Boffin Access

Open Access Journal | Volume: 2.2

Biomedical Research and Reviews

ISSN 2631-3944

The Establishment of Human Hookworm, Necator americanus in Hamsters - A Retrospective Look

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Keywords

Human hookworm; *Necator americanus*; Adaptation; Laboratory hamsters; Generations

Abstract

The developmental cycle of adaptation of human hookworm, *Necator americanus* into laboratory rodent host hamster, is being critically reviewed. This laboratory model is found to be reliable one and recommended for various biological experimentations. This model is undoubtedly representing all aspects of human hookworm biology, and for elucidating activity of chemotherapy and planning control experimentation. Adapted - hookworms are now available and can be sourced either from University of Nottingham, England or from Prof SH Xiao Shanghai, China. Further this model may prove useful for assessment of newer anthelminthics and to study the protective immune responses against the infection provided careful planning and windows of assessment be carried out under controlled conditions.

Introduction

More than1.5 billion people are infected with greatly-neglected soil- transmitted intestinal helminthic infections, caused by different species of parasitic worms for eg the round worms, (*Ascaris lumbricoides)*, the hookworms (*Necator americanus* and *Ancylostoma duodenale*), the thread worms, *(Strongyloides stercoralis*), the whip worms, (*Trichuris trichiura*), and the pinworms (*Enterobius vermicularis*); these infections are widely distributed in tropical and subtropical countries. About480 million suffer from hookworm infection alone which affect the poor and most deprived communities. They are transmitted by eggs present in human faeces, which contaminate the soil in areas where sanitation is poor, hookworm eggs hatch in the soil releasing larvae that mature into a form that can actively penetrate the skin. People become infected with hookworm primarily by walking barefoot on the contaminated soil [1]. Hookworms are major human pathogen and infected children are nutritionally deprived and physically impaired and they are affected mentally. These worms produce iron-deficiency anaemia and protein malnutrition in developing countries. Hookworms are commonly seen in most of the countries and usually more abundant in rural as opposed to urban communities [2]. Because they feed heavily on blood, infected persons suffer from iron deficiency anaemia.

Hookworms research has been greatly impeded by a lack of suitable animals for experimental work [3]. A laboratory model for hookworms is vital. Hookworms can be infected into large animals such as dogs pre-injected with cortisone and are expensive to maintain [4]. Alternatively, a rodent model is most -wanted for laboratory maintenance and controlled experimentations. *Necator americanus*-hamster model is well established one and used for several indications over many years. As early as 1967, Sen and his associates published an article in "Nature" on adaptation of human hook worm, *Necator americanus* in hamsters. Human derived NaL_3 were used to infect 2- to3-day old (neonatal) baby hamsters. The NaL_3 migrated inside the body, reached lungs and swallowed down the gullet and established at the gut level and later on differentiated into male and female worms. The male and female worms mate and produced eggs and thus completing the entire life cycle in hamsters. This full development of *N. Americanus* in the experimental host initiated with human derived infective larvae (NaL_3) is a great achievement indeed. Sen & Seth [5] concluded that the hamsters are suitable experimental hosts for human hookworm infection. Initially Sen [6] adapted human parasite, *Necator americanus* on injecting cortisone to experimental animals (up to 4 generations) and intensively made susceptible to *Necator* pathogen. The source of infective larvae (NaL_3) was originally derived from infected humans (as early as 1965). This human- derived NaL_3 formed the basis of adaptation in laboratory animals. As early as 1966 *Necator* was established in hamster. Hamster-adapted Generation No 1 started on May 31, 1966 and then on adapted parasite has been passaged in hamsters successfully. Sen [6] reported certain imbalances in earlier generations. Cortisone injections in to the hamsters were made altogether a different picture for worms to establish in the gut

Article Information

DOI:	10.31021/brr.20192112
Article Type:	Review
Journal Type:	Open Access
Volume:	2 Issue: 2
Manuscript ID:	BRR-2-112
Publisher:	Boffin Access Limited
Received Date:	02 October 2019
Accepted Date:	21 October 2019
Published Date:	23 October 2019

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Citation: Rajasekariah GH. The Establishment of Human Hookworm, Necator americanus in Hamsters - A retrospective L. Biomed Res Rev. 2019 Oct;2(2):112

initially for 5 generations; subsequent generation were successful, in absence of cortisone.

