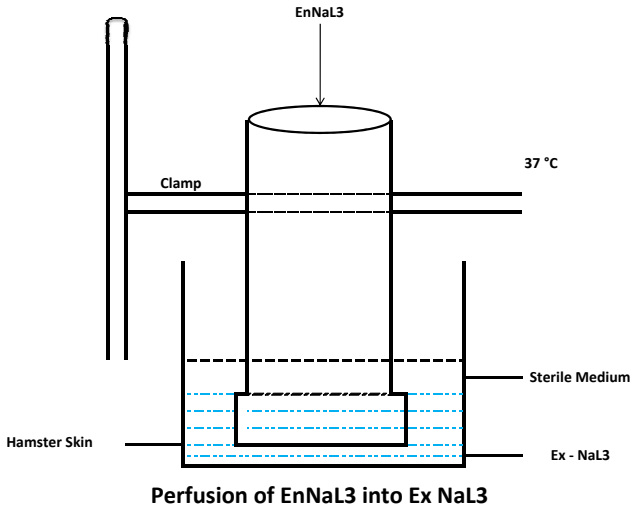

Figure 3. Simple condition for penetration of infective larvae of hookworms.

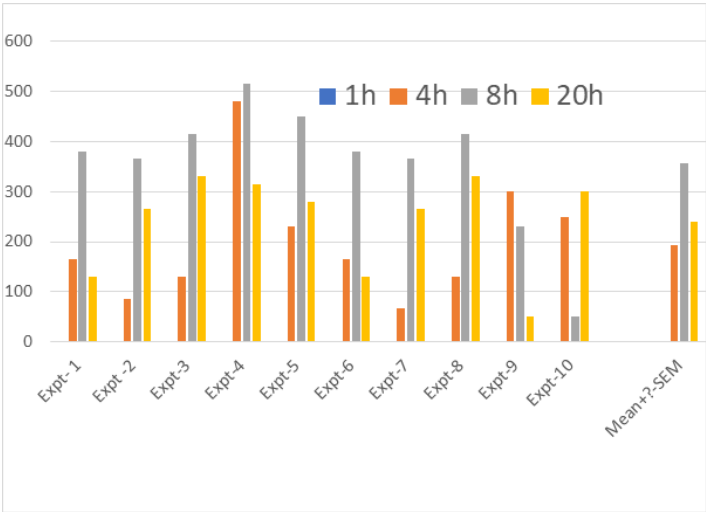

Figure 4. Skin penetration through hamster skin.

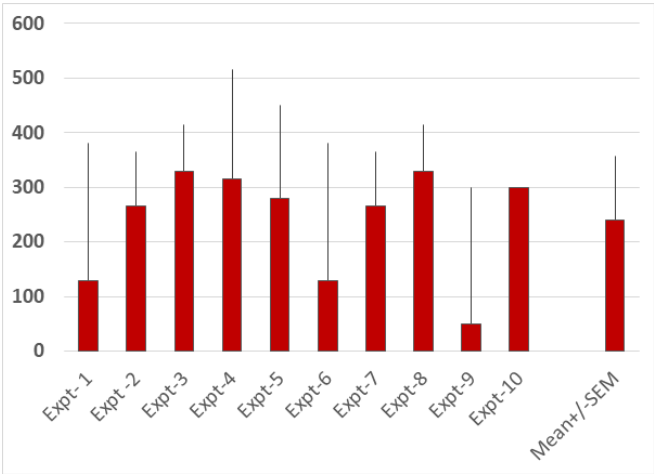

Figure 5. Skin penetration through hamster skin

Using such exsheathed larvae we can perform many experiments. For screening synthetic compounds in vitro will be useful. Their activity profile in presence of synthetic compounds as opposed to that reference compounds we can figure out the potential new compound. Screening the syn-

thetic compounds against active and ex-sheathed larvae *in vitro* is comparatively quicker than the in vivo screening. For immunological work, these experiments will be ideal to use as the target of protection experiments.

