

CURRENT TRENDS IN PARASITOLOGY

PROCEEDINGS OF THE
20TH NATIONAL CONGRESS OF PARASITOLOGY
SHILLONG, INDIA
(November 3-5, 2008)

Veena Tandon, Arun K. Yadav, Bishnupada Roy (Editors)

This book highlights the current trends and status of research relating to most common infections of parasitic origin in the tropics. It discusses recent advances in the emerging fields of Parasitology, namely molecular approaches to the identification of species, physiology and molecular biochemistry, functional biology and genomics of parasites, newer methods for the diagnosis of common parasitic diseases, advancement towards new antiparasitic drugs and issues pertaining to problems of emerging and re-emerging parasitic diseases.

The volume will be useful to Postgraduate Students and Researchers in Parasitology and teachers in the field and practitioners of medico-veterinary Parasitology.



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Foreword

Parasites have inflicted high morbidity and mortality to humankind since time immemorial. Although some of the parasitic diseases have been eliminated (e.g. Guinea worm) and many others are under effective control, bulk of these infections still continue to plague human and animal health. The impact is more pronounced in the developing tropical world. Parasitologists are confronted with complex problem of parasitic diseases in a rapidly changing environment that favors the survival and multiplication of these parasites. Changes in human ecology and climatic change may disseminate parasites far and wide, threatening global health. Chemical control of the parasites and their vectors may impede control efforts due to technical and economic reasons. Added to that, food-borne parasitic zoonoses have emerged as a major public health problem in many countries, tropical in particular, posing serious threat to human and animal health. Responsibilities on parasitologists to address these issues are far greater today than ever before. The organization of the 20th National Congress of Parasitology with the focal theme "Food-borne Zoonoses of Parasitic Origin: Molecular Taxonomy and Epidemiology", November 3-5, 2008 at the North-Eastern Hill University, Shillong (India), in collaboration with the Indian Society for Parasitology, has been a major step forward in pushing the agenda of a parasite-free world. The Congress received liberal financial support from several government and private funding agencies. During the 3-day program, eminent scientists from various Universities, national institutes and laboratories shared their knowledge and research findings on aspects such as molecular approaches to the identification of parasite species, physiology and molecular biochemistry of parasites and their vectors, besides their functional biology and genomics. A total of 18 plenary lectures were delivered, of which 7 submissions received have been published, besides the full-length articles of oral presentations. Scientists also participated in debates and discussions on topics related to newer methods of diagnosis of common parasitic diseases, advancement towards new anti-parasitic drugs and issues pertaining to problems of emerging and re-emerging parasitic infections. It is a matter of great satisfaction that special efforts were taken to compile recent research findings in a compendium form. The compendium volume, "Proceedings of 20th National Congress of Parasitology" contains 29 full-length articles of important oral presentations and keynote talks delivered during the conference, besides a brief conference summary. Some of the topics present in the volume include critical issues dealing with diagnosis, chemotherapy, vaccination and control of parasitic diseases. I am sure the information contained herein would provide ready reference of current information to the researchers to take the challenge of combating parasitic diseases forward with full enthusiasm.

V. P. Sharma

Dated: 5.10.2009

Dr. V. P. Sharma

Preface

Keeping in view the importance of parasite-borne infections, particularly those that prevail in the tropical regions of the globe, the 20th National Congress of Parasitology was organized at the North-Eastern Hill University, Shillong (India) under the auspices of the Indian Society for Parasitology from November 3-5, 2008. More than 125 distinguished delegates participated in the meeting, representing about 40 reputed universities and research institutes in the country and also a few from abroad. The focal theme of this Congress was “Food-borne Zoonoses of Parasitic Origin: Molecular Taxonomy and Epidemiology”. This compendium, comprising 29 papers including some invited talks and contributed research papers, is a part outcome of the deliberations held during the meeting and deals with the some of the recent advances in the field of parasitology.

No event of this magnitude is possible without the generous financial support from various sources. We wish to put on record our sincere thanks to the Department of Science and Technology, Department of Biotechnology, Ministry of Earth Sciences, Council of Scientific and Industrial Research, the North-Eastern Council (Government of India); The National Academy of Sciences India, the Indian Council of Medical Research, Indian National Science Academy, and North-Eastern Hill University for their financial support to program. Besides these public institutions, support from Lab India (Leica, Applied Biosystems), Kolkata; Labmate (Asia) Pvt. Ltd., Chennai; Premas Biotech Pvt. Ltd., Gurgaon; BSNL, Shillong; Panima Educational Book Agency, New Delhi; Laboratory Aids, Shillong; North-East Chemicals, Guwahati; Eureka Forbes, Guwahati; Bangalore Genei, Bangalore; Himedia Laboratories Pvt. Ltd., Mumbai; and Borosil, Mumbai is gratefully acknowledged and appreciated. Our grateful thanks to all our contributors for providing the manuscripts in time and for undertaking editorial changes. We also thank the Indian Society for Parasitology for entrusting us to hold this important event at North-Eastern Hill University, Shillong. Our special thanks are also due to various administrative authorities of NEHU and especially to the members of Organizing Committee of Congress for their active support rendered to the program.

We thank Mr. Manish Debnath for preparing the manuscripts in a camera-ready format and Panima Publishing Corporation, New Delhi for their cooperation in bringing out this compendium in print form.

Editors

Contents

Foreword	iii
Preface	v
List of Contributors	xi
1. Lipid binding nematode polyprotein allergen of the lymphatic filarial parasite, <i>Wuchereria bancrofti</i>: potential target for drug and immunoprophylactic development	1
<i>S. L. Hoti and G. Sivapragasam, Vector Control Research Centre, Puducherry</i>	
2. <i>Taenia solium</i> taeniasis and neurocysticercosis (NCC): experience from North Indian pig farming community	13
<i>K. N. Prasad, A. Prasad, A. Verma, R. K. Gupta and C. M. Pandey, Departments of Microbiology, Radiology and Biostatistics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow</i>	
3. Fish-borne zoonotic haplorchine trematodes: the question of differentiation of species	21
<i>R. Madhavi, Department of Zoology, Andhra University, Visakhapatnam</i>	
4. Cerebral malaria induced apo-necrotic mode of cell death in the mouse brain	33
<i>Meena Shukla, Anand Sripada, Padmini Arumugam and Prakash Babu Phanithi, Department of Biotechnology, School of Life Sciences, University of Hyderabad, Hyderabad</i>	
5. Application of recombinant proteins in molecular detection of <i>Toxoplasma gondii</i>	55
<i>A. K. Tewari and J. R. Rao, Division of Parasitology, Indian Veterinary Research Institute, Izatnagar</i>	
6. Animal trypanosomosis: <i>Trypanosoma evansi</i> – An emerging threat to human	61
<i>Veer Singh and H. R. Parsani, Department of Parasitology, College of Veterinary Science & Animal Husbandry, S. D. Agricultural University, Sardarkrushinagar</i>	
7. Development of vaccines against parasites: Current status and future directions	65
<i>C. L. Yadav and Stuti Vastya, Department of Veterinary Parasitology, G. B. Pant University of Agriculture and Technology, Pantnagar</i>	
8. Observation on one new species of the genus <i>Myxobolus</i> (Myxozoa: Myxosporaea: Bivalvulida), <i>M. naini</i> and redescription of <i>Myxobolus magauddi</i> (Bajpai, 1981) Landsberg and Lom, 1991 recorded from freshwater fishes of Kanjali Wetland of Punjab (India)	75
<i>Harpreet Kaur and Ranjeet Singh, Department of Zoology, Punjabi University, Patiala</i>	
9. Studies on infection and cross-infection of microsporidian spores of mulberry, tasar, eri and muga silkworm on economic parameters of silk to mulberry silkworm, <i>Bombyx mori</i> L.	81
<i>Satadal Chakrabarti and Buddhadeb Manna, Department of Zoology, University of Calcutta, Kolkata</i>	

10. **Detection of “Tim” gene of sheep *Giardia* by primers of “Tim” gene of *Giardia* of human origin** 95
Fateme Mirhadi, Mostafa Rezaeian and Mitra Zare Bavani, Faculty of Allied Health Sciences, Tehran University of Medical Sciences, Tehran, Iran
11. **RAPD-PCR assay for differentiation of *Sarcocystis bovicanis* and *S. bovifelis* in cattle** 101
Mamatha Gowribidanur Sanjeevamurthy, Placid Eugene D'Souza and Veluvorthy Venkata Sanyasi Suryanarayana, Centre of Advanced Studies, Department of Parasitology, Veterinary College, KVAFSU, Bangalore
12. **Prevalence of *Plasmodium falciparum* in Jodhpur (Rajasthan), India** 109
Aruna Swaroop Mathur and Barnali Majumdar, Department of Zoology, Jai Narain Vyas University, Jodhpur
13. **Platyhelminth parasite spectrum in edible freshwater fishes of Meghalaya** 113
S. Thapa, D. B. Jyrwa and V. Tandon, Department of Zoology, North-Eastern Hill University, Shillong
14. **Occurrence of the metacestode of *Taenia* sp. and the nematode, *Capillaria hepatica* in liver of wild rodents from bamboo growing areas of Mizoram** 127
C. Malsawmtluangi and V. Tandon, Department of Zoology, North-Eastern Hill University, Shillong
15. **Molecular characterization of *Lytocestus* spp (Eucestoda: Caryophyllidea) from *Clarias batrachus* (L.) in Meghalaya** 137
D. B. Jyrwa, V. Tandon and R. D. Lyngdoh, Department of Zoology, North-Eastern Hill University, Shillong
16. **Seasonal prevalence of intestinal cestodes of goats with respect to age, sex and breed in Kashmir valley, India** 147
F. Ahmad, K. A. Tariq and M. Z. Chishti, Post-Graduate Department of Zoology, University of Kashmir, Srinagar
17. **Molecular characterization of intestinal fluke, *Artyfechinostomum sufrartyfex* (Trematoda: Echinostomatidae) using PCR amplification of rDNA ITS regions** 153
V. Tandon, L. M. Goswami, P. K. Prasad and A. Chatterjee, Department of Zoology, North-Eastern Hill University, Shillong
18. **Observations on the occurrence of *Opisthorchis pedicellata* in *Rita rita* from Varanasi, India** 163
Sabhyata and Hare Govind, Department of Zoology, Harish Chandra P.G. College, Varanasi
19. **Two new species of xiphidiocercariae from the freshwater snail *Diagoniostoma pulchella* (Bithyniidae) in Palakkad, Kerala, India** 169
R. Brineth and K. P. Janardanan, Department of Zoology, University of Calicut, Calicut
20. **Population studies of *Camallanus fotedari* Raina and Dhar, 1972 in its host, *Nemachilus kashmiriensis* Hoar** 175
M. Y. Peerzada, Department of Zoology, Amar Singh College, Srinagar
21. **Pathogenicity and population dynamics of *Procamallanus spiculogubernaculus* Agarwal – a nematode parasite of fresh water** 187

	fish, <i>Heteropneustes fossilis</i> (Bl.)	
	<i>Anant Kumar Sinha and Chitra Sinha, Department of Zoology (P.G.), Jamshedpur Co-operative College, Jamshedpur</i>	
22.	Distribution and public health importance of <i>Anopheles minimus</i> Theobald, 1901 (Diptera: Culicidae) in India	193
	<i>S. Kamal and H. C. Mahanta, National Institute of Communicable Diseases (Regional Filaria Training & Research Centre), Varanasi</i>	
23.	Efficacy of <i>Eucalyptus</i> (a homeopathic medicine) in combination with artesunate to clear <i>Plasmodium berghei</i> infection in BALB/c mice	203
	<i>U. Bagai, A. Rajan and S. Chandel, Department of Zoology, Panjab University, Chandigarh</i>	
24.	<i>Setaria cervi</i> (Nematoda: Filarioidea): Absence of γ-glutamyl cyclotransferase activity	213
	<i>S. Gupta and A. K. Srivastava, Division of Biochemistry, Central Drug Research Institute, Lucknow</i>	
25.	Immunological markers for exposure, infection and infectivity of lymphatic filariasis for identifying transmission foci and evaluating the interventions	219
	<i>K. Krishnamoorthy, S. L. Hoti and K. Athisaya Mary, Vector Control Research Centre, Puducherry</i>	
26.	Nanotechnology based detection system for the control of infectious diseases – an overview	227
	<i>V. Vasuki, S. L. Hoti and K. Nagalakshmi, Vector Control Research Centre, Puducherry</i>	
27.	Structural analysis of purine nucleoside phosphorylase of <i>Schistosoma mansoni</i>	239
	<i>Dattatraya V. Desai and Archana V. Rao, Department of Biotechnology, B. V. Bhoomaraddi College of Engineering and Technology, Hubli</i>	
28.	Cost-effective medium for the production of mosquito pathogenic bacilli	245
	<i>Subbiah Poopathi and G. Rajeswari, Unit of Microbiology and Immunology, Vector Control Research Centre, Puducherry</i>	
29.	Compatibility of chemical pesticides with locally isolated entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from Meghalaya, Northeast India	261
	<i>Lalramliana and Arun K. Yadav, Department of Zoology, North-Eastern Hill University, Shillong</i>	
	Author Index	269
	Organizing Committee 20th NCP	271
	Congress Summary	273