Cortisone acetate is known to suppress the cell-mediated immune response as well as humoral response in animals [7] against a particular pathogen; effector macrophages migrate to the site of foci where larvae are migrating and kill them. Without cortisone, in hamsters, many larvae migrating inside the body succumb to immune attack before reaching the gut. Those avoiding this mechanism ultimately reach the gut and establish as adult worms. Hamsters, aged ~45days- old, carry about 60% of worms (out of 100 NaL_3used for infecting neonatal ones) and about 40% worms could not establish thereby eliminated by the immune attack possibly at the gut level. The early adults have the ability to resist the rejection force and possibly certain factors able to help them in establishment. Here at the gut level some resistance will come into force against the establishment of worms [8]. Worm expulsion was considerably reduced and animals continue to harbor a greater population of parasites by about 12th generations [6]. Recently, it has been demonstrated that Muc5ac, a mucin in the intestinal tract may be responsible for rejection of enteric nematodes [9].

Once adult worms developed in hamsters, the eggs produced from these adapted worms were used for culturing infective L_3 for further propagation. Eggs were uniformly cultured and the resultant L_3 was used for re-infect new neonatal baby hamsters for succeeding generations. This is how adaptation occurred and adapted worms were propagated for successive generation. Thus, the L_3 infection of adapted worms initiated in baby hamsters (neonatal) has produced adapted worms for succeeding generations into hamsters. This is how kinetics of adaptation occurred in permissive hosts. At least, Sen [6] has achieved the migration of *Necator* and resulted the adult worms to establish in the gut. With this, Sen [6] has shown that the hamsters as permissive host for human hookworms, *Necator.* It is a great achievement indeed!. A suitable model was required for gut helminths and its lacuna was filled successfully. A model parasite system in laboratory hamsters has achieved. Subsequently, over the years, this model has been extensively used for screening synthetic compounds and to study the activity of newer anthelmintics and identify the potential newer chemotherapeutic compounds.

It may be desirable to narrate certain specific details entailing the establishment of hookworm model in hamsters. Prior to *Necator*-hamster model, there was no suitable model for soil- transmitted nematode parasites. This could be used as a retrospective aspect. Once the model is set some of the details were not documented. How the adaptation came about and how the hamsters were considered as the suitable host for experimental purpose. The trend appears to be laboratory animals such as rats and mice which are not permissive host for soil transmitted nematodes and rather hamsters are relatively permissive and considered to be appropriate to use them for experimental purpose. Cortisone injections given to hamsters initially have provided the required condition for *Necator* worms to establish in the gut [6] and made any non-specific immune response nullified. In mice, they did not observe any such changes in the behaviour of the migration of larvae under the influence of cortisone [10]. In hamster, under the influence of cortisone, many worms could able to establish at the gut level [6,11]. The established adult female worms under the cortisone influence have laid eggs. These eggs became the source of adapted-NaL_3 which in-turn set the standard for infecting neonatal baby hamsters for future generation. The kinetics of adaptation occurred at all level such as the egg production, larval development and infection of L_3 to baby hamsters, larval migration inside the body and ultimately the establishment of adult worms at the gut level. These parameters have influenced the kinetics of *Necator* in hamsters.

Using this hookworm model well over 5000 synthetic compounds have been screened for antihelminthic activity.