Larvae migrating through the lungs

Hookworms undergo considerable development and pass-through histotrophic phase. We should take the advantage of this histotrophic phase and analyse the infected lungs for migrating larvae. Day 3 to 7 is critical phase for lung histotrophism. This period (Days 3 to 7 post infection) is very consistent and reliable. Depend upon the dose of infective stage larvae can be recovered from lungs and quantitative estimation can be done. This period could be used for chemotherapeutic evaluation or immuno-protective purpose. It is defined in *Necator* model. This phase is exclusive to migratory larval phase and not for *Ancylostoma* models and can be monitored and effective chemotherapeutic and immuno-protective experiments can be designed during this phase.

Possible in vivo experimentations on adult parasites

Adult parasites reach the intestine of hamsters during Day 9 post infection onwards. Experiments were planned to orally dose (gavage) with candidate synthetic compounds or proven anthelmintics. Keeping infected animals as control infection, (Fig 6) then equal number of infected animals were allotted randomly to the Treated Groups. Difference in parasite numbers can be evaluated in terms of percentage cure and percentage worm reduction (Table 1 and 2). These approaches were practically useful for screening candidate compounds. Recent review of current drugs against STH substantiates the usefulness of albendazole (cure rate 78.4%) mebendazole (22.9% cure rate?) Pyrantel pamoate (87.9% cure rate) against hookworms [10].

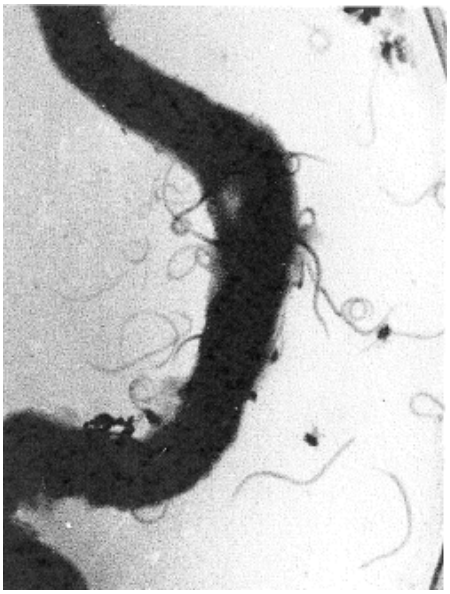

Figure 6. A piece of intestine from a hamster showing the hookworms *Necator americanus.* Many worms were seen in the intestinoal region.

Table 1: Anthelmintic activity against adult *Necator americanus.*

Evaluation in Necator-hamster model		Worm Burden (Average)	% Clearance (Cure rate)
CGI 13866 (benzimidazole)	Developed and not formulated	0.5	98.7
Mebendazole (benzimidazole)	In the market	6.5	82.7
Control		37.5	

Table 2: Effect on certain known anthelmintics on L-5 and early adult parasites of *Necator americanus.*

Anthelmintics	Dosage mg/kg	L-5 stage (4w after infection)		Early adults (5 weeks of infection)	
		PC	PWR	PC	PWR
Mebendazole	60 mgx1	40	83.5	40	88.1
Albendazole	50 mgx2	100	100	100	100
Amasconate (Go9333)	60 mgx1	70	98.8	40	88.6
New Benzimidazole (CGI 13866)	30 mgx1	20	78.5	80	99.3

PC=percentage cure; PWR= percentage of worm reduction
Background Information
Day 14-18 PI (post infection): Activity against L-4 stages
Day 21-27 Day PI: Activity against L-5
Day > 35 PI: Activity against adult worms

Discussion

Biologically speaking, for this type of work, appropriate models are required. Several preparations of anthelmintics are available such as albendazole, mebendazole and other benzimidazoles, and piperazine, pyrantel anthelmintics. Earlier on, scientists have used a basic rodent model, e.g., *Nippostrongyles brasiliense* and *Nematospiroides dubius* for screening compounds. *Nematospiroides* does not undergo lung migration whereas *Nippostrongyles* is very short lived in the intestine. These two rodent models were found to be unsuitable for research towards identifying candidate compounds and evaluation of potentially new anthelmintics for human indications. On that note, *Necator*-hamster model [6], is a unique model and solely useful for human intestinal worms and any newer anthelmintics are in need to improve the dosage and safety. In that point of view, *Necator americanus* - hamster model is relevant and greatly needed animal model for development of newer anthelmintics [7]. This model has been validated with known anthelmintics and found to be suitable for finding out new candidate anthelmintics. Well over 4500 synthetic compounds were so far trialled in this model at Hindustan Ciba- Geigy Centre in Goregoan, India and the activity of synthetic compounds reflected very well in this model. This model was found to be reliable for identification of potential drugs and GO 9330 (Amasconate) and CGI 13866 (newer benzimidazole) and other similar anthelmintics were identified and evaluated and the therapeutic dosage determined.

