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Exploring Antigenic Properties and Immune Responses in *Bufo himalayanus*: Implications for Conservation and Biodiversity Protection

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

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ABSTRACT

Background: The objective of the study is to investigate antigenic property of soluble antigens of Nematodes *Cosmocercoides dukae* isolated from infected *Bufo himalayanus* (Class Amphibia, order: Anura) and to investigate the nature of protective immune response in laboratory rats. *Bufo himalayanus* is a species found in the districts of Darjeeling, Sikkim and adjoining hills of the sub-Himalayan belt, at an altitude of 6000 ft. above the sea level and known to be heavily infested by natural gut dwelling nematode infections. The present study is a small step towards protection and conservation of this amphibian species.
Results: study of morphology of the nematode parasite confirmed it to be *Cosmocercoides dukae.* A crude whole body homogenate (antigenic protein) of the parasite was injected in healthy non-

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infected rats, spleen histo-architecture of treated rats differed from control rats. Humoral immune response assay by Gel Diffusion Precipitin (GDP) Test revealed that the parasite antigen invoked very little to no response. Study of the crude nematode antigenic protein extract concentration was 6.09 mg/ml and protein profile assay by SDS-PAGE showed seven major and several minor bands.
Discussion: Though humoral immune response assay of parasite in experimental rats evoked very little or no immune response, this would not mean absence of antibodies, rather the antibody titers were presumably very low. It is to be noted that spleen of treated rats showed a distinctive histological feature. GDP test with infected toad serum also showed negative result. But protein profile assay of antigenic protein by SDS-PAGE showed many bands of proteins, which could be isolated and tested for further characterization.
Conclusion: In order to maintain the Himalayan biodiversity, this amphibian species needs protection from such natural infective nematode parasites. If we are able to focus any light to this area, this knowledge might help the future research workers to plan for the protection of this species.

Keywords: Parasite; nematode; Bufo himalayanus; humoral immune response; Darjeeling; sub-himalayas.

1. INTRODUCTION

A number of nematode species are known to parasitize the vertebrates of all classes, including the human beings. Nematodes, being the parasites of vertebrates, are now becoming a major focus for imnunoparasitological research The basis for this impetus lies in the fact that the health problems they create to their host have no protective value for reinfection [1] Strictly speaking, the apparent absence of immune response in host body against the gut dweling nematodes has generated special interest among the researchers to know how the vertebrate hosts protect themselves from such natural infections It has been well documented that the gut- dwelling nermatodes release a variety of molecules during their interaction with host tissues and that may initiate an inflammatory response mediated primarily by the host immune system. However, as already mentioned, protective immunity against the nematodes is absent or atleast ineffective, often throughout the lifetime [2,3]. To understand this mechanism of immune response, laboratory animals like rodents in which immunity can be demonstrated readily, have been extensively used in research.

During a laboratory examination on the gut-content of the Himalayan toad, *Bulo himalayanus* a good number of nematodes were recovered along with the other parasites. These nematodes were lumen dwellers and careful observation had revealed that they remained partially embedded in the host's rectal mucosa. One might then expect antibody production by the host,. Since the excretions or secretions of those nematodes are in direct contact with the immune competent cells of the host (located in the rectal mucosa and mucosa associated lymphoid tissue).

The present project was undertaken to investigate whether antigenic preparations from these nematodes evoke any immune response, upon immunization to healthy, non-infected laboratory white rats.

2. MORPHOLOGICAL STUDIES OF THE PARASITE

(a) Collection of Toad (*Bufo himnalayanus*): 10 adult toads were collected from little water pockets/area near Lloyd's Botanical Garden, Darjeeling. The time and rate of collection is as follows:

List 1. Data collection

	Time of collection	Rate of collection
1	Day 1 Time- 5-6 p.m.	5
2	Day 2 Time-4-6 p.m.	2
3	Day 3 Time-4-6 p.m.	3

(b) Maintenance of Toad (*Bufo himalayanus*): Toads were kept in wire cage and agarose powder was sprinkled inside and around the cage, in order to attract insects, so that they can easily feed on these insects. Sometimes, earthworms were also given as food to those toads.