Development in Hamsters: The life cycle stages are thoroughly worked out in humans and there was indication of how they progress inside the body and how they will establish in the small intestine of humans. The development of human parasite, *Necator americanus* is not readily amenable to the hamster's system. It took some time to adapt itself to the bodily condition of hamsters. The sheathed infective larvae (NaL_3) were deposited on to the tender skin of 2-to 3 –day- old neonatal hamsters. On close examination, it was observed that deposited NaL_3were able to completely penetrate the skin and all larvae reached interior successfully for onward progress of development. After reaching the blood circulation, a period has reached that these developing larvae accumulate in the lungs. During the phase of migration, many succumbed the resistance mechanisms and only a few reaches the lungs. This period in the laboratory animals may takes place after 6 days of infection and sometimes it may take even longer. These excysted (sheath-free) L_4 larvae in the lungs are grown into size, larger in appearance and can be seen under naked eyes. The L_4 stages can be recovered from the lungs under fairly clean conditions and readily be used maintenance of *in vitro* condition. These stages are best suited for production of antigen. They do release the antigen into the suspended medium. These stages are excellent for recovery of antigens. These larvae are being coughed up and ultimately reach the gut to establish in the small intestines. As per Sen & Seth [5] adult worms measured (male 2.35 to7.75 mm; female 2.31 to11 mm). These are at 40 days after infection and need to grow little bit longer. (cf, *Necator* from humans: Male 5-11 mm Female 9 to 13 mm) (Figure 1 - 3) [Table 1].

Methodology followed

The 2- and 3- day old baby (Neonatal) hamsters along with

Parasites	Natural host	Experimental host	References
Nematospiriodes dubius	Mice	Mice	Mitchell&Prowse [12]
Nippostyronglyus brasiliensis	Laboratory rat	Rat	Ogilvie&Jungery [13]
Soil transmitted			
Strongyloides stercoralis	Humans,	Experimental	Grove [14]
Strongyloides ratti	Rat	Rat (extensively used)	
Ancylostoma brasiliense	Dogs		
A. caninum	Dog		
A. ceylanicum	Cat	Hamsters	Ray & Bhopale [15]
A. ceylanicum	Humans		Lane [16]
Necator americanus (adapted)	Humans	Hamsters	Sen & Seth [5]
Necator americanus.+ A. ceylanicum (Dual Infection in hamsters)	Humans	Hamsters	Rajasekariah et al. [17]

Table 1: Rodent Models

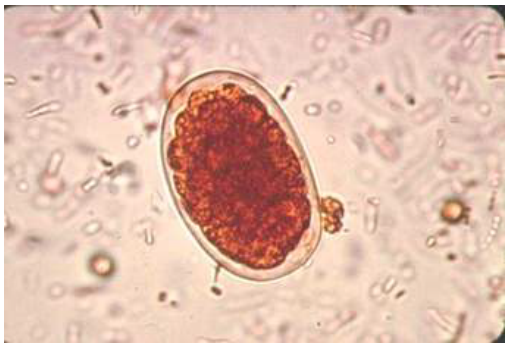

Figure 1: Egg of hookworm (down -loaded from Internet).

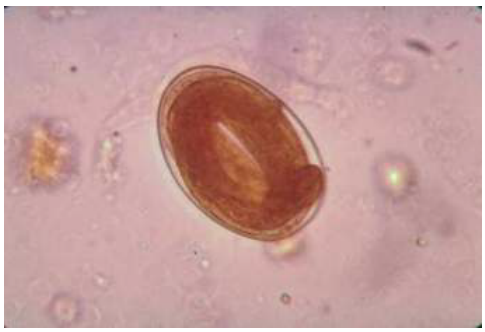

Figure 2: Embryonated egg with larva ready to hatch (Figure taken from Internet).

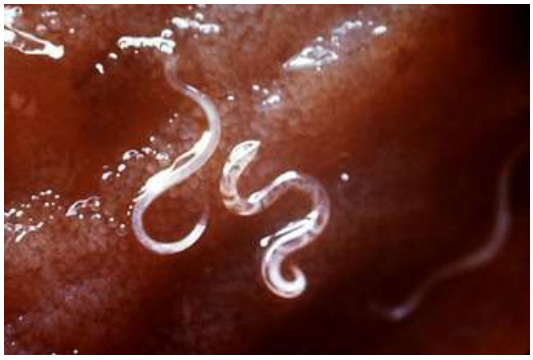

Figure 3: Adult worms attached to intestinal mucosa (Figure taken from Internet).