Dual Infection Model

Humans are naturally infected with two species of hookworms A*ncylostoma duodenale* and *Necator americanus* (Fig 7).

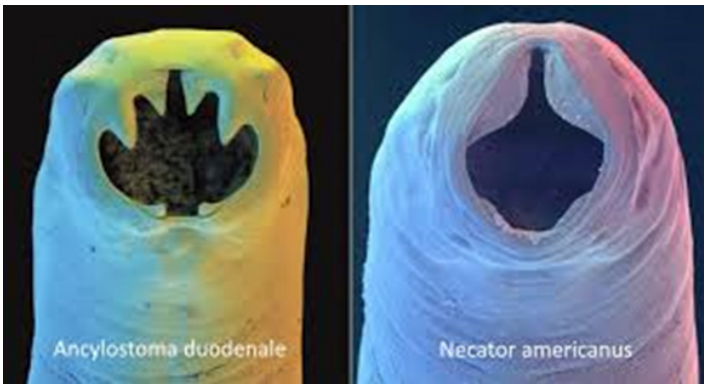

Figure 7. Two types of hookworms of human beings. From Internet downloaded from Facebook.com.

To mimic the situation in humans, an animal model for dual hookworm infection has been constructed in hamsters infected with both *A. ceylanicum and N. americanus* [8] for screening synthetic compounds. Two-day old hamsters were infected initially with infective larvae of *N. ameri-*

canus (NaL-3) and followed 3 weeks later the hamsters were reinfected with infective larvae of *A. ceylanicum* (as a duplicate for *A. duodenale* human hookworm). Three weeks later, hamsters were used for anthelmintics screening purpose. Equal number of hamsters were maintained with mono-infection of *N. americanus* as well as *A. ceylanicum*. The number of worms prevailing in the intestinal tract were identified, counted and their position was documented to establish the relevance of dual infection. Complete documentation is reported earlier by Rajasekariah et al. 1985 (Table 3). This model was used for screening purpose.

Soil transmitted helminths (STH) (A*scaris lumbricoides, Ancylostoma duodenale*, *Necator americanus* and *Trichuris trichiura*) are a group of helminths that are transmitted through soil contamination and are responsible for nearly one billion human cases in many parts of the world [15, 16]. In Bengal, Schad et al [9], observed that infective larvae of *A. duodenale* do not directly develop into adults but undergo arrested development. School children are required to be dewormed periodically to safeguard them from STH.

Single -dose albendazole and mebendazole are highly efficacious against round worms *Ascaris*, albendazole is superior to mebendazole against hookworms and mebendazole slightly out-perform Albenbazole against *Trichuris* [14]. Already through WHO and through Global Programme to Eliminate Lymphatic Filariasis (GAELF), nearly 90 countries have undergone mass drug administration which uses an anthelmintic, Albendazole, in this programme. This anthelmintic has its own effect in reducing the STH burden of residents who have undergone in 90 countries. This is beneficial in terms of reduction of worm burden (STH) in different parts of world. Such programmes are beneficial for controlling STH infection across the globe. Single dose of albendazole improves growth of Kenyan school children [11]. In China, hookworms are also important and nearly 39 million people are infected in 2006 [12], despite of improved living condition, economic growth and urbanization. Reinfection with hookworms is common and this is essential for continuation of hookworm infection [18].

Table 3: Dual infection model.

Necator americanus mono-infection	10	11	15	15	17	20	20	21	36
Dual Infection Na+ Ac	NA + AC	12	13	20	27	27	29	29	30
		39	19	15	13	21	10	12	31
Ancylostama ceylanicum Mono-infection		6	8	12	20	21	28	29	30

Hamsters received 50 *N. americanus* infective larvae percutaneously when hamsters were 2-days old and 50 *A. ceylanicum* infective larvae per os three weeks later.

