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Research Paper

Explicating the role of Immunization against Hookworm infection

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Abstract

Human hookworm infection is most perilous and common loam-transmitted helminthes infection worldwide infects nearly 750 million peoples, most of them are not peculiarly symptomatic. Hookworm infection is of cosmopolitan important, considering the zoonotic importance of mice and dog hookworm *Ancylostoma caninum*, which are in close in association and need for understanding the human immunological responses inducing by infective larvae. *A.caninum* infection in mice was taken as a suitable experimental model. In the present investigation immunization with larval antigen was carried out in experimental group (vaccinated infected) where as control group (unvaccinated infected) kept as unimmunized, later both groups were challenged with 500 *A. caninum* larvae. The recoveries of larvae were examined under different time interval as 6, 12, 18,24,36,48 and 72, hours. After challenge infection immunization with larval antigen (25µg/mice) result in the induction of a strong protective acquired immunity with great expulsion of larvae from the gastrointestinal track and also complete destruction within the other parts of body. Results of the investigation clearly show that adoptive or acquired immunity induced by *A.caninum* in mice is due to an immunological reaction in which cellular factor are also important in transferring resistance. There was no difference in the migration pattern of experimental and control groups, in both the groups' larvae followed the same pathway at same period after challenge. It has also been suggested that the elimination of larvae from gastrointestinal track and the destruction of larvae within the other body parts are also mediated by cellular and humoral immunity. This study will help in understanding in the immunology, epidemiology, public health significance and control of hookworm, and would aid to the study of this imperative parasite in the upcoming century. We envisage that understanding the interplay in the induced immune signaling by larval homogenate would spur identification of meaningful targets for useful therapeutic modalities and would help in designing a new anti-hookworm vaccine.

Keywords: Hookworm, Immunity, Vaccination, *A. Caninum*.

Introduction

The research studies on human hookworm infections initiate at the dawn of the 21th century. In recent years, there have been striking improvements in our understanding of many aspects of this globally widespread parasite. Progress in cutting edges technologies and molecular biology has lead to the identification of a variety of new molecules from hookworms, which have importance either in the molecular pathogenesis of hookworm infection or in the host-parasite relationship; some are also promising vaccine targets. Now comprehensive treatment programmes are currently in progress,

supported by health education and integrated with the provision of improved water and sanitation. There are also efforts underway to develop novel anthelmintic drugs and anti-hookworm vaccines [1].

Hookworm infection and disease is a significant threat to global health. Recent advances, particularly those at the molecular level, have provided a wealth of opportunities to better understand pathogenesis. This will likely allow for the development of novel measures such as vaccines to complement existing control method. Despite a century's worth of global economic and social development, the enduring problems of malnutrition, lack of sanitation, and poor education have provided fertile ground for the myriad parasitic diseases that afflict the world's most vulnerable populations. Hookworms, blood-feeding intestinal nematodes, currently infect over 700 million persons in developing countries [2, 3]. Substantial reduction of hookworm disease will likely require the joining of conventional control methods with novel drugs and/or vaccines. Toward that goal, the past year has seen notable advances in our understanding of hookworms that are likely to prove useful in future eradication efforts. Hookworm probably one of the most widespread infectious agents is usually found in the small intestine causing anemia to man and his domestic animals due to its voracious blood sucking habits. Though there is a still considerable controversy and lacuna in our knowledge concerning the pathogenesis of hookworm infection in man, there are virtually no substantiated evidence to prove that protective acquired immunity occurs in man, in animals particularly in dog and mice, there is however a wealth information available concerning various aspects of host parasite relationship. Vaccination would contribute immeasurably to improved public health, in much of the third world and improve the quality of life for many millions of people. The one vaccine to be used on any scale against an intestinal worm, that against the dog Hookworm *A.caninum* was withdrawn primarily because although protecting against Hookworm disease, it did not provide absolute protection against infection [4].