respective mothers were transported into the laboratory and baby hamsters were deeply anesthetised with warm ether. When baby hamsters become soft and unconscious (deeply anesthetised), they were taken out and restrained with tape onto a small metal plate with proper identification. In this way the baby hamsters were restrained and became motionless and are ready for infection. At this time the adapted NaL_3 were counted and arrange for infecting with precise number of infective larvae (NaL_3). The larvae were placed on a droplet of saline so that they can come in contact with the skin and able to penetrate the skin of the babies and establish themselves. The babies were held for a while until they woke up from anaesthesia and then they were transported to the cages and maintained with their respective mothers. They were held in the laboratory animals holding facility until maturity. The babies were weaned from the mothers and sexed at the appropriate time depending upon the labelled as infected hamsters.

Results and Discussion

The first lots of baby hamsters were infected with a dose of 300 Larvae in 50 to 60 µl saline. Saline droplet was left undisturbed until it dried up on the skin. During this process, all larvae penetrate the tender skin and reach interior and migrate inside the body. They reach the lungs and swallowed down into the intestine and ultimately reach the gut and differentiate into male and female adult worms and thereby establish in the small intestine region. This stage of infection is desirable and essential for knowing the effect of synthetic anthelmintic compounds on adapted- *Necator.* Sufficient number of control infected animals kept to know the worm burden. Control infection indicate the worm burden and those treated animals show the parasite number less indicating the positive effect of synthetic compounds. This model has been used as the primary screen of knowing the effect of synthetic compounds. Animals were autopsied 33 to 37 days post infection. The pattern of development is given below (Table 2). In this way generation of hamsters (Generation 18 to 27) received and there was wide variation in adult worms developed (Figure 4).

Later on, the dose of larvae slightly reduced to 250 L_3 per hamsters as the parasite slowly adapted itself to the hamsters. Generation 28 to 37 is improvement occurred (Figure 5).

The dose of 200 L_3 showed adult worms 35 to 60 with a further improvement as the generation (38 to 41) improved (Figure 6).

Further reduction in larval dose has greatly improved the final adult worms at the gut level. The adapted hookworm model has reached beyond Generation 75 on Nov 7 1984. By the time two positive compounds were identified Go 9333 and CGI 13866 (Figure 7).

Dose of 100 L_3 per animal was considered as satisfactory because the number of adult worms was fairly good and model has reached a

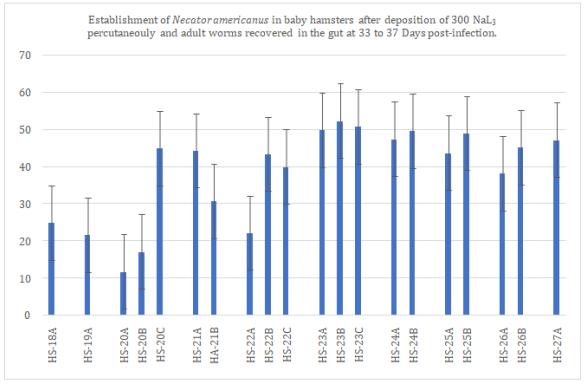

Figure 4: Development of *Necator americanus* from Generations 18 to 27.

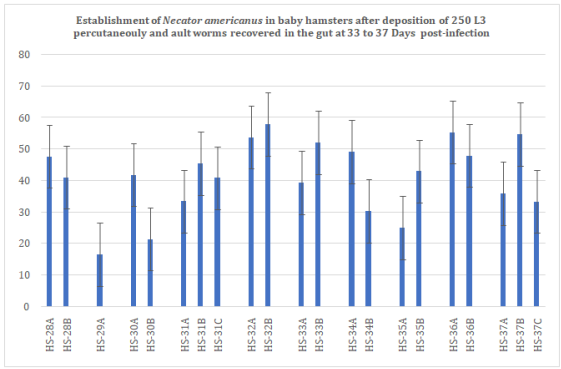

Figure 5: Development of *Necator americanus* from Generation 28 to 37.