(c) Collection of Parasites: Parasites were Collected in the Following Way: At first, a toad was anaesthetized by chloroform. The toad was then dissected and the hind gut (rectum) was taken out. After this, the isolated rectum was placed in a Petri dish containing vertebrate saline (0.89%NaCL). The nematodes were collected in a watch glass containing vertebrate saline one by one with the help of a fine brush.

List 2. Rate of collection

Day |	Number of Host Animals	Number of Nematodes isolated
Day 1	Toad 2	25+22= 47
Day 2	Toad3	17+12+21=50
Day3	Toad2	13+24=37
Day4	Toad1	15= 15
	Total=8	Total=149

(d) Light Microscopic Study of Parasites: In order to know the gross morphology, a few nematodes were fixed, stained dehydrated and mounted in the following way.

3. MICROSCOPIC OBSERVATION

At first, nematodes were fixed in 1% glutaraldehyde solution for 30 minutes. Then, the nematodes were washed in distilled water to remove the excess fixative solution. Fixed nematodes were then dehydrated in alcohol grades (50%-90%) and then stained with 1% eosin solution for 1-2 minutes. Next, the stained nematodes were washed in 90% alcohol to remove excess eosin solution and dehydrated in absolute alcohol for 30 minutes. They are cleared in xylene and mounted in DPX on a clean grooved glass slide.

1- Body Size: Usually, 5.0mm in length and 0.5 mm in breadth. Some are slightly long (6-7mm) and some are short (2-3 mm).
2- (Body Coloration: Nearly opaque white in colour.
3- Body Architecture:

(a) Usually long and rounded.
(b) Anterior end of the body is narrow and straight while the posterior end is curved ventrally and ends with a distinct spine.
(c) Alimentary canal is straight with lightly bulged esophagus. Alimentary c anal is distributed between the mouth at anterior end and cloaca at the posterior end.
(d) Body wall is thick and with striations.

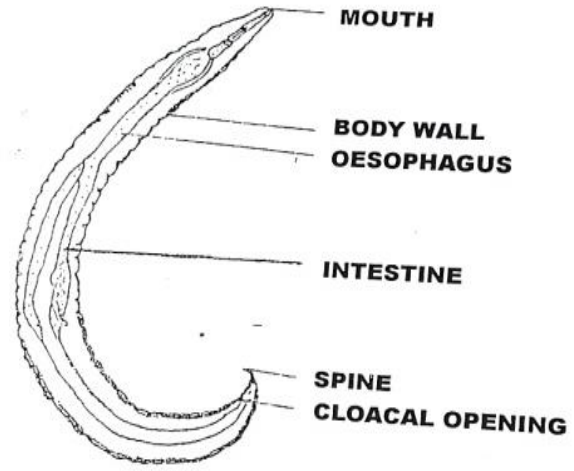

Fig. 1. *Cosmocercoides dukae*

Comments: The detailed morphological features of the said parasite suggest that it belong to the genus Cosmocercoides. sp. of Order -Ascaridea, Family-Cosmoceidae.

4. STUDIES ON ANTIGENIC PROPERTIES OF THE NEMATODE PARASITE

(a) Preparation of Crude Nematode Antigen: The isolated nematode parasites were kept in a watch glass containing little amount of vertebrate saline. After through washing with the help of a painting brush, they were transferred into a mortar containing a very little amount of lysis buffer. Then, with help of a pestle, they were macerated. After maceration, the nematode homogenate was centrifuged in a eppendorf tube at 5000 r.p.m. for 5 minutes. After centrifugation, the soup is collected in a small eppendorf tube and kept in refrigerator at -20^{o}C.

(b) Histo-Pathological Changes in the Spleen of Control and Immunized Rats: The spleen contains hematopoietic and lymphoid elements, is a primary site of extramedullary hematopoiesis, and removes degenerate and aged red blood cells as well as particulate materials and circulating bacteria from the blood supply. Lesions of this important component of the immune system may center on the red pulp, the white pulp or involve both compartments the spleen is the site of direct and indirect toxicity, a target for some carcinogens, and also a site for metastatic neoplasia. Many systemic or generalized diseases have splenic involvement [4].