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Lipid binding nematode polyprotein allergen of the lymphatic filarial parasite, *Wuchereria bancrofti*: potential target for drug and immunoprophylactic development

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Abstract

Nematode Polyprotein Allergens (NPA) play an important role of transporting small lipid molecules from the host to the nematode. Nematode parasites are unable to synthesize their own complex lipids and derive them entirely from the host. Found in many nematode species (e.g. *Onchocerca ostertagi*, *Dictyocaulus viviparus*, *Ascaris suum*, *Dirofilaria immitis*), these proteins are synthesized in the gut of parasites as large precursor protein complexes, comprising 10-50 tandemly repeated polypeptide units of 15 kDa, depending upon the species and having a short hydrophobic leader sequence. Individual units bind to a variety of molecules viz., haeme and divalent metal ions, arachidonic acid and its metabolites, lyso-platelet activating factor, lysophospholipids and retinoids, apart from sequestering pharmacologically active lipids. They also transport Arachidonic acid and its metabolites that are known to be targets for the action of antifilarial drug, Diethylcarbamazine and hence are of great significance to the control of lymphatic filariasis. Also, they elicit elevated levels of IgE response in the infected host, leading to Th2 type of immune response. We isolated a homologue of NPA, called gp15/400 polyprotein gene from lymphatic filarial parasites, *Wuchereria bancrofti* and *Brugia malayi* and investigated its polymorphism in 35 isolates of *W. bancrofti* collected from different geographic locations of India. The repeat subunit of the gene was found to be highly conserved in all the isolates with only two synonymous nucleotide changes at positions 369 (A-G) and 375 (C-T). The subunit of the gene was cloned and expressed as 15 kDa protein in a salt inducible host. Currently, the functional and immunological characterization of the recombinant protein is in progress. Since this molecule is highly conserved and has multifarious roles in the survival and pathogenesis of the parasite it has good potential as a target for drug, immunomodulation tools and immunotherapy development. The significance of this molecule in modulating the host immune response and evolutionary relationship with NPA of other nematodes are discussed in the foregoing sections.

Keywords

NPA; gp15/400; Polymorphism; *Wuchereria bancrofti*; Evolutionary relationship; Immunomodulation; Immunotherapy

Introduction

Nematode Polyprotein Antigens/Allergens (NPA) are found to be immunodominant antigens of nematode parasites and in some cases as allergens. The first NPA to be characterized was the ABA-1 protein of *Ascaris* sp. Since then they have been found in worms belonging to numerous species and have no counterparts in mammals. NPAs are extracellular in nature and are produced as large precursor protein complexes, comprising of 10-50 tandemly repeated polypeptide units, depending upon the species. The complex proteins are cleaved post-translationally at consensus cleavage sites (Lys/Arg-Xaa-Lys/Arg-Arg) to yield multiple copies of functional molecules of approximately 15 kDa (Kennedy, 2000a). These allergens are found in all stages of the parasite (Kennedy and Qureshi, 1986) and are located as the most abundant protein species in the body fluid of parasites and secreted into that of infected animals (Christie *et al.*, 1992; Kennedy and Qureshi, 1986; McGibbon *et al.*, 1990; Spence *et al.*, 1993; Tomlinson *et al.*, 1989). A homologue of this polyprotein, called ladder or gp15/400 protein, has been found in filarial nematodes, which is also a complex of approximately 400 kDa with 20 tandemly arranged repeat sub-units of 132 amino acid residues and encoded by a single gene. The gp15/400 protein of *Brugia malayi* is associated with the surface and also distributed in all tissues of the parasite (Tweedie *et al.*, 1993). Similar to its counterparts in other nematodes, it is associated with elevated levels of IgE antibodies, thereby inducing allergic type-II (Th2) response in elephantiasis cases (Hussain *et al.*, 1992) and exhibits strong genetic control of immune response (Kennedy *et al.*, 1990; Allen *et al.*, 1995).

Paxton *et al.* (1993) reported that the repeat sub-unit of the gene was highly conserved between brugian filarial parasites, *B. malayi* and *B. pahangi*, but exhibited small degree of divergence in *Wuchereria bancrofti* with 21 nucleotides (and 7 amino acid) variation (94% identity) and great degree of divergence in *Dirofilaria immitis* with only 57% identity. However, information on the diversity in the structure of lymphatic filarial antigens sequenced to date is extremely limited, both between closely related species and (presumably) between different strains of the same species (Paxton *et al.*, 1993). We, therefore, sequenced the repeat units of gp15/400 protein gene from 35 *W. bancrofti* isolates collected from different geographic areas of India in order to see the extent of nucleotide polymorphism, compared its nucleotide sequence with that of other filarial, non-filarial and parasitic nematodes and examined the evolutionary relationship, in relation to its host inhabitation and parasitic life. Principally, our interest in the polymorphism of this gene stems from the fact that it is involved in a) pathogenesis of lymphatic filariasis (Paxton *et al.*, 1993), b) transport of Arachidonic acid and its metabolites (Kennedy, 2000b), which, in turn, are involved in the action of antifilarial drug, Diethylcarbamazine (DEC). Polymorphism of this gene, if exists, would affect the disease outcome of the infection and the response of the parasite to DEC treatment. Further, the study also aimed to clone and over express the gp15/400 subunit gene of *W. bancrofti* and to evaluate the immunoprophylactic/drug target potential.

Materials and methods

Study areas, sample collection and purification of microfilaria and DNA

Blood samples were collected from microfilaria (mf) carriers residing in locations representing different geographic regions of India (Hoti *et al.*, 2003). A 5 ml of venous blood was collected from each mf carrier during night 20.00-22.00 h, after obtaining written consent from him/her. EDTA (final concentration 1mM) was added to the blood sample to prevent it from clotting and stored at 4°C. The mf were separated from the blood samples by membrane filtration technique (Dennis and Kean, 1971) followed by Percoll-Sucrose gradient centrifugation technique (Chandrashekar *et al.*, 1984). The genomic DNA from mf was extracted following the method described earlier (Hoti *et al.*, 2003).

PCR reaction condition and sequencing

Single repeat unit of Polyprotein Allergen (gp15/400) gene of isolates of *W. bancrofti* from different geographical regions was amplified using gene specific primers (Wbgp15f-5'-TGGCTTACGGATGCCCAAAGG-3' and Wbgp15r-5'-ACTTAGATGCGTCCGAAAATAG-3' (Paxton *et al.*, 1993). The PCR mixture consisted of 100 ng of purified DNA, 5µl of 10x Buffer, 20 pmoles of each primer, 20 mM dNTP, 2.5 mM and 2 units of Dynazyme II Ext Taq DNA polymerase (Finnzymes, Finland), in reaction volume of 50 µl. Amplification was carried out in a Master Cycler Gradient (Eppendorff, USA) and the temperature cycles consisted of an initial denaturation at 96°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min with a final extension step of 72°C for 7 min. The amplified products were resolved in 1.5% Agarose gel, stained in ethidium bromide and observed under UV transilluminator. The amplified product was purified using nucleotide removal kits (Qiagen, German) and sequenced in an automated DNA sequencer (ABI 7000). The nucleotide sequences of the isolates have been deposited in the GenBank and have the accession numbers from DQ 321502 to DQ321536. The sequences of the gene of other nematodes were obtained from the GenBank (Fig. 3). They were aligned using BioEdit programme (Hall, 1999), and the dendrogram was constructed using Mega 3.1 programme (Kumar *et al.*, 2004).

Nucleic acid techniques

Plasmid isolation, plasmid transformation, and standard DNA protocols were performed as described in Sambrook *et al.* (1989).

Cloning and overexpression of gp15/400 gene in E. coli

The gp15/400 gene corresponding to with 132 amino acids was amplified using forward primer Wbgp15f-5'-GAATTCTGGCTTACGGATGCCCAAAGG-3' and reverse primer 5'-GAATTCAGTATGCGTCCGAAAATAG-3'. In the forward and reverse primers. The under lined sequences represent the site of EcoRI. The PCR amplified DNA fragments were purified, digested with EcoRI, ligated into EcoRI digested pGEX4T-1, and transformed into *E. coli* BL-21 (DE3) competent cells to construct recombinant plasmid pGEX4T-1 gp15/400. The clone was streaked onto LB agar plate containing ampicillin (50 mg/ml) and the plate was incubated at 37°C for 16 h. A single colony was used to inoculate 5 ml LB broth containing ampicillin

(50 mg/ml) followed by its incubation at 37°C with vigorous agitation in a shaker incubator. One ml of overnight culture was used to inoculate 100 ml of LB broth containing ampicillin (50 mg/ml) in a 250 ml culture flask and the culture was grown at 30°C with vigorous agitation. When cells reached an optical density (OD) 0.6 at 600 nm, isopropyl β -D-thiogalactoside (IPTG; 1 mM) was added. After 4-5 h of induction at 30°C, cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and frozen.

Purification of recombinant protein

All steps were carried out at 4°C. After two freeze-thaw cycles, cells were resuspended in lysis buffer (50 mM Tris/HCl, pH 7.8, 1 mM EDTA) and homogenized by sonic disruption (60% duty cycle, 1 min; output control setting 6) using a 0.75 in. (approximately 1.9 cm) tip for a total of 20 min, with pulse and interval time of 1 min and 30 sec, respectively for each duty cycle. The mixture was centrifuged at 13,500 *g* for 10 min at 4°C to remove the unbroken cells and the supernatant. The pellet containing the soluble protein was washed in three volumes of wash buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1 M urea), kept at room temperature for 5 min, and centrifuged as above. The supernatant was passed through the Sepharose 4B column and the GST portion of the recombinant protein was cleaved using thrombin protease. The gp15/400 protein was eluted in 1x PBS. Protein quantification was done by the Lowry method with bovine serum albumin as standard.

Gel electrophoresis

Protein analysis was performed on 12% Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli (1970). Protein samples were reduced by boiling for 5–10 min in loading buffer containing 5% β -mercaptoethanol and then centrifuged at 10,000 *g* for 5 min prior to loading the gel. Electrophoretic analyses were performed using a Mighty Small Unit II system (model SE 250; Pharmacia Biotech Asia Pacific Ltd, Hongkong). Vertical slab gels, containing 12% (w/v) resolving gel and 5% stacking gel concentrations of acrylamide, were run at a constant current of 15 mA for 6 h. The proteins on PAGE gels were fixed in 45% methanol and 10% acetic acid in distilled water, stained in 0.25% Coomassie brilliant blue R-250 dissolved in 10% acetic acid and 50% methanol in water. The gels were destained for 3 h in 5% methanol and 7% acetic acid in distilled water and documented using photo gel documentation system.

Western blotting

After SDS–PAGE, gels were soaked in the transfer buffer (25 mM Tris-HCl, 192 mM Glycine, 30% methanol, pH 8.4) and electrotransferred at 0.8 mA cm⁻² for 50 min with a semi-dry blotting apparatus Semiphor Transphor Unit (Pharmacia Biotech Asia Pacific Ltd., Hongkong) onto a 0.45-l m nitrocellulose membrane (Advantech MFS Inc., CA, USA) as described by Towbin *et al.* (1979). The Western blot was saturated in Tris NaCl buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 3% gelatin and incubated for 3 h with a 1:1000 dilution of mice antiserum against gp15/400 in the same buffer. The membrane was washed once in Tris NaCl and twice in Tris NaCl, 0.05% Tween-20 (10 min each) and incubated for 30 min in a 1:5000 dilution of alkaline phosphatase labelled goat anti-mouse IgG. Detection was

achieved with the chromogenic substrates, 5-bromo-4-chloro-3-indolyl phosphate and Nitrobluetetrazolium (BCIP/NBT tablets, Sigma Chemical Co. MO, USA), according to the manufacturer's instructions.