	Generation HS 18-27	Generation HS 28-77	Generation HS 38-41	Generation HS 41-51	Generation HS 18-27
Dose of infection	300 L_3	250 L_3	200 L_3	150 L_3	100 L_3
Neonatal hamsters	Initiated	Initiated	Initiated	Initiated	Initiated
	Infected	Infection	Infection	Infection	Infection
	Percutaneously	Percutaneously	Percutaneously	Percutaneously	Percutaneously
Development	Normal	Normal	Normal	Normal	Normal
Autopsy	33-37 days post-infection	33-37 days post-infection	33-37 days post-infection	33-35 days post-infection	35-40 days post-infection
Adult worms	Both male and female worms seen	seen	seen	seen	seen

On a larger usage, neonatal hamsters are routinely infected with 100L_3 percutaneously

Table 2: Different generations and development of *N. americanus* in hamsters

satisfactory level. Generation 55 to 84 yielded a range of adult worms (25 to 45) at 35 to 40 days after infection. Here the main objective is to screen synthetic chemical compounds against the adult worms of *Necator.* We were interested to study the compounds exerting action on the adult worms and thereby clearance of adult hook worms from the gut of hamsters. A fairer indication of number of adult worms present in the control infected animals provided the effect of synthetic compounds. The hamster model is used for screening synthetic compounds. This model has been used extensively to screen compounds in identifying the activity against target anti-parasites activity. There are no other situations to predict the activity of these compounds. Out of so many synthetic chemical compounds screened, Sen [18] identified 4-isothiocyanato-4"-nitrodiphenylamine (C 9333-Go 4540) a new anthelmintic with potent anti-hookworm activity. They found several hundred isothiocyanates tested in the primary screen of *N. americanus*-hamster model. Repeated tests have revealed that both adult and immature stages of *Necator americanus* are highly susceptible to these class of compounds Single oral dose of 30-60 mg/kg administered to hamsters harbouring a non-patent 37 -day old infection eliminated 94 to 99 of the total parasites while a single oral dose of 25 mg/kg completely eliminated the worms in adult mature patent infections [18]. The minimum effective dose against *Necator americanus* is 10 mg/kg (ED_{50})and the maximum tolerated dose in laboratory animals (mouse, rat, hamsters, dog cat, rhesus monkey is greater than 5000 mg/kg giving a therapeutic index of over 500. Phase 1 tolerability and searching dose studies were conducted by Vaidya et al. [19]. Further he observed that with tetrachlortheylene, iodothymol, and bitoscanate made us to wonder whether it would be ever possible to achieve zero post-treatment egg counts in a large population with a single course of anthelminthic. There was no evidence of any toxic effects and the only side effects is drug related giddiness in one patient out of total 30 patients and found to be effective against both *Necator americanus* and *Ancylostoma duodenale*.

Pharmaceutical industry with its approach of random screening has regarded the *Necator* model as a reliable *in vivo* model for identifying active compounds (Figure 8).

Biomedical research work is so valuable and cannot be carried out always on target population. Alternatively, biomedical models are highly desirable. For infectious disease, animal models require careful observation and results can be checked and re-checked for validation. Infectious diseases involved, in particular bacteria and parasite, and the way these organisms behave in animal model. Interested parasite stages are not easily grown in the laboratory without the involvement of experimental animals. Laboratory animal model is very important and highly desirable in pursuing growth of the organisms, and to study the bionomic of disease. The animal model is also essential for basic work carried out in animals. Biological aspects and in particular screening of unknown compounds, designing therapeutics and vaccine research [20].