Much of the progress that has come from experimental studies in model system and from work directed toward the production of defined molecular vaccines against gastrointestinal parasite in domestic animals, both offer paradigm for the development of vaccine in human and will be used to discuss that, might be in the field. Attention will be focused on data relevant the immune control of those species against which vaccination is most necessary namely the major gastrointestinal parasite. In experimental oral infection *A.caninum* larvae migrates though the intestine liver, lungs and ultimately reach to the various muscles region and the brain of the mice [5,6,7] and persisting in muscles for more than one year [5]. In abnormal host mice majority of the larvae undergo lungs migration eventually accumulate in the viscera and subsequently development does not occurs [7, 8] found that when mice had been injected one year previously with *A.caninum* larvae were fed to uninfected pups patent infection were established in the latter. Kerr (1936)[9] established beyond doubt that *A.caninum* was able to induce a true acquired immunity using mouse as the experimental host. In oral infection of *A.caninum* larvae in laboratory rat, [10] demonstrated periodically expulsion of larvae without attaining maturity they could have however, developed further if they had been in normal host. The migration route of *A. duodenale* and *A.caninum* larvae in the abnormal host rat had also been studied by numerous other workers [11-17].

Who found that about half of the inoculated larvae bored through the intestinal wall and migrated via the liver, heart, lungs, trachea and pharynx to the muscles, the other half were readily expelled out of the system, 57% of the larvae which penetrated the wall of the host stomach or small intestine eventually reached the muscle about ten days after infection [17] despite the demonstration of acquired immunity against many parasites and advanced in the understanding of the immune response that the parasite generate in their host, protection to man and animal against parasitic diseases by vaccination has proved an elusive goal [18-20] in case of nematode infection, limited success has been achieved using live vaccine [21-24] but vaccination with nonliving nematode material has been generally unsuccessful. Vaccination of man and his domestic animals is primarily aimed at prophylactic immunization against infectious disease, due to fact that helminthes disease remains among the major health problem of today. Many of the major vaccine candidates are shared between these blood-feeding species, not only those from the blood-feeding stages but also those expressed by infective L3s in the early stages of infection. Challenges for the future include: exploiting the expanding genome information for antigen discovery, use of different recombinant protein expression systems, and formulation with new adjuvant [25]. Although protection was

apparent following vaccination with antigen prepared from the posterior region and from whole larvae worms. The dog hookworm *A.caninum* has been one of the most suitable experimental model for the study of host parasite relationship because of its cosmopolitan occurrence, convenient cultural technique and zoonotic importance; as accidental infection to man may cause cutaneous larval migrants.

Materials and Methods

Source and collection of A.caninum larvae
Dog is the larval host of *A.caninum* hence, faecal sample of dog was collected from low sanitary area where most the dogs are infected with *A.caninum*. The feacal sample serves as the source of *A.caninum* larvae, eggs of *A.caninum* present in dog feacal sample.

Experimental animal
The Swiss albino mouse *Mus musculus albinus* was selected as an experimental animal for the present study. They are brought from the College of Veterinary Science and Animal Husbandry Mhow. Kept in the animal house under ideal condition of light, temperature, ventilation and food. The 3-4 weeks old mice of either sex selected for experimental design and kept in different well labeled cages.

Cultural techniques of A.caninum larvae
Infective filliform larvae of *A.caninum* were obtained by the petridish method of [26] faecal sample of dog containing *A.caninum* eggs, mixed with sterilized sand 1:3 (1 part of faecal sample and 3 part of sand). The mixture was kept in small petridish containing a moisturized filter paper at the bottom. The small petridish was then placed in larger petridish containing 100ml of culture solution, which contained Sodium chloride (2 gm), and concentrated hydrochloric acid (0.1ml) in 1000 ml of distilled water (pH -5). The outer petridish was covered by another petridish to prevent evaporation. This set was then incubated at 25 to 28 ºC for 10 to12 days in dark. At end of this period, most of larvae had migrated in the culture solution, which was carefully collected in clean large beaker and allowed to settle for 1to 3 hours.

Preparation of inoculums
The supernatant was sucked with help of suitable pipette, leaving the bottom residual solution to about 25ml the suspension were transferred to clean centrifuges tubes, subjected to slow centrifugation at 700 rpm for 5 minutes. The supernatant was discarded and larvae were washed thrice in fresh distilled water. This process repeated for three times. Finally the entire sediment was transferred to a clean glass stopper 100ml measuring cylinder and made up to the 80 ml mark with distilled water.

Method for counting of larvae
The number of actively motile larvae counted by dilution method of [10] after vigorous shaking, 1ml of the suspension was pipetted out and transferred to several grease free slide with squares already made on the reverse side with a glass making pencil. Actively motile larvae in all the squares were carefully counted with a hand tally counter under dissecting microscope(X10). The average of three such counts was multiplied by 80 to get the total number of larvae.