(i) In the normal control sections of spleen, there were few cortical nodules interspersed amongst the tissues of the red pulp (medullary Cords) (Fig. 2).

(j) In the treated rats (that received immunization), the spleen histo-architecture was clearly different from the controls in that the cortical nodules, appeared more extensive, occupying over a large area of the medulla. The proliferated cells of the cortical nodules, were slightly dispersed and appeared faintly basophilic. There was no apparent change in spleen micro vasculature (Fig. 3).

Inference: The observed histological changes in the immunized rats is perhaps due to the exposure to nematode antigens, thus warranting proliferation of B- lymphocytes within the cortical nodules (white pulp) to tackle foreign antigens.

(c) Estimation of Crude Nematode Antigenic Proteins: Protein concentration of crude nematode homogenate was assayed by total protein kit (Biuret and BCG Dye binding method), (BCG = Bromo cresol Green) manufactured by Dr. Reddy's Diagnostic Laboratories Division, Bachu Palli, Hyderabad, A.P.

(i) Features of the Total Protein Assay:

Reaction type: End point with std.;
Wave length :555 nm (yellow green filter);
Incubation time: 10 minute;
Incubation temp. :370C;
Standard :6.4 gm. %

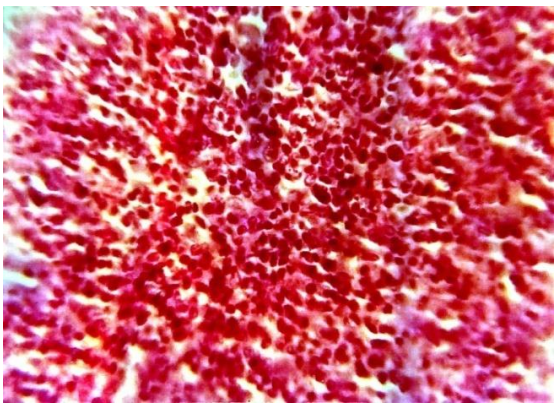

Fig. 2. SITE I

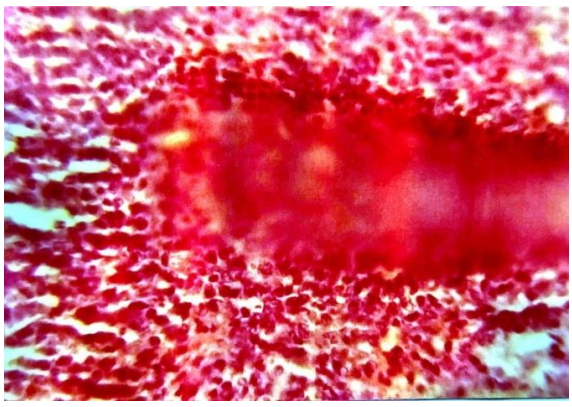

Fig. 3. SITE II

(ii) Pipeting Scheme: Three test tubes marked as (D) or blank, (S) or Standard and (T) or Test were taken and reagents mixed in each test tubes as follows:

List 3. Pipeting scheme for analysis

	(B)	(S)	(T)
Biuret reagent	1.0 ml	1.0 ml	1.0 ml
Dist. Water	2,0 ml.	2,0 ml.	2,0 ml.
Standard	-	0.05 ml	-
Crude Nematode Homogenate	-	-	0.05 ml

(iii) Procedure: Biuret reagent and distilled water were mixed into (D) or blank marked test tube Standard or (8) marked lest tube was filled with Biuret reagent, distilled water and standard solution Test or (T) marked test tube was filled with Biuret reagent, distilled water and serum. Volume of the reagents, mixed in each of these 3 test tubes are shown in the above table.Reagents taken in these 3 test tubes were mixed well and incubated at 37^{o}C for 10 minutes. The absorbance of standard (S) and test (T) Against blank (D) on a plhoto colorimeter with yellow greon filtler was moasured, at 555 nm (Hg540 nm).