Antibody Dependent Cytotoxicity Assay (ADCC)

Antibody Dependent Cellular Cytotoxicity (ADCC) is believed to be one of the principal immunological mechanisms functional in man and animals and the disappearance of circulating parasite is mainly attributed to this phenomenon (Chandrasekar *et al.*, 1985a, b; Mehta *et al.*, 1981). The cytotoxicity assay was carried out as described by Chandrasekar *et al.* (1985, 1990). Briefly, 100 mf or 10 L3 in 50 μ l of RPMI-1640 were incubated with 50 μ l of peritoneal exudates (PEC) cells (2×10^3 cells for mf or 5×10^4 cells for L₃ larvae) and 50 μ l of control or gp15/400 immune mice serum in the 96-well culture plate. The plate was incubated in 5% CO₂ incubator at 37°C for 48 and 72 h. At different periods of incubation (48 and 72 h), the samples were examined microscopically for cellular adherence and cytotoxicity was expressed by considering the number of immobile or dead parasites.

Results and discussion

Amplification using gp 15/400 gene specific primers (Paxton *et al.*, 1993) yielded a ladder like profile on gel with amplicons ranging from approximately 400–1200 bp, in multiples of 400 bp (Fig. 1) from all the *W. bancrofti* isolates collected from different geographic locations of India. This is similar to that reported for Indonesian isolate of *W. bancrofti* and other filarial nematodes, *B. malayi* and *B. pahangi*, by other workers (Paxton *et al.*, 1993). These results thus showed that the polyprotein gene had no size polymorphism among *W. bancrofti* isolates originating from wide geographic areas of India. The amplicons of the gp15/400 gene from geographical

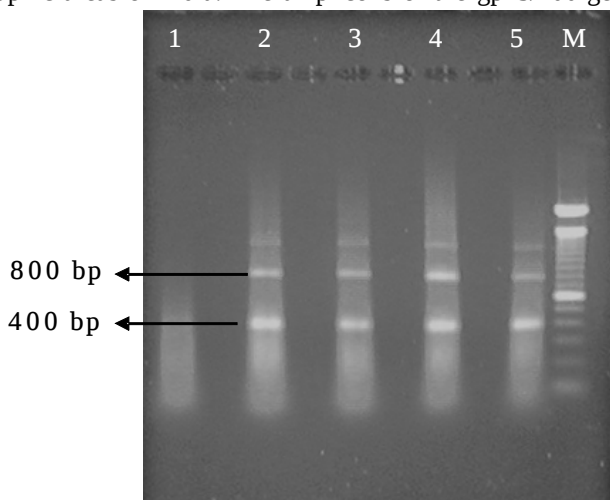


Fig.1. Amplification of gp15/400 repeat sub-unit of polyprotein allergen gene from *Wuchereria bancrofti* Mf collected from different geographic regions of India (1: Negative control, 2: Kozhikode, 3: Rajahmundry, 4. Varanasi and 5: Jagadaldpure. M100 bp ladder).

isolates were sequenced and compared after alignment. Since sequences of many isolates were truncated at 5' and 3' ends, sequences of nucleotide positions between 144 and 359 only were compared. It was evident from the comparison that the subunits of the gene are highly conserved within *W. bancrofti* isolates, with very minimum nucleotide variation. The variations were mainly at nucleotide positions 286 (A-G) and 337 (C-T), which are synonymous. Thus, nucleotide sequences of individual repeat units of the gene, although obtained from 35 different isolates of *W. bancrofti*, were found to be highly conserved between sub-units of an isolate, as well as between sub-units of different isolates.

The nucleotide sequence alignment of the repeat sub-unit of gp15/400 gene of *W. bancrofti* from Pondicherry (PD8) isolate with that of Indonesian isolate (Paxton *et al.*, 1993) showed that the latter also had only three additional nucleotide variations, at positions 62 and 63, which lead to a change in amino acid (from K to I) and at position 369 with no amino acid change (Fig. 2). The consequence of change of an amino acid at this position needs to be investigated for functional (phenotypic) changes at protein level and at this stage it is difficult to predict such changes. But for these variations the gp15/400 gene of *W. bancrofti* might be conserved in Pan-Asian region. In the present study, a sub-unit of gp15/400 gene of *W. bancrofti* was investigated for polymorphism among parasite isolates collected from wide geographic areas of India, spanning about 3000 km. The Indian isolates, as also the Indonesian isolate differed from one another only at two nucleotide positions, thus, indicating that the gene is highly conserved across geographic boundaries. Since the nucleotide variations are synonymous the protein structure of gp15/400 of *W. bancrofti* isolates from different Indian regions is highly conserved and this also is in agreement with that reported for *B. malayi* and *B. pahangi* (Paxton *et al.*, 1993).

The phylogenetic tree (Fig. 3) constructed using nucleotide sequences of NPAs of other nematodes, along with that of filarial nematodes exhibited two clades, with one branching earlier and consisting of *Strongyloides* species and the other consisting of rest of the nematodes falling under in two sub-branches. One of the sub-branches had lone free living member, *C. elegans* and the other with all other parasitic nematodes. Within the latter branch, lymphatic filarial parasites formed a distinct and most recent clade, while animal filarids formed early branching clades. Interestingly, within lymphatic filarial parasites the zoonotic *B. pahangi* and *B. malayi* formed a group distinct from the strict human parasite, *W. bancrofti*. Human eye dwelling parasite *Loa loa* and intestinal parasite *Ascaris lumbricoides* grouped along with the animal filarids. The second major clade comprised parasites that infect rodents, canines, cattle, equines as well as humans. Generally, latest branches showed tendency towards parasitizing humans. Further, it appears that there is a similarity in the trend of evolution of polyprotein genes and small sub-unit rDNA genes of parasitic nematodes.

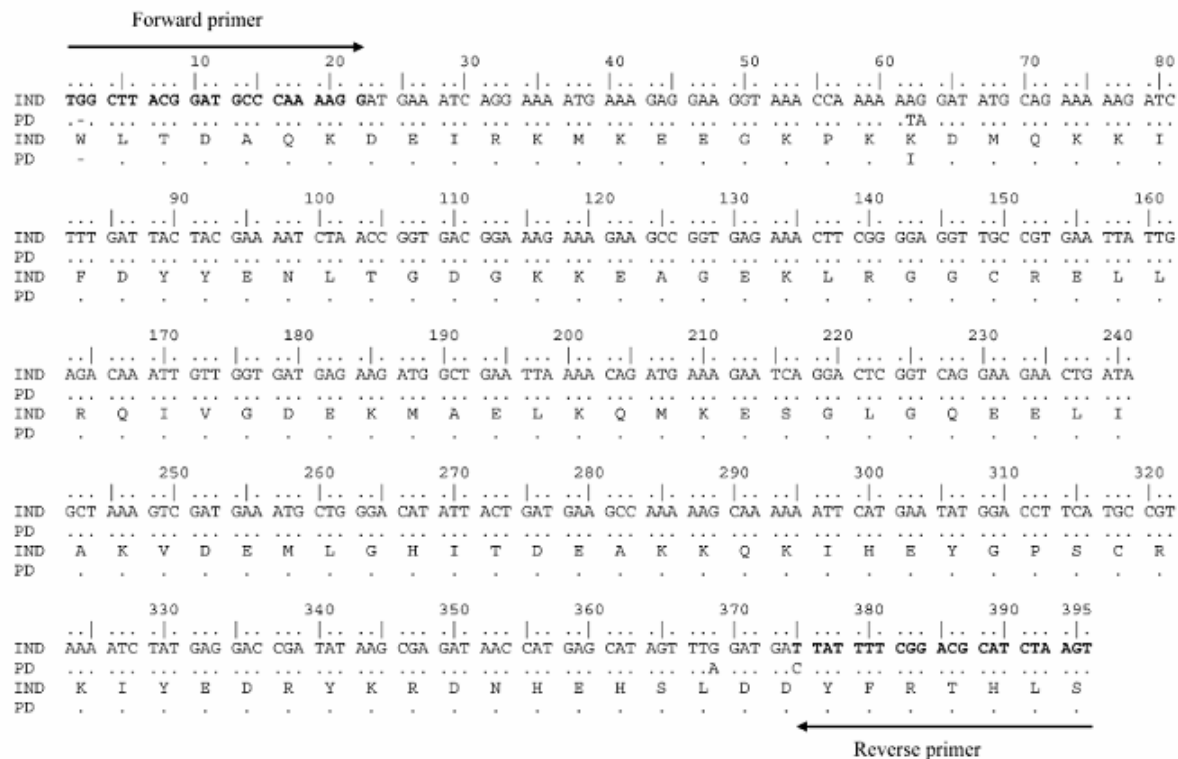


Fig. 2. Alignment of nucleotide and derived amino acid sequences of gp15/400 repeat sub-unit of *W. bancrofti* from Jakarta, Indonesia (IND) and Pondicherry (PD), India.

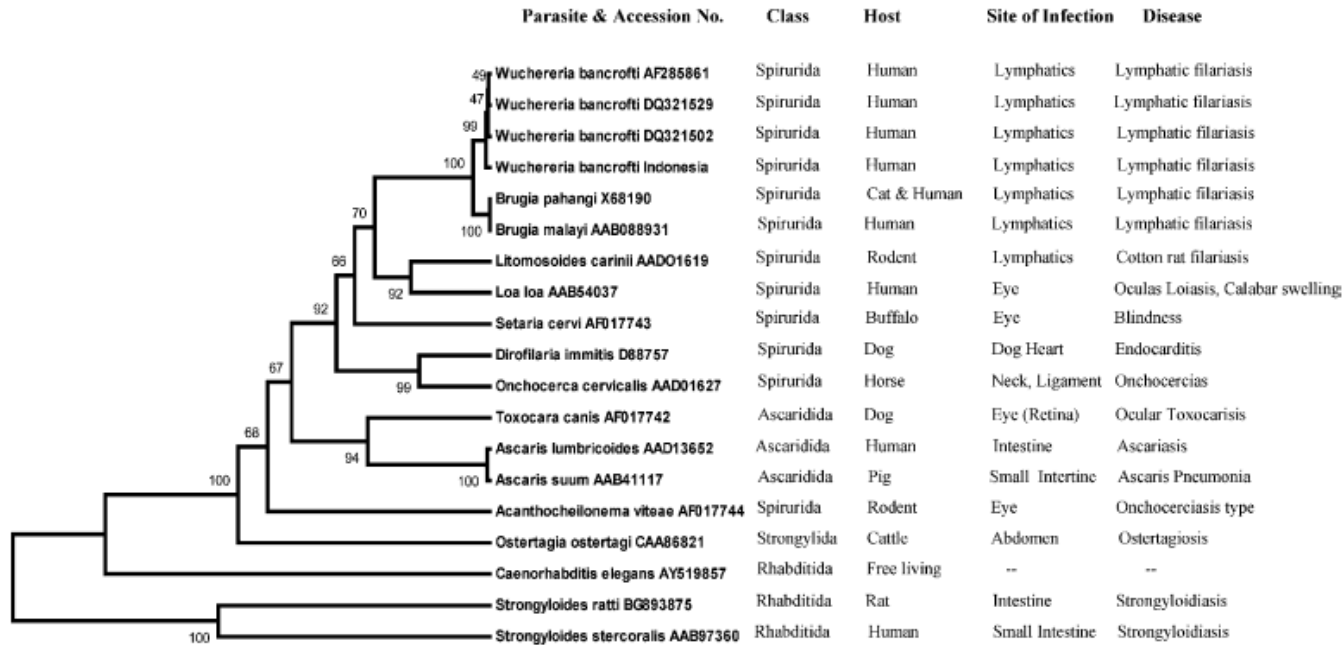


Fig. 3. Dendrogram of nucleotide sequences of NPAs from different parasitic nematodes and free living *Cenorhabditis elegans*. It was constructed using MEGA 3.1 programme and with 1000 replicates and employing unweighted pair-group method with arithmetic mean (UPGMA).

The gp15/400 gene subunit was cloned into pGEX4T1 expression vector and transformed into BL21 (DE3) cells. The fusion recombinant protein was over expressed using 1mM IPTG and purified using Sepharose-4B affinity column chromatography. The gp15/400 protein was cleaved from the recombinant protein using thrombin protease and used for investigating the levels of Wbgp15/400 specific IgG and IgE antibodies and Antibody Dependent Cellular Cytotoxicity (ADCC) assay.

The levels of Wbgp15/400 specific IgG and IgE in the sera samples collected from 10 each of asymptomatic amicrofilaraemic individuals residing in an area not endemic for human lymphatic filariasis (NEN), asymptomatic amicrofilaraemic individuals residing in an endemic area (EN), asymptomatic microfilaraemic individuals (mf carrier) and asymptomatic amicrofilaraemic individuals with chronic irreversible lymphoedema (CP) were determined by ELISA. The IgG and IgE showed that individuals with CP exhibited significantly higher levels of anti-Wbgp15/400 specific IgG and IgE antibodies. A significant difference between the mf carrier and the EN groups was also noticed (Figs. 4 & 5).