Preparation of dose
Inoculums (with the desired no. of motile larvae) of 0.2ml per mouse was orally administrated into the stomach with suitable sized syringe fitted with a blunt 2" 18 gauge feeding needle. The mice were usually infected before there feeding time to minimize the chances of regurgitation.

Preparation of living larval antigen
Deadly packed 2 ml of *A.caninum* larvae were homogenate with homogenizer, the solution is centrifuged at 5000 r.p.m. and supernatant is sucked out and its protein was estimated with help of Lowry's method. Protein injected according to experimental design.

Larval recovery in various organs in mice

Mice from both the group were scarified under ether anesthesia at various intervals according to the experimental design, Stomach, small intestine, large intestine, liver, lungs, heart, kidney, spleen, brain, muscles of different region viz. head and neck, abdomen, fore limbs and hind limbs were collected in separate watch glass, minced into small pieces packed separately in small pieces of surgical gauge and immersed in beakers containing artificial gastric juice (5 gm pepsin, 8.5gm NaCl, 0.7ml conc. HCl in 1000 ml distilled water) and kept for digestion at 37 ºC for 3 hours. After digestion, contents of beaker were poured into separate Bearmann's apparatus for 4 hours and larvae settled at the bottom of funnel stems were collected into tubes. Aliquots of 1ml of the sediments were then pipetted and spread on clean glass slides and actively motile larvae counted under a dissecting microscope.

Study Design

Studies were conducted at different time course (n=3) ranging from 6 to 72 h. The final immunization dose of larval homogenate used for the treatment was 25µg/mice. Experiment was undertaken for the larval recovery of *A.caninum* (Hookworm) in mice. Mice were immunized intra-peritonealy with larval homogenate and there after challenged with 500 *A.caninum* larvae to assess the effect of immunization. There are two groups (A-unvaccinated infected & B-vaccinated infected) of mice each group comprises 21 mice; the larval recovery was done at different time intervals (6, 12, 18, 24, 36, 48 and 72h).

Statistical analysis

Student's t-test was employed for statistical analysis using SPSS software (SPSS Inc. Chicago, IL, USA) package.

Results

6 Hours: All the larvae recovered from mice of experimental group A (79.2%) and control group B (94.0%), in both groups most of the larvae recovered from gastrointestinal tract. Stomach and small intestine yielded 25.2% and 54.0% respectively in experimental group and in control group its 52.0% and 42.0% respectively (Figure 1A).

12Hours: The percentage of larvae decreased to 68 %(A) and 86.0% (B) in both groups, the percentage of larvae recovered from stomach was 24.0% (A) and 50.0% (B) in small intestine 44.0%(A)and 36.0%(B), but no larval migration in any others organs (Figure 1B).

18Hours: In both the group A and B there was suddenly a great decreased in the larvae recovered to 46.0%(A) and 86.0%(B).The percentage of larvae recovered from stomach was 16.0%(A) and 58.0%(B), from small intestine 30.0%(A) and 28.0%(B). Larvae did not migrate into any other organ and muscles (Figure 2A).

24Hours: Maximum larval recovery was made in both the groups A and B, where it was 41.6% (A) and 85.0% (B). This time larvae migrate to liver, lungs, heart and muscles of body. Stomach yielded 5.6%(A) ,41.0%(B) small intestine 19.6%(A) and 6.6%(B), liver 4.8%(A) and 23.0%(B) lungs 10.0%(A), 14.0%(B), heart .4%(A), 0.0%(B). Larvae were recovered from muscles of head and neck also 1.2 % (A) and 0.4 % (B) (Figure 2B).

36Hours: The total recovery was 40.6 % in group A and 84.6% in group B. The larvae continue to invade in others organs. The percentage of larvae recovered from stomach 5.0%(A), 18.0%(B) in small intestine 10.4%(A), 2.4%(B) in liver 5.0%(A), 4.0%(B) in lungs 8.0%(A), 11.0%(B) heart 0.2%(A), 1.0%(B). muscles of head and neck yielded 12.0%(A), 1.0%(B) respectively for experimental and control groups the larvae recovered from abdomen was 24.0%(B) from fore limb and hind limb was 10.0%(B) and 13.0%(B) was only from control group (Figure 3A).