1. Result: (Absorbance) B – 0
S-0.21
T-0.02

2. Calculation:

Total protoin in gm. %
Absorbance of T / Absorbance of S x Standard concentration= 0.02 /021 x 6.4 = 0.60952 gm % or or, 609 mg /100m! or, 6.09 mg /ml
Crude protein extract concentration is 6.09 mg / ml.

(d) Protein profile assay by SDS-PAGE: In order to know the different protein species present in the crude nematode homogenate, a SDS-PAGE analysis was done in the following way:

(i) Procedure:

(i) Preparation of 10% resolving gel-The glass plates were fixed with the help of spacers, clips and 1% Agarose as the sealing agent. Then 10% resolving gel is prepared by adding -Distilled water-1.9 ml.

30% Acrylamide solution-1.7 ml.
1,5 M Tris -HCI (pHB.8) -1.3 ml.
10%APS- 0.05 ml
10% SDS- 0.05 ml
TEMMED – 0.002 ml

After thorough mixing, gel solution is placed in the cassette of glass plates. Some water is added at the top of the gel. After solidification of the gel, water from the top is removed.

(ii) Preparation of 4% stacking gel and wells in stacking gel (by using comb). After resolving gel preparation, 4%stacking gel is prepared by adding

30% Acrylamide solution - 1.25 ml.
Stacking gel buffer (TRIS-Hc, Ph 6.8)-2.5 ml.
10% SDS-0.1ml
1.5%APS-0.5 ml
D. H_2O -5.65 ml
TEMMED- 7.5 µl

After thorough mixing, gel solution is placed on the top of the resolving gel and comb was placed for making wells. After solidification of the gel, comb is removed and wells were cleared by the reservoir buffer.

(iii) Preparation of sample: Equal amount of parasite homogenate and sample loading buffer is added in an eppendorf tube. Then the tube Containing sample is placed on a boiling water bath for 5 min.

(iv) Loading of sample 100 µl sample is loaded on a gel well and 2 µl of standard protein marker is also loaded on another well forcomparision. (Lane A= Protein markers and lane B= Nematode homogenate)

(v) Electrophoretic run

After loading the samples and protein markers on respective wells, the apparatus was connected with a power pack. A current of 60 volt was applied for about 1to 1.5 hr. for migration through stacking gel.When the tracking dye touches the resolving gel, 120 volt is applied for the next 2.5 to 3 hr. for total migration of the samples through the end of the resolving gel.

(vi) Fixation, staining and destaining of the gel: After the completion of Electrophoretic run,

the gel was removed from the cassette and placed in a container of fixative. After 1 hr. of fixation, the gel was placed in staining solution for about 30 minutes. After completion of staining the stained gel was placed in a separate container containing destaining solution for several hours.

5. RESULTS

Lane A showed five distinct polypeptide bands at 14 kDa, 25 kDa 68 kDa, 97 kDa, 118kDa of standard protein markers.

Lane B showed several distinct polypeptide bands of crude nematode homogenate between the range of 14 kDa to 125 kDa.

Among the several bands, on lane B, seven major bands were observed at 30 kDa, 45 kDa, 85 KDa, 88 KDba, 97 kDa. And 125kDa regions respectively. Apart from these major polypeptide bands, several minor bands were observed at the region of 14 kDa, 16 kDa, 18kDa, 20kDa, 25kDa, 28kDa, 32kDa (Fig. 4).

(i) Studies on humoral immune response assay of nematode homogenate: Immunization of experimental rats and collection of antiserum -

In our project two healthy and non infected rats were immunized with crude whole body homogenate of nematode parasites in the following immunization schedule to see whether the parasite derived antigens evoked any humoral imnmune response to rats. Nematode surface coat (SC) proteins and excreted–secreted products (E–S) are likely to play important roles in the host–parasite interaction and considerable similarities can be found in SC proteins and E–S products from certain plant and animal parasitic nematodes [5].