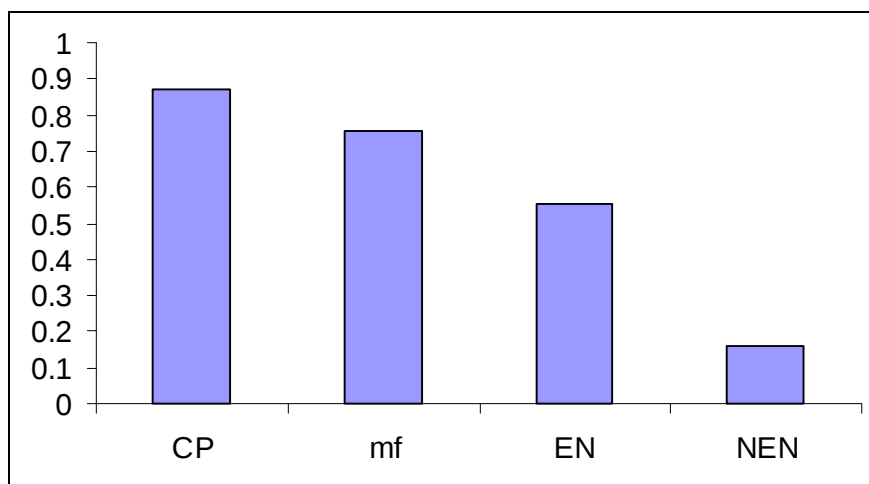


Fig. 4. Anti-Wb gp15/400 total IgG levels in different clinical groups. Each bar represents the arithmetic mean of 10 OD values.

ADCC assay was carried out in order to evaluate the immunoprophylactic potential of the gp15/400 protein. In this study ADCC assay with anti-sera raised against recgp15/400 promoted the adherence of peritoneal exudates cells (PEC) and were effective in inducing cytotoxicity and killing of *W. bancrofti* mf in *in-vitro*. The cytotoxicity induced by the mice anti-recgp15/400 was found to be significant compared to that observed in control. Antibody and complement mediated effector mechanisms are believed to play a role in inducing *in vitro* cytotoxicity to the mf.

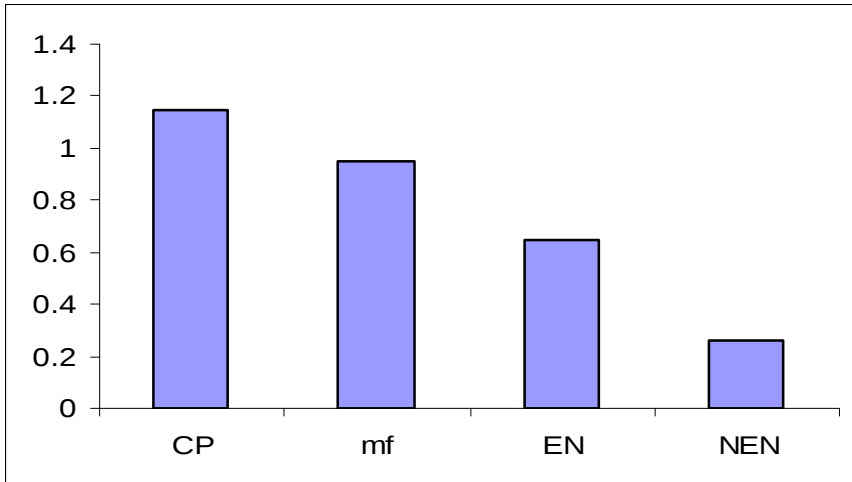


Fig. 5. Anti-gp15/400 IgE antibody levels in different clinical groups. Each bar represents the arithmetic mean of 10 OD values.

The *in-vitro* serum dependent cellular cytotoxicity assay showed that the anti-gp15/400 sera promoted the adherence of PEC to *W. bancrofti* by 64% cytotoxicity, respectively in 48 h.

The preliminary study revealed the conserved nature and protective potential of gp15/400 protein of *W. bancrofti*, as a prospective immunoprophylactic candidate in antifilarial therapy and further studies required to evaluate the induced protection mechanism in experimental models and characterization of immune response may lead to development of effective vaccine strategy for the prevention of human lymphatic filariasis.

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***Taenia solium* taeniasis and neurocysticercosis (NCC): experience from North Indian pig farming community**

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Abstract

Neurocysticercosis (NCC) caused by *Taenia solium* larva is a major public health problem, especially in the developing world. It is identified as the most common parasitic infection of the central nervous system and the single most common cause of community acquired active epilepsy. Population based studies to estimate the disease burden in India are lacking. We conducted a systematic study to estimate the disease burden in 294 pig farming families from 30 villages of Mohanlalganj block, Lucknow district, Uttar Pradesh. The families and villages were chosen based on 30 cluster sampling approach recommended by World Health Organization. Demographic, clinical and epidemiological data were collected from all the subjects. Stool specimens were examined for *Taenia* and other parasitic infections. Individuals with active epilepsy were identified on door-to-door survey and such patients underwent magnetic resonance imaging (MRI) of the brain. Asymptomatic family members of the symptomatic NCC related active epilepsy patients were also encouraged to undergo brain MRI. Pigs slaughtered for human consumption in the community were examined for cysticercal infections.

On stool examination, the prevalence of *T. solium* taeniasis was 18.5% (172/924). Factors associated with taeniasis were- age above 15 years, consumption of undercooked pork, passage of *Taenia* segments in stool and poor hand hygiene. During the survey, 5.8% (95/1640) subjects were found to have active epilepsy. Based on MRI, clinical and epidemiological criteria, NCC related active epilepsy was identified in 48.3% (44/91) of patients. Interestingly, 31 of 107 (29%) asymptomatic family members were also found to have NCC by MRI. Epilepsy in the family and no separate place for pig were identified as risk factors for NCC clustering in a family. Thirteen (26%) of 50 pigs slaughtered had cysticerci in their skeletal muscles; 5 (38%) of them also had NCC.

The study shows very high prevalence of *T. solium* taeniasis, NCC related active epilepsy and porcine cysticercosis in the community where pigs are raised. Possibly NCC is grossly under reported in India especially in its northern part. It emphasizes the need for further population-based studies. Since cysticercosis is a

preventable and potentially eradicable disease, appropriate interventional strategies play important role to control the menace of the disease.

Keywords

Taenia solium; Taeniasis; Pig farmer; Neurocysticercosis; India; Prevalence

Introduction

Taenia solium infections, taeniasis and neurocysticercosis are widely prevalent in humans in the developing countries, especially where pigs are raised as a food source. These infections are reported to be major health problems in South Africa, Eastern Europe, South America and South East Asia (Chopra *et al.*, 1981; Cruz *et al.*, 1999; Garcia- Noval *et al.*, 1996; Sarti *et al.*, 1994). *T. solium* infection is increasingly diagnosed in affluent countries owing to human migration from endemic areas (Garcia and Del Brutto, 2000). Human is the only definitive host and harbour adult tapeworm in the intestine (taeniasis), whereas both man and pig can act as intermediate hosts and harbour the larvae in different internal organs (cysticercosis) including brain (neurocysticercosis). Most of the carriers of *T. solium* remain asymptomatic; hence they do not seek medical care and remain potential risk of neurocysticercosis (NCC) for themselves, the family members and the population at large. Therefore, identification of *T. solium* taeniasis and its associated risk factors are important to control the menace of the disease. Neurocysticercosis, caused by larval stage of the tapeworm, *T. solium*, is the most common parasitic infection of the central nervous system (CNS) and the single most common cause of seizure/epilepsy in the developing countries (Garcia *et al.*, 2003). NCC is common in communities where pigs are allowed to roam freely, the residents consume insufficiently cooked pork and the basic sanitary facilities are lacking (WHO, 1983). However, community based studies on NCC related epilepsy are lacking throughout the world because diagnosis of NCC with accuracy requires expensive neuro-imaging tools such as computed tomography (CT) or magnetic resonance imaging (MRI). Majority of the community studies are based on serological tools. In India, pig breeding has been a traditional occupation of some communities and pork constitute part of their food. Pigs raised in the community are allowed to roam freely in the field, and human faeces form an important part of their diet. The co-existence of poor sanitary conditions and free roaming of domestic pigs play an important role in circulation of *T. solium* infection in the community. We reviewed the published and unpublished data from studies carried out in a pig farming community of Lucknow district by our centre. We also reviewed the literature to identify the prevalence of *T. solium* taeniasis, NCC and their associated risk factors in human along with the disease burden in swine with special emphasis on Indian studies. The aim of this review is to enhance our knowledge and create awareness about the disease, which is potentially preventable.

Taenia solium taeniasis and associated factors

Occurrence of taeniasis and cysticercosis has been reported from most parts of the world, including the developed countries due to frequent migration of persons with disease and *Taenia solium* carriers from endemic area (White Jr., 2000). In a pilot study on pig farmers of Lucknow district, India, we observed that the intestinal *T.*

solium infection rate was very high (almost one third of the volunteers had intestinal taeniasis) (Prasad *et al.*, 2002). This observation prompted us to undertake a systematic study to determine the disease burden in this community. Thirty cluster sampling approach suggested by World Health Organization (Lwanga and Lemeshow, 1991) was adopted to estimate the prevalence of taeniasis and NCC. A questionnaire was developed to collect the information that included age, sex, occupation, family income per month, food habit, personal hygiene, household facilities (type of house, electricity, cooking fuel, drainage and toilet etc.) and symptomatic morbidity such as abdominal pain, history of passing tape worm segments in stool, and diarrhoea preceding one month, seizure etc. A team of trained field workers and research fellows collected the information through personal interviews. The stool specimens were microscopically examined for the eggs of *Taenia*; morphologic analysis of expelled *Taenia* segments was also carried out for species identification. Further, taeniasis positive stool specimens from randomly selected individuals and expelled segments of *Taenia* were subjected to multiplex PCR following standard protocol for species identification (Yamasaki *et al.*, 2004).

A total of 1181 subjects belonging to 210 households (families) of 30 villages were enrolled for the study. Stool samples from 924 subjects were received. The eggs and/or segments of *T. solium* were demonstrated in 172 (18.6%) subjects by stool microscopy; 134 (14.5%) of them were having the *T. solium* infection alone. Among 172 taenia positive cases, 109 (63.4%) had the history of passing taenia segments in stool. The factors that were found to be significantly associated as risk for *T. solium* taeniasis were abdominal pain, passage of tapeworm segment in stool, headache, consumption of undercooked pork, frequency of consumption of pork, income of the households and age on univariate analysis. Multivariate analysis revealed that age (>15 years), history of tape worm segment passed in stool, post defecation hand wash with clay/water and consumption of undercooked pork were significantly associated with taeniasis (Prasad *et al.*, 2007).

Banerjee *et al.* (1994) had found that 78% of children of pig farmers passed taeniid eggs in their stool. In an earlier study from Uttar Pradesh, India, the overall incidence of faecal adult *T. solium* was reported to be 2% in community (Pathak and Gaur, 1989). In foot hills of Himalaya (Uttarakhand, India), 30.3% of the NCC patients were having the *T. solium* ova in their stool (Kumar *et al.*, 2006). Copro antigen based study from North Vietnam reported the occurrence of *T. solium* taeniasis in 0.3%, 1.8% and 0% among the rural mountainous, rural coastal and peri-urban population, respectively. Garcia *et al.* (2003) had found 0-6.7% (median 2.5%) taeniasis cases by copro antigen ELISA among the rural populations of Peru.

Although abdominal pain and diarrhoea have been attributed to tapeworm infestation in an earlier report (Garcia *et al.*, 2003), these symptomatic morbidities were not associated with taeniasis on multivariate analysis in our pig farming community. The *T. solium* carriers in the community largely remained symptom free except for off and on passage of segments in stool. These *Taenia* carriers are potential threat to community, as in a study from Peru, Garcia *et al.* (1993) showed that chances of getting sero-positive for taeniasis/ cysticercosis were 3.4 times higher in individuals living with tapeworm carrier.