48Hours: By this time larval burden in both the groups, was reduced in comparison to that at 18 hours and 24hours, most of the larvae from the gastrointestinal tract were either expelled or migrated to other organs

in both the groups. Stomach yielded 3.2%(A) and 3.0%(B), small intestine 6.2%(A) and 7.0%(B), large intestine 0.8%(A) and 2.4%(B), liver 3.8%(A) and 6.0%(B), lungs 8.2%(A) and 12.0%(B), heart 0.4%(A) and 0.6%(B), muscles of head and neck was 13.0%(A), 0.8%(B).Larvae recovered from abdomen, fore limb, hind limb in group B were 32%, 12.2%, 1.4%respectively(Figure 3B).

72Hours: The total larvae recovered from both the groups were 33.2 % (A) and 49.2 % (B). Percentage of larvae recovered from brain was 2.0 % (A), 6.0% (B). In control group B larvae recovered from liver 0.4%, lungs 2.6% and heart 4.0%. Most of larvae distributed among muscles of head and neck yielded 14.2%(A), and 20.0%(B), Abdomen 15.0% (A), 14.0% (B) fore limbs 1.4%(A), 2.2%(B) hind limbs 0.6%(A) (Figure 4).

A

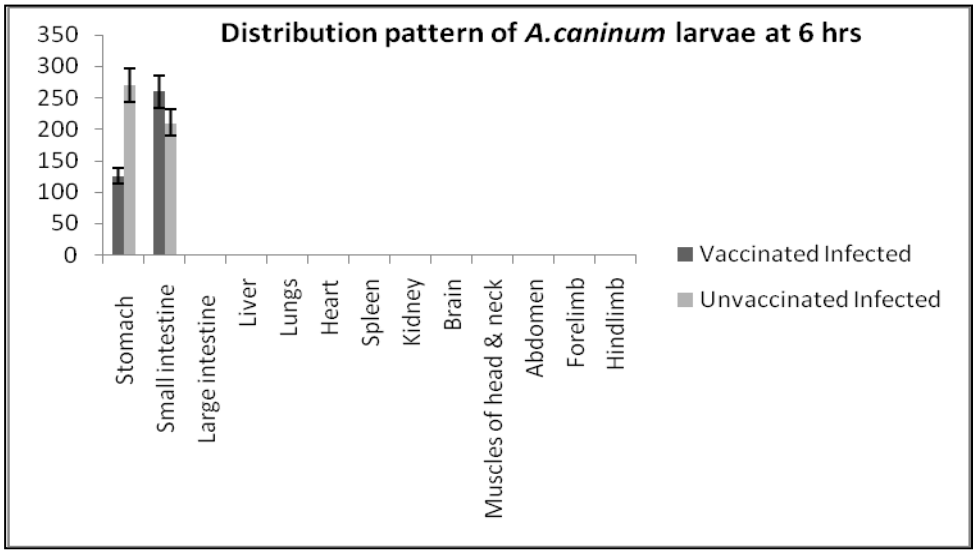
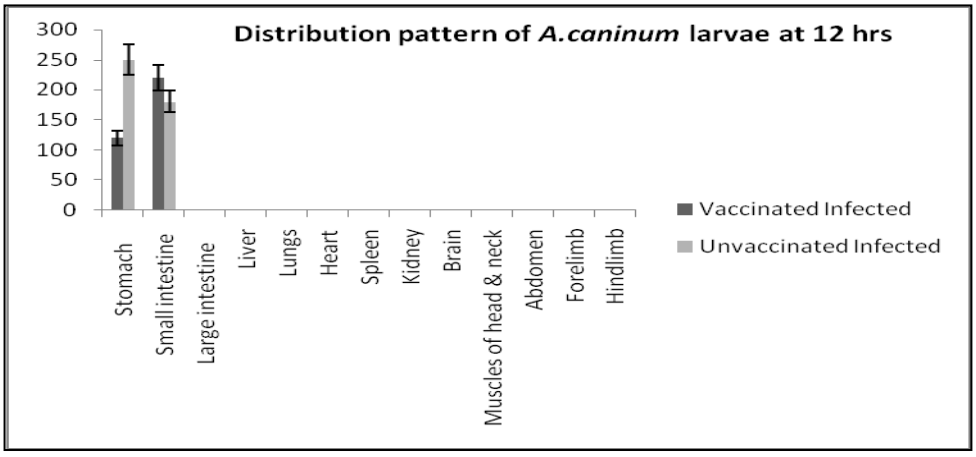
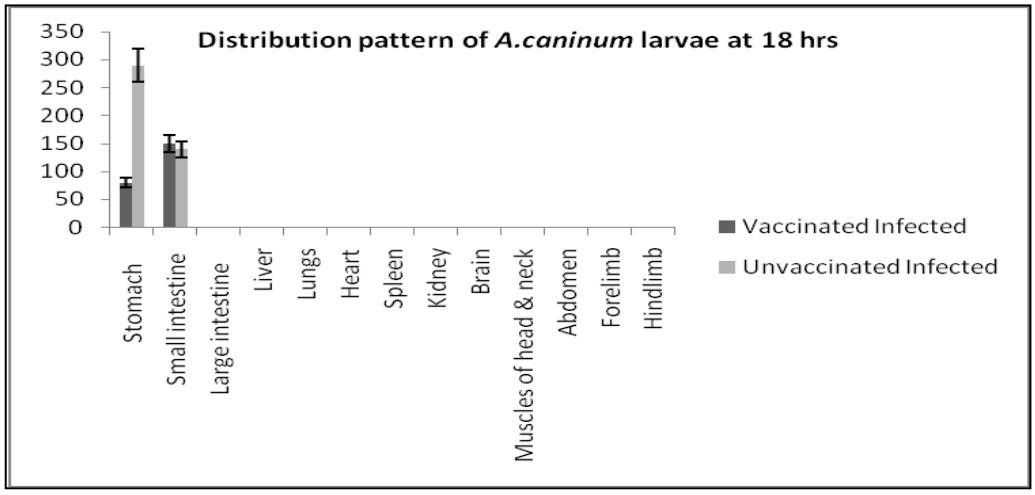