There was considerable interest on *Nippostrongylus a*nd *Nematospiriodes* as rodent animal models, and these parasites presented considerable limitation in the rodents. Dr Bridget Ogilvie had worked extensively on *Nippostrongylus* and she identified limitation of *Nippostrongylus* [13]. *Nippostrongylus* parasites do not survive for a long time and they were naturally cleared (expelled) from the gut on their own due to elicitation of immunological response. This phenomenon, similar to the self-cure in domestic animals was a practical limitation of *Nippostrongylus*rodent model. In comparison, the adapted *Necator americanus* survive for a long time in the gut of hamsters (up to 60 - 65 days post infection). Ogilvie etal. [21] also studied the development of *Necator americanus* in hamsters for 14 generations over a period of 3 years. They were not successful in adapting the worms to hamsters in any consistent manner. They used similar techniques of Sen [5] infecting neonatal baby hamsters percutaneously with 200 infective larvae. First seven passages were made with cortisone. The first passage from man in hamsters appears to produce a good infection and even after 14 generations the capacity of our adapted strain to infect hamsters is no better and may still be less than this first passage from man. They made attempts to infect a number of species of laboratory rodents including mice, rats and gerbils but have not succeeded in establishing a patent infection even in immune suppressed or thymus cell deprived rats and mice. Thus, at present the hamster appears to be the only feasible small rodent host for *N. americans* [21].

Later on, Dr Ogilvie from Wellcome Trust made the University of Nottingham to show some interest on *Necator americanus*. Dr Jerzy M. Behnke of University of Nottingham expressed interest in this model. They were keen on using this model for experimental purpose. They expressed interest in the establishment of this model at the University with the grant from Wellcome Trust. They have undergone training on the establishment of this model at Hindustan Ciba Geigy Research Centre, Goregaon, Bombay and complete training was provided and co-operated in providing hamster -adapted infective larvae and other materials for their study. Then this model has been established successfully at University of Nottingham under the guidance of Dr Behnke. Initially infected animals were provided as seed colony and as later on infective L_3 were provided to inoculate hamsters at University of Nottingham. This group included Dr David Pritchard produced many publications based on this hamster- adapted human hookworms. At long last, hamster-adapted *Necator* was successfully handled by the Nottingham research group and they are highly successful in using it independently outside the Ciba Company.

Then, there was also another request came from Rockefeller University, New York, USA (Dr Peter Hotez) for working on proteolytic enzymes of hookworms for his doctoral thesis. We supplied only the infected male hamsters to him. Nothing came out on this effort and the model was not established in USA.

For carrying out any work on human hook worms, appropriate model is required. Using *Necator*, a robust metazoan parasite, it is difficult to establish the In vitro experimentation because of non-availability of developmental stages of hookworms. Although the

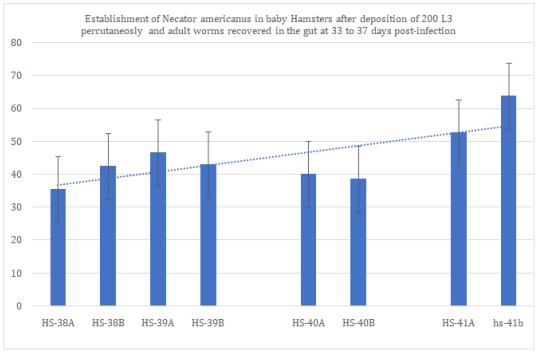

Figure 6: Development of *Necator americanus* from Generation 38 to 41.

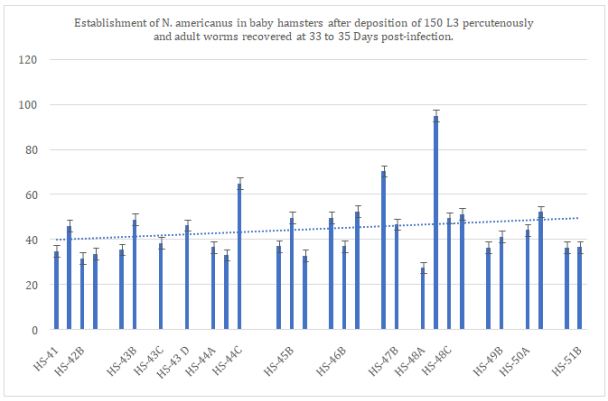

Figure 7: Development of *Necator americanus* from Generation 41 to 51.