Neurocysticercosis and associated risk factors

In an earlier pilot study among the volunteers from the pig farming community of Lucknow district, we observed that 9.7% of them reported with seizures (Prasad *et al.*, 2002). Such high rate of seizure had not been reported in literature. This interesting observation prompted us to plan a systematic study in the community. We conducted a study to know the true prevalence of active epilepsy in the community and also to estimate the disease burden of active epilepsy related to NCC in the pig farming community of North India (Mohanlalganj, Lucknow, UP), and also to identify the socio-epidemiological determinants associated with the disease. Active epilepsy was defined in a patient who had two or more episodes of seizures, one of which had occurred in the previous 5 years, regardless of antiepileptic drug treatment. Again, thirty cluster sampling method was used to enroll the subjects from the 30 villages. A questionnaire was developed to collect the epidemiological information and to identify individuals with seizure/ epilepsy in the family. The screening questionnaire for seizure and epilepsy was similar to the one used in previous studies in South India (Radhakishnan *et al.*, 2000) and was modified from the WHO questionnaire for neurological disorders (WHO, 1981). All patients with seizures between ages of 2 and 60 years were brought to the community hospital and examined by the neurologist to confirm the diagnosis of active epilepsy. Diagnosis of NCC in patients with active epilepsy was made following the proposed criteria based on clinical, neuro-imaging (MRI), immunological and epidemiological data (Anonymous, 1993; Del Brutto *et al.*, 2001; Garcia *et al.*, 2005). During door-to-door survey of the community, 112 individuals with active epilepsy were identified from 49 households based on the questionnaire; 107 individuals turned up for the clinical evaluations. A total of 95 were clinically confirmed to be having epilepsy and 94 of them consented to undergo brain magnetic resonance imaging (MRI) for the presence of cysticerci and immunological evaluation by EITB. The prevalence of active epilepsy in the community was 5.8%. Either definitive or probable diagnosis of NCC was made in 44 patients (48.3%) with active epilepsy; 36 (81.8%) of them fulfilled the diagnostic criteria for definitive NCC. Among NCC cases partial seizures with secondary generalization was most common finding 29/44 (65.9%). Single cyst was present in 21 (47.7%), two cysts in 11 (25.0%) and multiple cysts (>2) in 12 (27.3%) cases. Occurrences of vesicular (viable), colloidal/degenerating, calcified and multiple stages of cystic lesions were detected in 2 (4.5%), 9 (20.5%), 21 (47.7%) and 12 (27.27%) individuals respectively. On MRI all 44 NCC diagnosed patients had cysticerci in their brain. Socio-economic factors and morbidity were analyzed as risk for occurrence of NCC. Age (above 15 years), epilepsy in the family and no separate place for pigs were found to be associated with NCC on univariate analysis. On multivariate analysis, epilepsy in the family and no separate place for pigs were found to be strongly associated with the occurrence of NCC (Prasad *et al.*, 2008a).

Population based studies had shown substantially higher epilepsy rates (almost twice) in developing countries than in industrialized world and NCC is identified as a major contributor of increased rates of epilepsy in the developing world (Placencia *et al.*, 1992; Garcia *et al.*, 1993). In a community survey of 50,617 individuals from South India, the prevalence of active epilepsy was 3.83 per 1000 and NCC was detected in 28.4% of them by CT (Rajshekhar *et al.*, 2006). The incidence of epilepsy had been reported 2.3% in rural Honduras (Medina *et al.*,

2005) and 11.4 per 1,000 in Ecuadorian rural community (Cruz *et al.*, 1999). Studies from different countries have reported that 26.3% to 53.8% active epilepsy cases in the developing world including India and Latin America are due to NCC. Like other Indian studies (Krishnaswamy, 1912; Rajshekhar *et al.*, 2006), single cyst infection was of the most common occurrence in the pig farming community of Lucknow district; calcified lesions were more frequently seen [alone in 21 (47.7%) and with other stages in 9 (20.5%)] in the study corroborating other Indian studies. Previous studies from Central and Latin America had identified consumption of uncooked or infected pork, passing proglottides in stool, drinking unboiled water and not proper washing of hands before and after eating food as potential risk for cysticercosis (Garcia *et al.*, 1995; Sarti *et al.*, 1994). Another study by Del Brutto *et al.* (2005) in rural Ecuador reported raising pigs were strongly related to cysticercosis seropositivity ($p = 0.002$).

Cysticercosis in swine

The local slaughter houses were surveyed to determine the infection in the pig. During the survey, 13 of the 50 swine (26.0%) slaughtered had obvious metacestodes (cysticerci) in their skeletal muscles (Prasad *et al.*, 2002). Cysticerci were also seen in the brain of 5 of these pigs, in the liver of 3, and in the cardiac muscle of one. Both large and small cysticerci were demonstrated in both muscles and brain of one pig. In earlier studies, cysticercosis appeared to be widely spread in swine in India. In and around Chandigarh, 8-10% of the pigs slaughtered had cysticerci in their muscles and around 0.5% of the pigs reared in Government farms were found to be infected (Mahajan *et al.*, 1982). Another survey in slaughter houses of Kolkata (West Bengal) revealed cysticercosis in muscles of 7% of the slaughtered pigs (Ratnam *et al.*, 1983). So far no clinical signs of cysticercosis in swine have been reported. Recently, we reported the clinical signs for the swine infected with cysticercosis; these signs are excessive salivation, excessive blinking and tearing, and subconjunctival nodules (Prasad *et al.*, 2006).

Diagnosis of neurocysticercosis

The diagnosis of NCC is impaired by its pleomorphic clinical presentations. A definitive or probable diagnosis of NCC can be made on the basis of proposed clinical, radiological and epidemiological criteria (Del Brutto *et al.*, 2001; Garcia *et al.*, 2005). Definitive diagnosis of NCC is made by direct demonstration of the parasite in tissues or radiological demonstration of scolex in cystic lesions using neuroimaging modalities. The neuroimaging procedures (MRI and CT) are most reliable tool but their availability is restricted. Different immunodiagnostic tests like enzyme-linked immunosorbent assay (ELISA), haemagglutination tests (HA), HA-CL, HA-Cc, complement fixation tests, indirect haemagglutination tests (IHA), electroimmunotransfer blot (EITB) etc. for NCC had been described in literature (Mahajan *et al.*, 1982). However, the only tools included in the diagnostic criteria by the International Working Group on NCC are glyco-protein based EITB in serum (Tsang *et al.*, 1989) and ELISA performed with cerebrospinal fluid (CSF). But the sensitivity of the EITB for single cyst infection, which is very common in India (Rajshekhar *et al.*, 2006; Prasad *et al.*, 2008a) is poor and CSF collection requires lumbar puncture, which involves certain risk and is not routinely practiced until the patients have raised intracranial pressure. Recently we had reported a *Taenia solium*

cyst fluid based lymphocyte transformation test (LTT) as a diagnostic tool for NCC (Prasad *et al.*, 2008b), with sensitivity of 93.7% and specificity of 96.2%. Even for the single cyst infection the sensitivity of the test was 87.5%, which was much higher compared to EITB or ELISA. This test has future potential provided the test can be made user friendly based on colorimetric method.

Conclusion

Cysticercosis is a global public-health problem, especially so in the developing countries including India. It is considered as a “biological marker” of social and economic development. Recent MRI based Indian studies suggest that the disease burden in India surpasses many other developing countries. Possibly NCC is grossly under reported in the country, especially its northern part because all socioeconomic determinants responsible for the transmission of the disease exist in India. It emphasizes the need of systematic population-based studies to estimate the disease burden and to develop simple, cost effective diagnostic kits. Since cysticercosis is a preventable and potentially eradicable disease, appropriate interventional strategies play important role to control the menace of the disease. The present day need is to render due attention by health care authorities to this old resurgent disease.

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Fish-borne zoonotic haplorchine trematodes: the question of differentiation of species

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Abstract

Among the various fish-borne zoonotic trematodes, an important but neglected group is the haplorchine trematodes, the human infections of which are quite prevalent in many SE Asian countries and cause severe pathogenicity. The life cycle of these flukes involves thiarid snails as first intermediate hosts, fish as second intermediate hosts and birds and mammals, as definitive hosts. Human infections result from consumption of raw or semi-cooked fish. The metacercarial infections occurring in cultured fish also cause pathogenicity and have serious economic consequences.

Infections with metacercariae and adult stages belonging to this group occur fairly commonly in a wide range of freshwater fish and birds in the Indian region. Several species of haplorchine trematodes belonging to the genera *Haplorchis* and *Procerovum* have also been recorded from birds and mammals. However, taxonomic differentiation of the various species based solely on the adult morphology especially the structure of ventrogenital complex has caused a great deal of confusion. In the light of this it is necessary to employ alternative taxonomic tools as supplements to morphological criteria for the differentiation of the species in these genera.

The following account compares the morphology, biology and ecology of two species of haplorchines, *Haplorchis pumilio* and *Procerovum varium* and shows how differences between the two species in details of the life cycle, behaviour of larval stages, surface ultrastructure of adult flukes and alignment of chromosomes in the karyotype serve as supplementary to morphology and prove valuable in their taxonomic differentiation. It is also emphasized that introduction of these modern taxonomic methods such as molecular, cytological and morphometric tools as well as investigations on ecology and behaviour of free living stages and surface ultra structure for differentiation of the species would give a boost to taxonomy research and save the science of taxonomy from the present crisis.

Keywords

Haplorchiinae; *Haplorchis pumilio*; *Procerovum varium*; Fish-borne intestinal trematode zoonoses; Differentiation of taxa; Trematoda

Introduction

Fish-borne zoonotic trematodes (FZT) are recognized as an important group of emerging human pathogens (Chai and Lee, 2002). As per the estimates provided by WHO (2004), the number of people currently infected with FZTs exceeds 18 million and number of people at risk worldwide is estimated at more than half a billion. The major contribution to this load is by liver fluke infections represented by *Clonorchis sinensis* and *Opisthorchis viverrini* and these infections received global attention because of their association with intrahepatic cholangiocarcinoma. However, food borne intestinal zoonotic trematode infections (FIZTs) are also becoming common in recent years and their impact on human health is also realized. Over 50 species of intestinal digeneans spread over families Heterophyidae and Echinostomatidae are identified as zoonoses and the infections are particularly prevalent in South-East Asia. In countries like Thailand, China, Korea, Laos and Vietnam, the human infections with FIZTs although the prevalence is low, have assumed public health significance.

Among the various Fish-borne intestinal zoonotic trematodes, an important but neglected group is Haplorchinae (Heterophyidae) the human infections of which occur fairly commonly in many South-East Asian countries and cause severe pathogenicity involving gastrointestinal disturbances and even cardiovascular complications. About a dozen species of haplorchines belonging to the genera *Haplorchis*, *Stellantchasmus* and *Procerovum* are reported from human beings, the most common being *H. pumilio*, *H. taichui* and *H. yokogawai*. Human infections are a consequence of consumption of raw or semicooked fish or fish sausages, infections are therefore, common in countries where fish is a preferred food item. In the life cycle of these flukes the snail *Thiara tuberculata* widely distributed in various freshwater bodies serves as the first intermediate host, a wide range of freshwater and brackish water fish act as second intermediate hosts. Birds and mammals often carry heavy infections with adult flukes in endemic regions and serve as reservoir hosts playing a major role in dissemination of infection as well as maintaining heavy infections in snails and fish. Thus these are snail and fish mediated intestinal infections. The foci of pathogen transmissions and natural reservoirs are widespread. Indeed intensification of aquaculture, increased consumption of fish, changes in social pattern of humans and environmental changes are responsible for increased prevalence of human infections with these flukes.

Haplorchine trematodes have also received significance because of their implications to the aquaculture with metacercarial infections causing pathogenicity to the cultured fish (Sommerville, 1982b). Heavy infections result in mass mortalities in fingerlings. In view of the public health and economic significance, this group of trematodes deserves sustained investigations with emphasis on epidemiological surveys.

From India, infections with metacercariae and adult stages belonging to haplorchine group have frequently been reported from a wide range of freshwater fish and birds (Nath and Pande, 1970; Pande and Shukla, 1972; Nath, 1973a, b; Pande and Premvati, 1977) although reports of human infections are rare. However, epidemiological studies dealing with occurrence of infections in snails and fishes, identification of endemic foci and possible presence of human infections have not

received the attention of either medical professionals or parasitologists. The small size of the flukes, transient nature of the infections and difficulties in the diagnosis have been the main obstacles for undertaking investigations on these infections.

For planning and undertaking epidemiological studies on haplorchine zoonoses, the major requirements are the accurate taxonomic identification of the species and the knowledge about their life cycle and transmission pattern. Information available on these aspects from the Indian region on haplorchine trematodes is meager. Several species belonging to the genus *Haplorchis* have been described from birds and mammals in India. But differentiation of the various species based solely on morphological characteristics especially the structure of ventrogenital complex has caused a great deal of confusion as is evident from the fact that many of the Indian species have been relegated to synonymy or considered as species of uncertain taxonomic status (Pearson, 1964). In the light of this prevailing situation, it is necessary to employ alternate taxonomic tools as supplements to morphological criteria for differentiation of the species. In recent years molecular methods have emerged as important tools and as supplements to traditional taxonomic methods in solving taxonomic problems and for determining phylogenetic relationships. Evidently these modern methodologies should be extended to haplorchine trematodes.