Figure 1: Graph depicting the distribution pattern of *A.caninum* larvae in mice, in vaccinated infected and unvaccinated infected group, at 6 hours (A) and 12 hours (B) after a challenging dose infection of 500 larvae given 15 days after last immunization dose of antigen

B



A



B

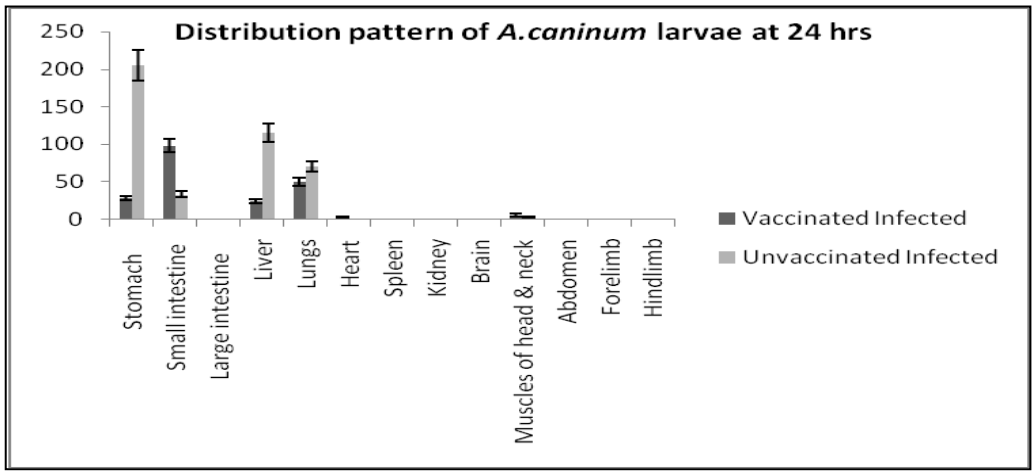
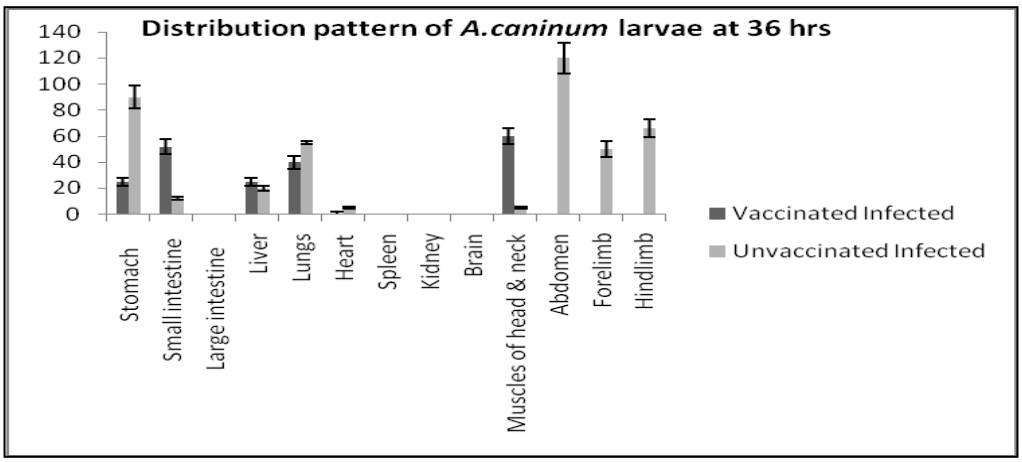