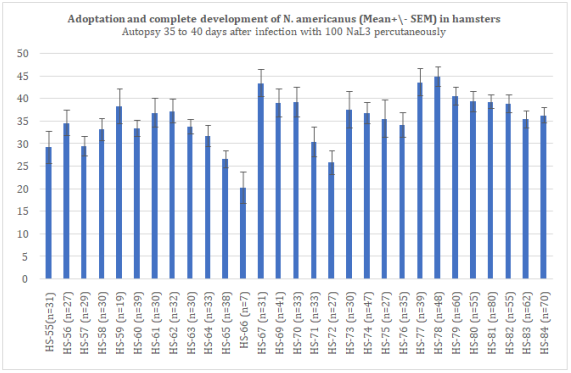

Figure 8: Development of *Necator americanus* from Generation 55to 84.

L_3 stages can be obtained quite easily but L_3 are sheathed and not represent the pathogenic stages. That is why the infected hamsters are preferred and the effect of anthelminthic can be studied and clearance of worms can be quantitatively monitored. That is where the value of Sen's adaptation of hookworms comes in [5] and this model is so valuable to characterise the synthetic compounds for its new anthelmintic activity.

There are elegant reviews written on human hook worms [22-27] and much emphasis laid on the protective immune response and possibility of developing worms- derived antigens as vaccines. Even sub-unit vaccines have been described. These points are beyond the scope of this article.

There are other works indicating one-hundred generations passaged in golden hamsters by Jian X et al. [28-30] and stating that this unique adaptation resulted with no need of exogenous steroids. But they have given steroids during the course of adaptation. When they have given the steroids and how much they have given, no details are reported. They have stated that they gradually removed of steroids and the passage reached up to 100 adapted generations. With this strain, recent workers [31] have used steroids in maintenance of *Necator americanus* from Prof SH Xiao Shanghai, China (see below). Whatever may be the fact that it is possible to adapt human parasite to suitable experimental model. Now in the hands of biologists there are two strains of adapted Necator; Sen's [5,6] and Jian X 's [28,29]. Here is the comparison that Sen and his associates [5,6] have administered steroids for first four generations and subsequently they stopped using it because they saw more adult worms establish in the gut. Subsequently this group used for screening the synthetic compounds. The methodology they adopted were highly suitable for them because the infected hamsters at the time of having adult worms (about 40 to 45 days old) in the gut, these animals are well suited for screening with synthetic compounds. But Sen [6] stated clearly that he has given the cortisone for first 4 generations and subsequently stopped. Administration of cortisone has helped to establish greater number of adult worms in the gut of hamsters. Parasites were successfully maintained by serial passages in hamsters for a period of 3 years (up to 12 generations). Parasites were surviving in the gut up to beyond 50 days from the date of infection.

Ideally, age and size of hamsters considered, a range of 30 to 50 adult worms should be more desirable in the gut (by the time 40 days). These are expected at the time of dosing the compounds to infected animals. The worm burden can be visualized in the control infection group. It is more or less achieved with *Necator* model by the Sen's group. But with Jian X model, it is anticipated an erratic uptake of worms due to usage of 9- 10- week old hamsters. The innate resistance of hamsters at the time of infection may limit the worm burden at the gut level [8.] Ogilvie et al. [21] also observed that majority of larvae or early adults do not establish well.

When I joined this laboratory, this adapted *Necator* parasite was continuously maintained in hamsters. We have donated this adapted strain in 1983 (at the 69th generation)to the University of Nottingham who successfully carried out several types of research work on this strain and published well over 15 publications [32].With this, one can conclude that the strain derived from Hindustan Ciba Geigy Research centre is well accepted and proven *Necator* strain in hamsters.