An investigation was undertaken in our laboratory on morphology, biology and ecology of 3 species haplorchine trematodes: *H. taichui*, *H. pumilio* and *Procerovum varium*. The study revealed that subtle differences that occur between the various species in the life cycle pattern, the behaviour of larval stages; the surface architecture of adult flukes and the alignment of chromosomes in the karyotype serve as supplementary to morphology and prove valuable for the differentiation of various species. This account deals with differences in these feature in the two species *H. pumilio* and *P. varium*, infections of which are common in snails, fish and birds in a stream near Visakhapatnam.

Observations and discussion

Haplorchine trematodes

Adult morphology: Characteristics include small, oval body, 1-2 mm long, 0.5-0.8 mm wide, densely spined tegument, eyespot pigment in forebody, short caeca, poorly developed suckers, single testis, ventrogenital complex armed with spines, single ovary acinous vitellaria and well developed uterine coils filled with numerous oval operculate eggs.

Species of *Haplorchis* and *Procerovum* reported from India

Haplorchis taichui (Nishigori, 1924)
H. pumilio (Looss, 1896)
H. yokogawai (Katsuta, 1932)
H. butei Chatterji, 1953
H. rayi Saxena, 1954
H. solus Simha, 1964

H. gyanpurii Jain, 1968
H. tagori Nath, 1973
H. vagabundi Nath, 1973
Procerovum varium (Onji & Nishi, 1926)

***Procerovum varium* (Onji and Nishio, 1926)**

Pearson (1964), in an exhaustive review dealing with hosts, geographic distribution and morphology of different species of the genus *Haplorchis*, synonymized the various Indian species reported under this genus with one or the other of following 3 species *H. pumilio*, *H. taichui* and *H. yokogawai*. This proves the confusion prevailing in the taxonomy of the group and the need to adopt authentic methods for their identification.

Comparison of the morphology and biology of *H. pumilio* and *P. varium*

Adult morphology:

Morphologically the adults of *H. pumilio* and *P. varium* reflect many similarities but they could be differentiated by the presence of an expulsor in *P. varium* and the details of the structure of genital complex (Figs. 1, 2).

Life cycle: Life cycle follows the same pattern in the two species, with the snail *T. tuberculata* as the first intermediate host, and a wide range of freshwater fish as second intermediate hosts and birds as definitive hosts. However, differences occur in the minute details of the life cycle of the two species as shown in Table 1.

Cercarial emergence from the snail host:

One of the important aspects of the cercarial behavioural studies is the emergence pattern of the cercaria from the snail host. It is considered an adaptation favouring transmission of the cercaria from the snail host to the next target host. The emergence pattern varies from species to species and can be used for differentiation of species of cercariae.

Cercariae of *H. pumilio*: Emerge throughout the day, exhibit a distinct diurnal periodicity with peak output between 10 am and 12 noon, released in small numbers during night (Figs. 3, 5, 6) (Umadevi and Madhavi, 1997a).

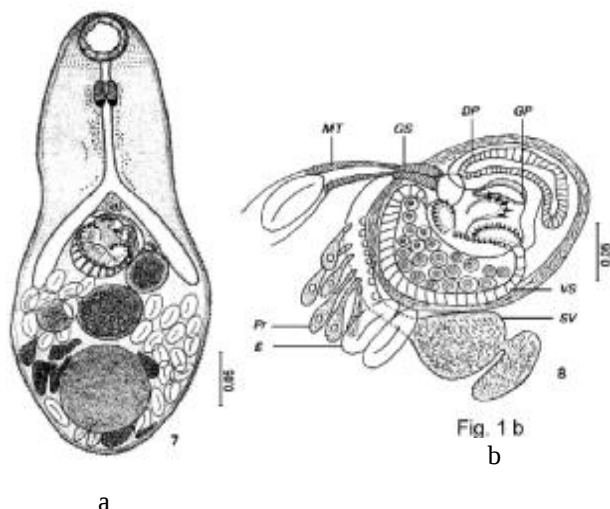


Fig. 1. *Haplorchis pumilio*. a. Adult fluke. b. Terminal genitalia, magnified.

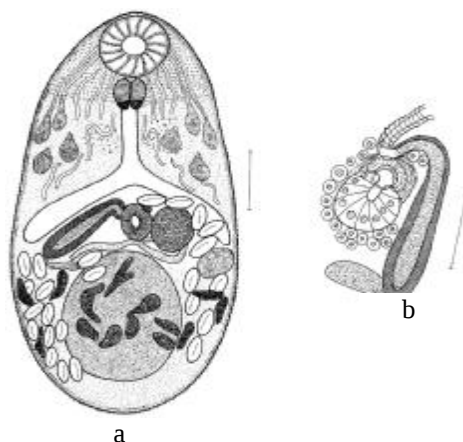


Fig. 2. *Procerovum varium*. a. Adult fluke. b. Terminal genital region magnified.

Table 1. Comparison of the stages in the life cycles of *H. pumilio* and *P. varium*

Character	<i>H. pumilio</i>	<i>P. varium</i>
Definitive host	<i>Ardeola grayii</i>	<i>Ardeola grayii</i>
First intermediate host	<i>Thiara tuberculata</i>	<i>Thiara tuberculata</i>
Second intermediate host	<i>Channa</i> spp., <i>Gambusia affinis</i>	<i>Oryzias melastigma</i>
Location of metacercariae	Caudal muscles, pathogenic	Viscera, nonpathogenic
Cercaria	Pleurolophocercous	Pleurolophocercous
Cercarial morphology		
Body size	213 x 91 µm	153 x 65 µm
Body pigmentation	Heavily pigmented	Transparent
Cystogenous glands	Few (Fig. 3)	Dense (Fig. 4)
References	Martin (1958); Sommerville (1982a); Umadevi and Madhavi (2004); Diaz <i>et al.</i> (2007)	Hsu, 1951; Umadevi and Madhavi (2000)

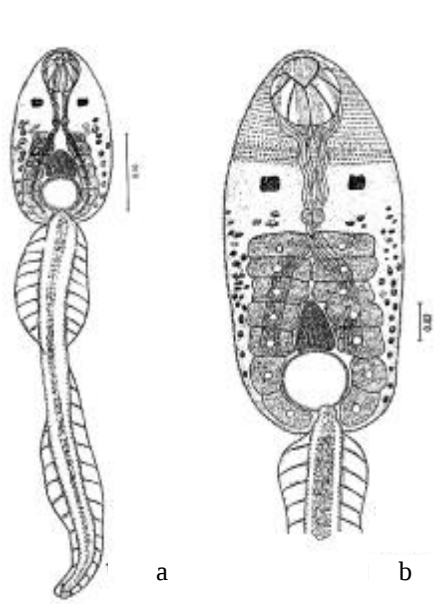


Fig.3. Cercaria of *H. pumilio*. a. Whole larva. b. Forebody, magnified showing cystogenous glands.

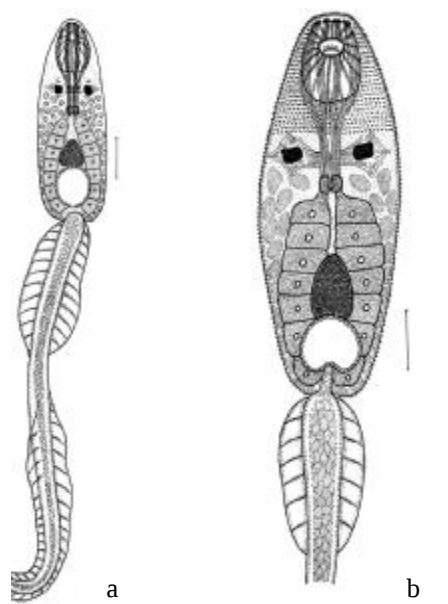


Fig.4. Cercaria of *P. varium*. a. Whole larva. b. Cystogenous gland cells in forebody.

Cercaria of *P. varium*: exhibits a distinct diurnal pattern, peak emergence between 12 noon and 2 pm. Emergence totally suppressed during night (Figs 4, 7, 8).

The cercaria of *P. varium* is more sensitive to light than that of *H. pumilio*.

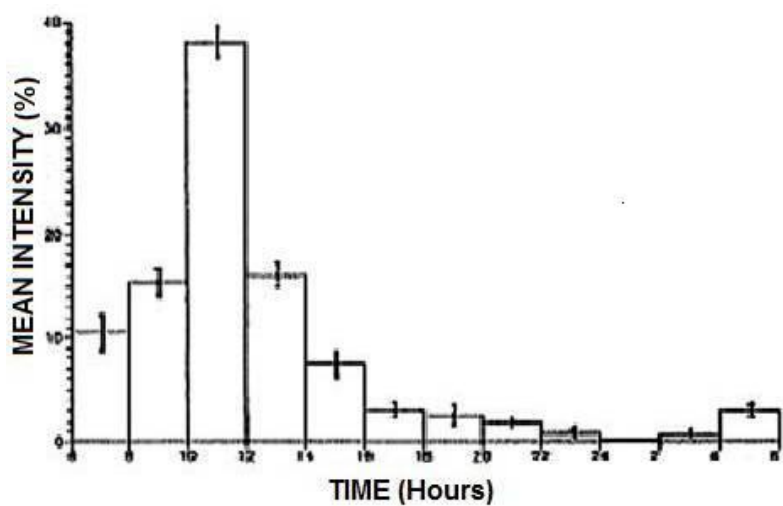


Fig. 5. Periodicity in the emergence of the cercaria of *H. pumilio* from the snail host.

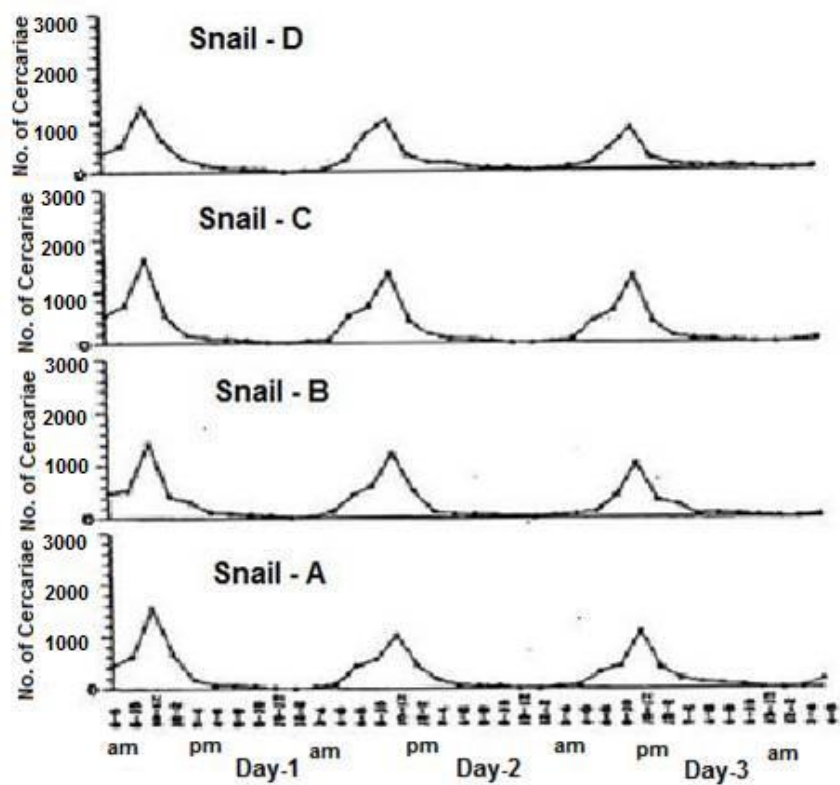


Fig. 6. Shedding pattern of the cercaria of *H. pumilio* from *T. tuberculata*.

Argentophilic papillar pattern: (Umadevi and Madhavi, 1997b, 1998) Cercariae possess sensory papillae (argentophilic papillae) on the body. Their distribution follows a definite pattern and varies from species to species and the pattern is of taxonomic significance. The papillar pattern of the two species is compared in Table 2.