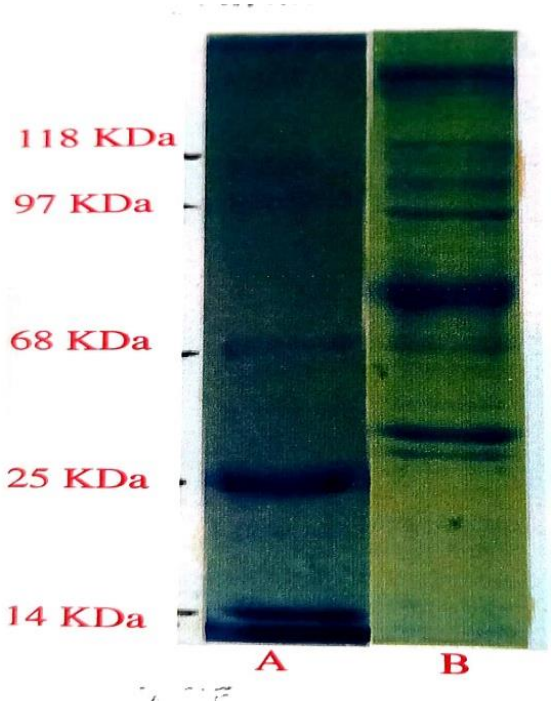

Fig. 4. SDS-PAGE Analaysis of crude homogenate of isolated Nematode
Lane A: Standard Protein Marker Lane B: Crude Nematode Homogenate

(ii) Immunization schedule: In the immunization schedule experimental rats received injections at repeated intervals of 3 to 7 days followed by booster injections In case of nematodes (used as immunogen) effective immunization can be a with simpie depot adjuvant such as Freund's complete and incomplete adjuvant, using 4 doses of about 250 µg antigen given for a period of 21 days. Three consecutive booster doses was given with 250µg of antigenalone at an interval of 3 days till 30th day of immunization.

Day	Dose	Rat I	Rat II
Day 1	1 dose	Antigen +buffer+FCA (200.ug+0.5 ml+0.5 ml)	Antigen +buffer+FCA (200.ug+0.5 ml+0.5 ml)
Day 5	2 dose	Antigen +buffer+FCA (200.ug+0.5 ml+0.5 ml)	Antigen +buffer+FCA (200. ug+0. 5 ml +0.5 ml)
Day 9	3 dose	Antigen +buffer+FCA (200.ug+0.5 ml+0.5 ml)	Antigen +buffer+FCA (200. ug+0. 5 ml +0.5 ml)
Day 13	4. dose	Antigen +buffer+FCA (200.ug+0.5 ml+0.5 ml)	Antigen +buffer+FCA (200. ug+0. 5 ml +0.5 ml)
Day 16	5 dose	Antigen +buffer (250 µg+0.5 ml)	Antigen +buffer (250 µg+0.5 ml)
Day 19	6 dose	Antigen +buffer (250 µg+0.5 ml)	Antigen +buffer (250 µg+0.5 ml)
Day 22	7 dose	Antigen +buffer (250 µg+0.5 ml)	Antigen +buffer (250 µg+0.5 ml)
Day 25	8 dose	Antigen +buffer (250 µg+0.5 ml)	Antigen +buffer (250 µg+0.5 ml)

(iii) Collection of Blood from Immunized Rats: The immunized rats wore anaesthetized with chloroform, kept in a wax tray and dissected. After that blood was collected from the heart of the dissected rat into 2 eppendorf tubes, one from rat I and the other from rat II. These2 tubes were kept in incubator at 370c for 1hr for bettor coagulation of blood and then the tubes were kept in refrigerator at 4^{o}C.

Collection of Serum: The serum was collected from the coagulated blood (kept into eppendort tubes in refrigerator) By micropipette and then centrifuged at 5000 r.p.m. for 3 minutes. The supernatant solution was used as an antiserum.