Figure 2: Histogram shows the distribution pattern of *A.caninum* larvae in mice, in vaccinated infected and unvaccinated infected group, at 18 hours (A) and 24 hours (B) after a challenging dose infection of 500 larvae given 15 days after last immunization dose of antigen

A



B

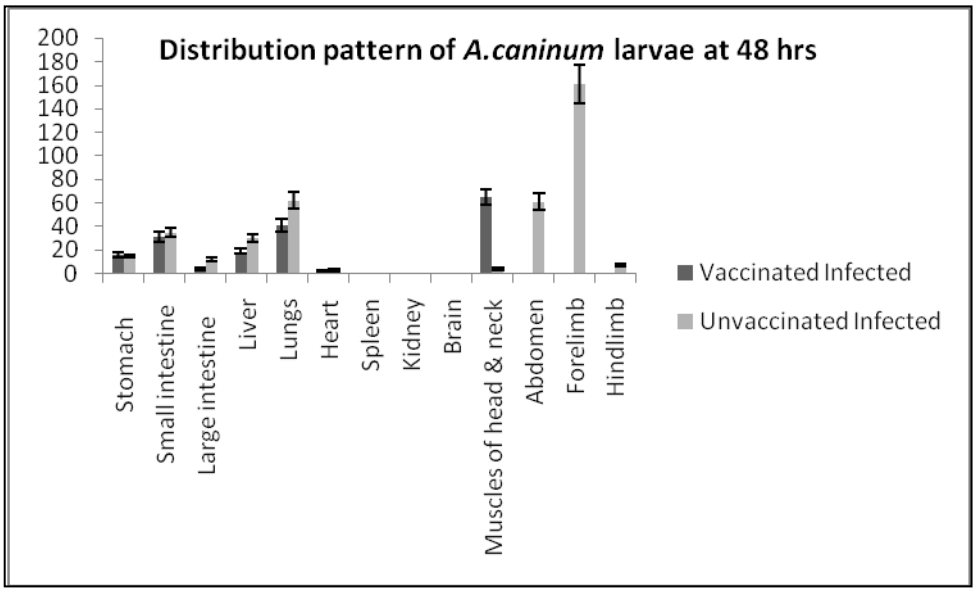


Figure 3: Histogram demonstrate the distribution pattern of *A. caninum* larvae in mice, in vaccinated infected and unvaccinated infected group, at 36 hours (A) and 48 hours (B) after a challenging dose infection of 500 larvae given 15 days after last immunization dose of antigen