Table 2. Comparison of the argentophilic papillar pattern of the cercariae of *H. pumilio* and *P. varium*

Region	No. of papillae in <i>H. pumilio</i>	No. of papillae in <i>P. varium</i>
Total cephalic papillae	27	30
Dorsocephalics	12	20
Anterior ventrals	12	12
Anterior dorsals	0	0
Laterals	10	8
Dorsomedian	2	2
Ventromedian	4	3
Posteroventral	4	8
Posterodorsals	4	0
Posterolaterals	6	6
Total	81	89

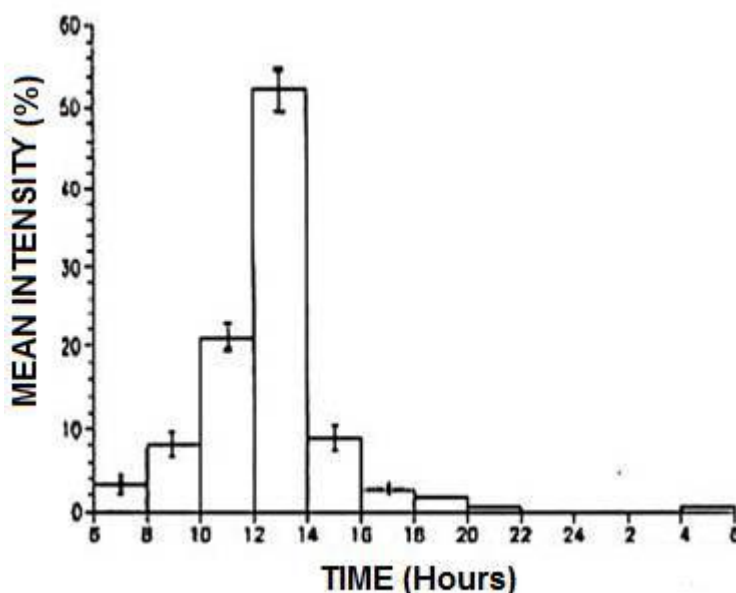


Fig. 7. Periodicity in the emergence of the cercaria of *P. varium* from the snail host.

The cercariae of the two species can be easily differentiated by counting the number of papillae on posterodorsal side of cercarial body. There are 4 papillae for *H. pumilio* and none for *P. varium* (Figs. 9 & 10).

Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) has a long and distinguishing history in the study of helminth parasites and has been used as a powerful tool for multidisciplinary approaches to parasitological problems. It has proved invaluable in helminth taxonomy. SEM study of *H. pumilio* and *P. varium* adult flukes has brought out distinct differences in the surface architecture of the two species.

***H. pumilio*:** Spines are pectinate with 7-9 serrations. The serrations are deep so that the spine appears like a fish scale. Spines are closely set, almost overlapping each other (Fig. 11a).

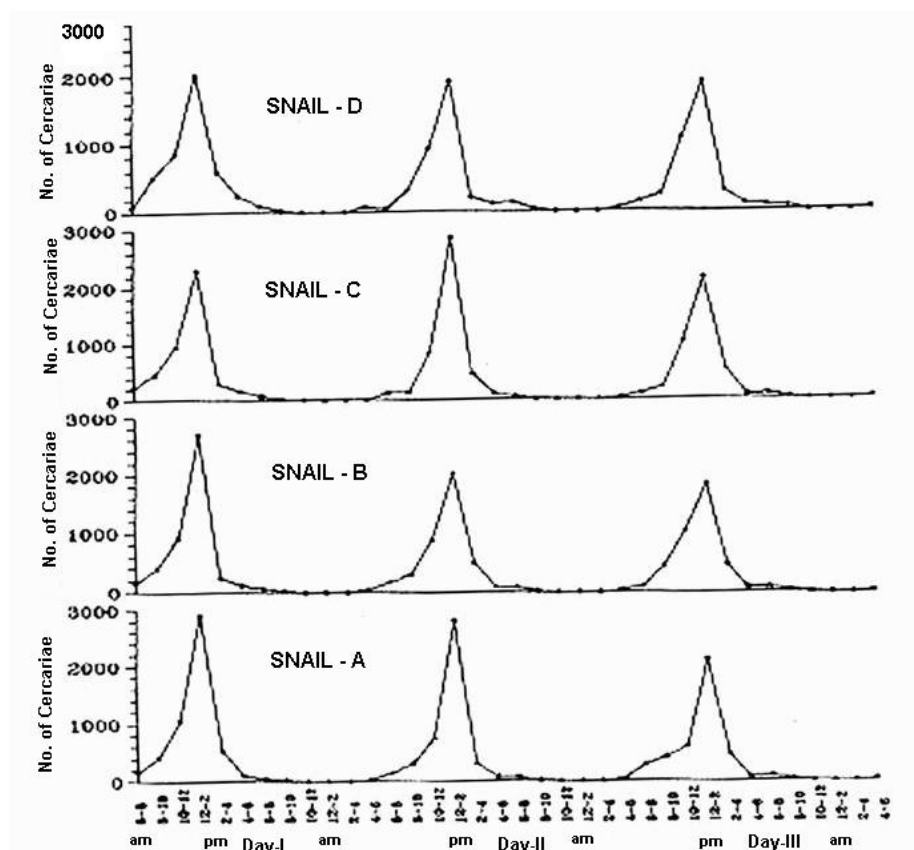


Fig. 8. Shedding pattern of the cercaria of *P. varium* from *T. tuberculata*.

***P. varium*:** Spines are smaller and triangular, loosely arranged, there is intervening parenchyma and wide gaps exist between rows (Fig. 11b).

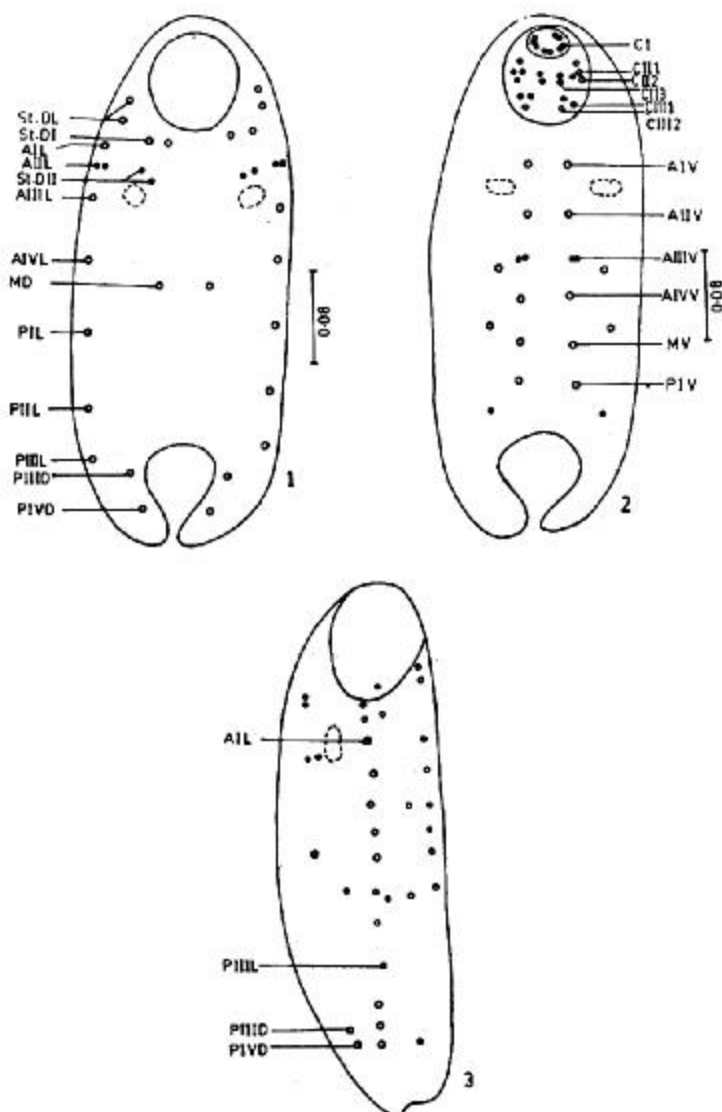


Fig. 9. Argentophilic papillae on the body of the cercaria of *H. pumilio*. 1. Dorsal view 2. Ventral view 3. Lateral view. CI: First cephalic cycle; CII: Second cycle of cephalic papillae; CIII: Third cycle of cephalic papillae; St.DL: Subterminal dorso laterals; St.D: Subterminal dorsals; AV: Anterior-ventrals; PV: Posterior-ventrals; AD: Anterior-dorsals; MD: Mid-dorsals; PD: Posterior-dorsals; AL: Anterior laterals; PL: Posterior laterals.

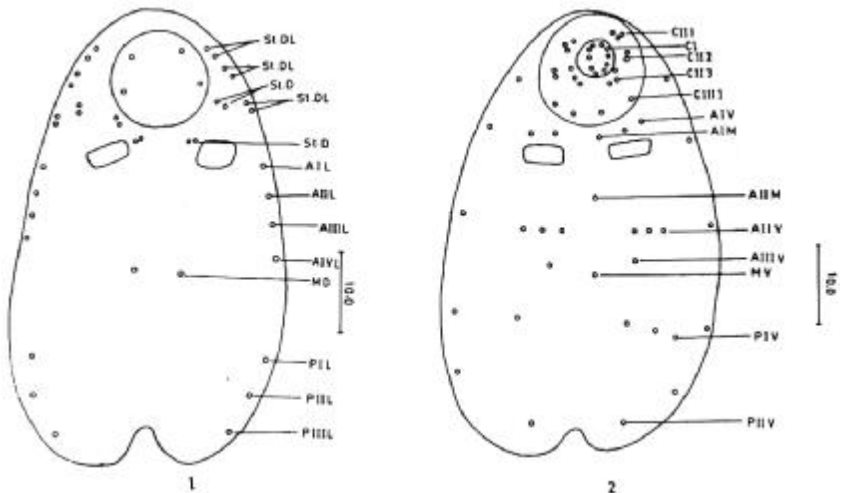


Fig. 10. Argentophilic Papillae on the body of the cercaria of *P. varium*. 1. Dorsal view 2. Ventral view. CI: First cephalic cycle; CII: Second cycle of cephalic papillae; CIII: Third cycle of cephalic papillae; St.D: Subterminal dorsals; St.DL: Subterminal dorso laterals; AV: Anterial-ventrals; PV: Posterior-ventrals; MV: Medians; MD: Mid-dorsals; AL: Anterior laterals; PL: Porterior laterals.

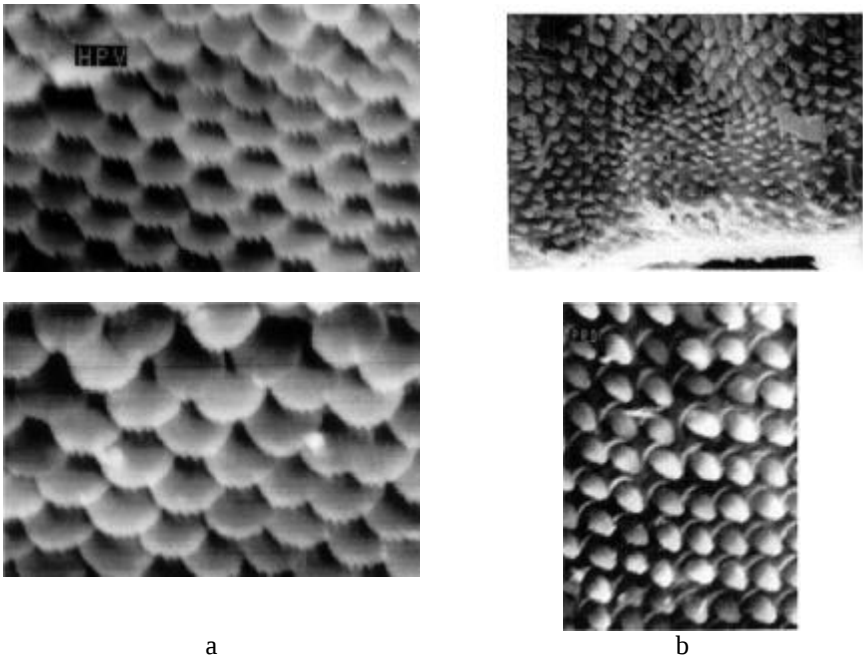


Fig. 11. Pattern of surface spination - SEM photomicrographs. a. *H. pumilio*; b. *P. varium*.

Conclusions

Investigations on life cycle patterns, enzyme profiles, SEM studies of the tegument, cercarial behaviour and chaetotaxy, cytotaxonomy and molecular taxonomy should be undertaken on different species of *Haplorchis* so as to identify valid criteria for their taxonomic differentiation. Epidemiological studies involving surveys on prevalence of haplorchine infections in snails, fishes and birds and the possible occurrence of human infections especially in the tribal villages, their mode of transmission and pathogenecity and the local factors should be undertaken on a priority basis.