(iv) Humoral Immune Response Assay by Gel Diffusion Precipitin Test in order to know the immune-reactivity of the crude nematode homogenate a GDP test was performed in the following ways-

Procedure:

i) 1 gm. of agarose was boiled in 100 ml. of vertebrate saline (0.89% NaCl), till the solution become transparent.
ii) 4 grease free clean slides were taken and 3 ml. or agarose was poured on each slide very carefully, and slowly by taking care that the solution did not overflow from the slide.
iii) After 15-20 mins, when the solution had become opaque and partly solidified, small wells were made, on the gel.
iv) In first 4 slides, 2 wells were made at the left hand side and 2 wells on the right hand side. One well at each side is meant for antigens and other one is for antisera Wells were made usualy at a distance of 0.2cm
v) In the 5th slide, 3 wells were made and this slide was used as control. Among these 3 wells the central well was used for vertebrate saline and the two side wells were used for antisera collected from Rat 1 and Rat 2.
vi) The antigen used for immunization schedule was diluted 1 /2, 1/4, 1/8 and 1/16 of the original concentration with vertebrate saline.
vii) For test first 4 slides were loaded with antigen and antisera in respective wells. For control, on 5th slide, central well is dispersed with vertebrate saline and side wells were dispersed with antisera from Rat I(left) and Rat II (right).
viii) All sides were then placed in a moist plastic chamber, which is then left overnight at room temperature.
ix) Then all sides were stained with coomasie blue for 15 minutes and then to remove excess dye solution, destaining was done by washing readily with 5% acetic acid solution.

Initially, before staining the slides with coomasie blue a faint whitish band was found at middle position between the 2 wells (one well was filled with serum and the other was with antigen) of a slide. No positive result (1.e., formation of band) was obtained in the case of central side. But after performing staining and destining the faint bands become disappeared and no band was visible in any of these slides. From the GDP test analysis, it would seem, apparently that the parasite antigens evoked very little immune response to

rats (hosts) or simply absence of specific antibody against these crude nematode antigens. This does not, however mean that no antibodies were formed during the immune response to nematode antigens rather the antibody titers presumably are so low that these are somewhat difficult to demonstrate by a test like GDP test which is not highly sensitive, as it is for ELISA or western immunoblot technique which can demonstrate the presence of antibody even at a very low titer. GDP test with infected toad serum was performed in the same manner as in the case of rat, but no positive result was obtained.

6. DISCUSSION

Morphologic features tend to suggest that the nematode parasites were rhabditoid in nature, corresponding to the morphology of *Cosmocercoides dukae* (Holl, 1928) Taxonomy ID: 646688 (for references in articles NCBI:txid646688) was confirmed by scientists, at the Zoological Survey of India, Kolkata.

In the present experiment, crude whole body homogenate of nematode parasites as immunogen was injected into healthy non-infected rats, in an attempt to see whether the parasite derived antigens evoked any humoral immune response to rats. Similarily, rabbits were infected with Dermatophilus congolensis and tested for humoral immune response by indirect haemagglutination and for cell-mediated immune response to crude antigens of D. congolensis [6]. In the immunization schedule, the experimental rats received injection at repeated intervals of days followed by booster injection. No adverse effect was noticed in the immunized rats following injections and animals appeared completely normal till completion of the immunization schedule following this, their blood samples were utilized as source of antisera to perform the GDP test. However, a crude whole body homogenate (antigenic protein) of the parasite injected in healthy non-infected rats revealed distinct difference of spleen histo-architecture as compared to control rats.

SDS-PAGE study revealed several distinct polypeptide bands of unidentified nematode homogenate in the range of 14 kDa to 125 kDa. It was not known to us which particular polypeptide (s) elements were immunogenic to the hosts. In order to have information on this aspect, GDP test was performed. From the GDP analysis, it would seem apparently that the parasite antigens evoked very little immune response of the rats (hosts). This does not mean, however, that no antibodies were formed during the immune response to nematode antigens, rather the antibody titers presumably are so low that these are somewhat difficult to demonstrate by a test like GDP.

7. CONCLUSION

A thorough knowledge of parasite antigens and of the immune response that they elicit in the host is a prerequisite for understanding the factors which determine whether the outcome of the host parasite interaction will be resistance or susceptibility, immune-suppression, pathologic or asymptomatic. In essence, this involves determining which parasitic components are immunogenic (either surface molecules or metabolic products, enzymes, receptors etc) and identifying the interactions within the complex network of immunological phenomena.The antisera that were being isolated from the immunized rats can now be tested by immune-electron microscopy to see whether they bind to the nematode surface or to any other parts of its body. If there is a positive result, it would definitely be an indication of the generation of immune response in the experimental rats in response to nematode antigen.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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