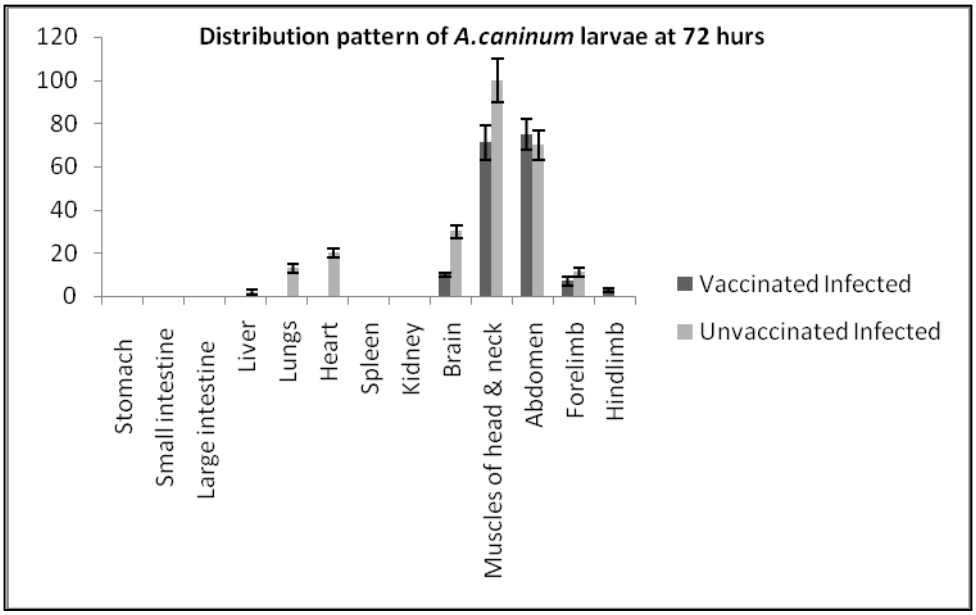


Figure 4: Graph shows the distribution pattern of *A.caninum* larvae in mice, in vaccinated infected and unvaccinated infected group, at 72 hours after a challenging dose infection of 500 larvae given 15 days after last immunization dose of antigen

Discussion

Through a lot of work has been carried out on various aspects of *A.caninum* in Swiss albino mice, many still remain to be explored. In case of nematode, limited success has been achieved using live vaccine [21,22,23,27,28,29] but vaccination with nonliving material was generally unsuccessful. An important finding in the present investigation is that lungs migration by the larvae took place from 24 hours onwards in the experimental group and indicating lungs migration in adoptively immunized mice. The earliest detection of the induction of immunity in mice by vaccination with somatic larval antigens of *A.caninum* was made at 24 hours after challenge resulting in a drastic reduction (74.2%) of larval burden in the host system. The migration of *A.caninum* larvae to the muscles occurred earlier in the experimental group comparison to controls, such an earlier migration was also observed by Vardhani and Johri (1981a) in adoptively immunized mice. Though the muscles offer a greatly favorable environment for the larvae as is evident from the greater and longer larval retention in control group where they may live for even more than one year [5] this does not happen in case of immunized recipient where destruction seems to proceed rapidly. Vardhani and Johri (1981a) have similarly recorded less *A.caninum* larvae from the actively and adoptively immunized mice.

In present study total worm recovery in both, experimental and control group generally decreased with the lapse of time after challenge, the rate was; however, higher in experimental group than control group. The maximum destruction and consequent expulsion of larvae took place in the intestine of experimental group up to 18 hours demonstration that the gastrointestinal tract plays an important role in the immune response to the mice to *A.caninum* larvae. Most of the larvae initially reaches to stomach and small intestine after 24 and 36 hours, after that most of larvae migrated towards liver and lungs and some of were toward muscles, after 48 hours larvae was abundant in muscles. But they were reduced subsequently at 72 hours. Most of the larvae were seen in brain, muscles, forelimb, hindlimb and abdomen. From the observation it is clear that in case of experimental group larval reduction is high than that of control group. It is due to the immunity produced by the host. The results reveal that cell mediated immunity or delayed hypersensitivity plays an important role in both expulsion and destruction of *A.caninum* larvae with an abnormal host, the swiss albino mice was used as an experimental model in the present investigation. In present study most of the larvae initially reaches to stomach and small intestine after 24 and 36 hours. Most of larvae migrated towards liver and lungs and some of were toward muscles, after 48 hours larvae was abundant in muscles. But they were reduced subsequently at 72 hours. Most of the larvae were seen in brain, muscles, forelimb hindlimb and abdomen.

From the observation it is clear that in case of experimental group larval reduction is high than that of control group. It is due to the immunity produced by the host, after immunization with larval homogenate of *A.caninum*. Moreover, underway control programmes have been indicates that morbidity caused by hookworm can be considerably reduced through usual chemical therapies. We envisage that understanding the interplay in the induced immune signaling by larval homogenate would spur identification of meaningful targets for useful therapeutic modalities and would help in designing a new anti-helminthes vaccine.

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