Different hookworms present in humans and animals; they are enlisted in Table -4 and generally compared. Although the table depicts different species present in different hosts and the conditions and attributes are the same for most of the hookworms. Exceptions are in location- most species use small intestine as the seat of predilection; only one species- *Grammocephalus* - present in the stomach of elephants and worms burrow cavities within the gastric mucosa. That is the exception otherwise the behaviour of the hookworms is the same.

Table 4: Type of Hookworms seen in Man and animals

	Human being	Domestic	Pet animals	Wild animals	Geographical distribution
Ancylostoma duodenale	Primarily		Experimental		Tropical and temperate climate
Necator americanus	Primarily		Experimental		Tropical environs
A.caninum	Model for human hookworms CLM		Dogs and cats. Larvae Arrests in the body and result in prenatal infection.	Very much common in all	Ubiquitous
A, braziliense	important CLM in humans		Dogs and cats		
A. ceylanicum	Rare, complete development in humans. Also produce CLM,- less frequently		Cats and dogs	Wild caniids	Asia
A. tubaeforme	Main cause of Cutaneous larva migrans (CLM)		Cats and other felids		Temperate climate
Bunostomum phlebotomum	CLM	Cattle			
"B. trigonocephalum"	CLM	Sheep and goats			Tropical climate
Uncinaria stenocephala	CLM less frequently		Occasionally in Dogs and cats	Fox and wild caniids	Colder climate Canada northern USA
Grammocephalus	N/A	N/A		Elephants	Ubiquitous

The world population has reached up to 7.9 billion, of which the bulk of the people living in the tropical environment. There are as yet no practically useful and effective preventative and control measures against wide variety of tropical parasites. Living conditions and sanitation determine the prospects of these infections. The only answer remaining is the usage of anti-parasiticals (anthelminthics) as effective and curative tool. The magnitude of hookworms can be visualised in the attached map (Fig 8). Vaccines are still in experimental stages and are not yet available. It takes years to develop suitable vaccines against metazoan infection. As the bulk of affected people are continuously exposed and infected throughout the year exposing to a wide variety of infectious diseases. The only practical way is to carryout mass drug administration (MDA) and recently WHO and other bodies have successfully carried out MDA for control of lymphatic filariasis and which has its own effect on gastro-intestinal parasites. This control of parasitic infection is being clearly shown to be practically effective through mass drug administration (MDA) against some of the endemic infections. Such a population live in the tropical and subtropical regions of the world. Anti-worm or chemotherapeutic approach in the form of deworming school children is very useful [13]. As there are anthelmintics such as Mebendazole, and Albendazole as effective chemical cure and periodically these anthelmintics are routinely used in eliminating these worms. Developing new anthelminthics is a core business of pharmaceutical industries. But they have realized that there is limited or no reward in the business of identifying or developing newer anthelminthics. That is the main reason why premier companies are not interested in this venture because it takes at least 10-12 years to develop an anthelmintic drug.

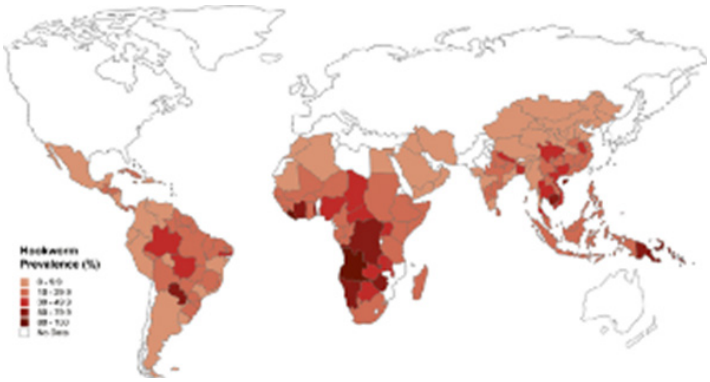

Figure 8. Geographical distribution of hookworms. (From Hotez PJ et al. 2005 Hookworm "the great infection of mankind PLOSMEDICINE 2 e67).

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