In China, Jian X [28,29] passaged *Necator americanus* in hamsters up to 100 generations over a period of 26 years. When they have initiated the infection and when they have administered the steroids and when they have eliminated, it is not clear. There is no clear write-up in their report. It is a monumental work and took so many years to adapt *Necator* to hamsters. They simply stated that they have "gradually removed steroids". Its time course is not clearly stated. They used 9- to 10- week-old hamsters and subcutaneously injected with 250 L_3 per animal to set up infection. The worm burden (6 to 81 on Day 27 and 24-34 pre-adult hook worms on day 34 post infection in female hamsters as well as 60-81 on Day 27 and 44-45 pre-adult worms on Day 34 in male hamsters). Adult worms were present in the gut at about 60 days (maximum burden of worms) and beyond this, many worms were expelled gradually. There is erratic "uptake" in the worm burden because of the age of hamsters at the time of infection. Injection of L_3 s/c would lead to erratic uptake of worms. Their intention of this model was to test the recombinant vaccines in hamsters.

Both these two strains of *Necator* survive reasonably well for a while unlike the other models of gut helminths which (viz *Nippostrongylus* and *Nematospiriodes)* were cleared pretty easily due to self - cure phenomenon. *Necator* parasites were resilient to Self-

Stages		Skin (Few hours)	Blood/Vascular	Lungs (Day 3-7)	Intestine (Day 9 onwards)	
L3	Initiate infection	Penetrate skin Exsheathment				
L4			Transient stages (Trophism)	Migrate through lungs (trophism) initiate tracheal migration (isolate larvae by lung perfusion)		Window 1: Larval counts can be made.
L5/Adult worms					Reach intestine (Triggers non-specific response)	
Adult worms					male and female worms (manual count). Expulsion after day 50 onwards	Window 2: Adult worms can be counted during Day 35 to 40 post infection by opening the intestine region.

Table 3: Trapping of stages of *Necator americanus* in hamsters

cure. The most desirable situation is to obtain a consistent burden of hook worms at the gut level (may be ideal will be 30 to 50). For meeting these criteria, it may be desirable to infect the young adult hamsters (preferable 3-4-week-oldones) with a smaller number of L_3 to avoid the competition in migration and elimination at the gut level.

Recently Hawdon [33] has reviewed and commented that *N. americanus*-hamster model as problematic one. No model is satisfactory for all experimentations and every model got its own requirements and limitations. One has to be careful in the interpretation of efficacy of these models. In case of Sen's adapted hookworms' model, it is completely satisfactory from his point of view where he has tested the activity of synthetic compounds. It is ideal that animals were infected and carried adult worms by the time of dosing with synthetic compounds thereby the anthelminthic activity can be elucidated. There seems to be no unsatisfactory points on this model instead there are many attributes. In case of Chinese, Xue 'model, it is being tested for the ability of recombinant sub-unit antigens for inducing protective immune response against adult worms. By the time adult worms establish in hamsters the worm burden is naturally erratic (see my comments above) and only a few residual worms exist in control group. There is considerable non-specific activity prevailing for the establishment of adult worms at the gut level (Ogilvie et al. 1973; Rajasekariah et al. 1985).Therefore, this model is unsuitable for monitoring the development of adult worms and assessing the induction of protective immune response. Instead, if they observe the developing the larvae in the lungs, this model may be useful. There are many larvae go through the lungs. At lungs the larval load is discriminatory and they can pinpoint the value of recombinant antigens inducing protective immune response against larvae of *N. americanus*. Then, the potential of recombinant antigens can be assessed in this model. Fig 6 illustrates how to overcome the problems of erratic "take" of adult worms. Trapping of larvae migrating at lung level is better way for rationalising the model for assessing the protective ability of recombinant antigen. In this way one can overcome the limitation of this model [Table 3].

Acknowledgment and Appreciation

The work reported is performed at **Hindustan Giba -Geigy Research Centre**, Goregaon East, Bombay. Many are involved in the adaptation of human hookworms, *Necator americanus*to laboratory hosts, hamsters. Great appreciation is expressed for the efforts and documentation of adaption and pains taking generation studies conducted under the direction of Dr Himamshu Sen, Bierendra N. Deb, K.R. Dhage, and Shukla Bose. Great acknowledgment is also expressed for the Corporate Hindustan Ciba –Geigy Pty Ltd for the Laboratory Animals Facility and other services provided during this investigation.

Author of this article has no conflict of interest on the adaptation of *Necator*into Hamsters.

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