Acknowledgement

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Cerebral malaria induced apo-necrotic mode of cell death in the mouse brain

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Abstract

Cerebral malaria (CM) is associated with high mortality and morbidity as a certain percentage of survivors suffers from persistent neurological sequelae. The mechanisms leading to cell death and functional impairments are not fully understood. This study investigated biochemical and morphological markers of apoptosis and necrosis in the brains of mice infected with *Plasmodium berghei* ANKA. Basic histopathological studies and TUNEL staining revealed a variety of morphologies ranging from apoptosis to necrosis. Further, increased immunoreactivity of active Caspase-3 provides unequivocal support for apoptosis. Cleavage of PARP into its characteristic signature fragments reveals a role for necrosis. Ultrastructurally infected cortex and cerebellum exhibited co existence of both apoptotic and necrotic features. In conclusion, our results suggest that pathology of fatal murine cerebral malaria results in the existence of apoptosis and necrosis in both cortex and cerebellum.

Keywords

Cerebral malaria; Apoptosis; Necrosis; Caspase-3; PARP; NF- κ B; COX2

Introduction

A major cause of morbidity and mortality of *Plasmodium falciparum* malaria is cerebral malaria (CM) presenting as a diffuse encephalopathy with alteration of consciousness, ranging from drowsiness to deep coma frequently accompanied by seizures (Warrell *et al.*, 1990; Marsh *et al.*, 1995). Clinical manifestations of CM are numerous, however, three primary symptoms generally common to both adults and children: (1) impaired consciousness with non-specific fever; (2) generalized convulsions and neurological sequelae; and (3) coma that persists for 24-72 h, initially rousable and then unrousable. Further, malaria is a major cause of death in people infected with *P. falciparum*, and approximately 1% of *P. falciparum* infections progress to CM (WHO, 2000; Snow *et al.*, 2001). The great majority of cases occur in young children (coma is very often accompanied by fever, convulsions, seizures, metabolic acidosis and hypoglycaemia) in sub-Saharan Africa,

and 10-20% of children who develop CM die, and many survivors are left with permanent neurological damage including cognitive impairment and speech disorders, motor abnormalities and cortical blindness (WHO, 2000; Snow *et al.*, 2001). Importantly, in terms of prevention, high blood parasitemia is significantly correlated with risk of developing CM (Molyneux *et al.*, 1989). CM was long thought to result from blood vessel obstruction by parasitized RBC (pRBC), leading to ischemia and petechial haemorrhages. However, it is now clear that CM also involves systemic inflammation that includes the increased expression of adhesion molecules on cerebral microvascular endothelial cells (MVECs) leading to cytoadherence of pRBC, activated leukocytes and platelets, and subsequent damage to the BBB. CM is also likely the common endpoint for several pathways leading to pathogenesis. After the circulation of pRBCs to the microvasculature and the establishment of physical contact with Endothelial cells (EC), an endothelial stress process characterized by cell apoptosis is initiated. The damage to the ECs caused by the direct interaction between pRBC and EC is the main factor triggering the development of CM (Brown *et al.*, 1999; Pino *et al.*, 2003). Loss of cell junction proteins such as Zo-1 and occlusion seem to cause the leakage at the Blood Brain Barrier (BBB). Serum proteins and liquid diffuse in massive amounts into the brain tissue which leads to coma and further damage to the nervous system. Macrophage activation has been speculated to contribute to the loss of barrier proteins, however, further studies are required. Recently, in a study (Medana *et al.*, 2002) demonstrated axonal injury in the autaptic brain of CM patients. However, the exact mechanisms by which the brain cell death occurs in CM remains less well understood.

Two hypotheses were proposed to explain the pathogenesis of cerebral malaria. The mechanical (or sequestration) hypothesis, first proposed in 1894 by Marchiafava and Bignami, postulates that *P. falciparum*-parasitized erythrocytes bind to the post capillary venules, causing considerable obstruction of blood flow, decreased tissue perfusion and decreased removal of waste products (lactic acid) (Miller *et al.*, 2002; Idro *et al.*, 2005). There are concerns about the mechanical hypothesis reviewed in Eling and Kremsner, 1994; Clark and Cowden, 2003). Briefly, this hypothesis predicts that the greater the parasitemia, the greater the likelihood of vascular obstruction by parasitized erythrocytes. However, clinical studies indicate a low correlation between parasitemia and mortality; individuals with high parasitemia are sometimes asymptomatic, and symptomatic patients can have low parasitemia. The inflammation hypothesis, proposed by Maeagraith in 1948, states that malarial parasites and other infectious agents elicit a systemic inflammatory response that causes multi-organ failure and death. Malaria toxin (presumably glycosphosphoinositol) binds to pattern recognition receptors of the innate immune system and activates monocytes to secrete proinflammatory cytokines, including interleukin IL-1, IL-6, macrophage colony-stimulating factor (M-CSF), tumor necrosis factor (TNF- α) and lymphotoxin (LT), and produce superoxide and NO (Nebl *et al.*, 2005). The proinflammatory cytokine TNF- α is proposed to upregulate intercellular adhesion molecule (ICAM1) and other endothelial cell adhesion molecules (eCAMs) during cerebral malaria. In ECM, interferon IFN- γ instead of TNF- α is required to elicit increased ICAM1 levels in brain, and the cytokines regulating increased eCAMs are cell adhesion molecule-specific and tissue-specific (Bauer *et al.*, 2002). Overwhelming evidence using knockout mice and inhibitory monoclonal antibodies (mAbs) indicates that the inflammatory response is required for the development of disease (Schofield and

Grau, 2005), but the relevance of ECM to human malaria has been questioned [Miller *et al.*, 2002]. Moreover the role of inflammatory mediators such as COX2 (Ball *et al.*, 2004; Xiao *et al.*, 1999) and iNOS (Nahrevanian and Dascombe, 2002) and the activation of NF- κ B pathway in regulation of these inflammatory mediators during the pathology of murine cerebral malaria is well established (Kumar *et al.*, 2003).

Apoptosis is a complex biological process of cell death characterized by morphologic features that are distinct from the changes occurring during necrosis. These morphologic alterations include preservation of organellar structure, cell body shrinkage, and plasma membrane blebbing, followed by nuclear chromatin condensation that is often accompanied by internucleosomal DNA cleavage through the action of activated endonucleases (Wyllie *et al.*, 1980; Enari *et al.*, 1996). Caspases are cell death proteases which play an important role in the commitment and execution phases of apoptosis (Villa *et al.*, 1997). Importantly, caspase-3 acts as a major downstream executioner in mediating neuronal apoptosis (Kuida *et al.*, 1996; Beer *et al.*, 2000). In a recent study, Caspase-3 activation has been demonstrated in autaptic brains of Vietnamese CM patients (Medana *et al.*, 2001) and lately, study by another group has shown the role of caspase3 in mediating the neuronal cell death during the pathology of murine cerebral malaria. (Lackener *et al.*, 2007). In contrast to apoptosis, necrosis is a relatively untouched area and its role in the cerebral pathology has not been studied till now. Necrosis is often observed under severe pathophysiological conditions, such as Hypoxia, Ischemia, Hypoglycemia, exposure to reactive oxygen metabolites and toxins. This type of cell death has also been described in neurodegenerative disorders like Alzheimer's disease, Huntington's disease, Parkinson's disease, Amyotrophic lateral sclerosis, and Epilepsy (Syntichaki and Tavernarakis, 2002; Proskuryakov *et al.*, 2003). Moreover, autophagic and cytoplasmic types represent alternative necrosis-like forms of programmed cell death that normally occurs during development (Kitanaka *et al.*, 1999). In contrast, to apoptosis, necrosis has been considered a passive and disordered process leading to accidental and unavoidable death of cells exposed to extreme insults. However, new findings have revised this concept. Thus, execution of necrotic death depends on activation of Ca^{2+} -dependent proteolysis that employs proteases different from caspases. Moreover, similar morphological and ultrastructural features of necrotic cells of different origin suggest, existence of a conserved execution program. Moreover, the contribution of necrosis to the neuronal cell death during the pathology of murine cerebral malaria is not well understood. Taking this drawback into consideration, the present study is aimed at determining the contribution of necrotic and apoptotic mechanisms to the pathophysiology of CM in an established model of CM.

Materials and methods

Induction of cerebral malaria in mice

All the protocols followed for the use of animal experimentation were approved by the institutional as well as national ethical committee guidelines (CPCSEA, 2003). *P. berghei* (ANKA) strain was obtained from MRC (Malaria Research Centre), New Delhi, India. Swiss Albino mice were procured from National Institute of Nutrition, Hyderabad, India. Six to eight weeks old Swiss albino mice of either sex (~20g body

weight, $n = 4$ per each group) were inoculated intraperitoneally with 10^6 parasitized red blood cells, suspended in 200 μL of phosphate buffered saline (pH 7.4). Uninfected mice of same age and sex were used as negative controls ($n = 4$). The animals infected with *P. berghei* ANKA strain showed behavioral changes around day 5 after inoculation followed by cerebral symptoms like paralysis, hemiplegia, convulsions and coma eventually leading to cell death. The parasiteamia was monitored by preparing periodic blood smears from the 3rd day of parasite inoculation and was typically between 40-50% at the time of sacrifice. The duration between parasite inoculation and sacrifice of terminally ill animals was approximately 14-16 days.

Preparation of tissue sections for H&E and toluidine blue staining

Swiss albino mice were anesthetized with isoflurane and perfused with buffered 4% paraformaldehyde when they developed cerebral symptoms (PbA infected, $n = 4$; control mice, $n = 4$), which occurred on day 14 postinoculation (p.i.) Brains were removed, cortices and cerebellum were dissected, and immersion fixed in the same fixative for 24 h at 4°C. Coronal tissue sections (5 μm) were prepared from infected mice and control mice after dehydration and embedding in paraffin. Sections were stained with hematoxylin and eosin stain (H&E stain) and toluidine blue for light microscopic examination.

Sub-cellular fractionation of brain

Brain was dissected from control and terminally ill animal after decapitation and stored at -80°C until use. Cerebral cortex and cerebellum regions were separated and preparation of cellular fractions and whole tissue lysate was done essentially as described earlier (Kumar *et al.*, 2002). Homogenization was carried out in ice-cold isolation buffer (0.32M sucrose, 10 mM Tris-HCl buffer (pH 7.2), 1mM EDTA, 1 mM EGTA, and 1.5 mM MgCl_2 , 1mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin). The homogenate was centrifuged twice at 1000 $\times g$ (3000 rpm) for 15 min and the pellet was used as crude nuclear fraction. The supernatant was further centrifuged at 100,000 $\times g$ (36,000 rpm). The pellet obtained from the spin was used as cellular membrane and the supernatant as pure cytosol. For whole tissue lysate the cerebral cortex and cerebellum were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Deoxycholate, 1% TritonX-100, 10% Glycerol, 2 mM EDTA, 1mM PMSF, 2 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatinA, 1mM Sodium orthovanadate, 20 mM b-glycerophosphate, 20 mM Sodium fluoride). Cell lysate was centrifuged at 14,000 g for 15 min. All the steps were carried out at 4°C. Protein content in the cellular fractions was quantified by the method of Lowry (1951).

Western Immunoblotting

50 μg of cytosolic/nuclear tissue protein was separated by reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membrane overnight at 30V in Towbin buffer (25 mM Tris-HCl (pH 8.3), 192 mM Glycine, 20% methanol). The nonspecific binding sites were blocked with 5% w/v non-fat milk for 2 h. The blots were probed with primary antibodies overnight. The primary antibodies were 1:1000 dilution of mouse monoclonal antibody against Cyclooxygenase-2, (COX-2) (Santacruz Biotech, USA) 1:1000 dilution of rabbit polyclonal antibodies raised against Caspase-3 (R&D Systems,

Minneapolis), PARP (Cell signaling Tech, USA) NF- κ B p-50, p-52, p65 (Santacruz Biotech, USA). The blots were further processed with either ALP-conjugated anti-rabbit or anti-mouse secondary antibody for 1 h at Room Temperature (RT). The protein bands were visualized using BCIP/NBT as substrates dissolved in ALP buffer. Quantitative densitometric analysis of Western blots corresponding to the respective protein bands was performed using a color scanner (HP Scan Jet 3200C) and the NIH Scion Image Beta 4.02.

Caspase-3 Activity Assay

Caspase-3 activity was measured by a spectrofluorometric assay. The assay was performed in a final volume of 500 μ l containing [20 mM Tris-HCl (pH 7.5), 0.1 mM NaCl, 5 mM DTT, 10% sucrose]. Aliquots of cytosolic fraction containing 100 μ g of protein were incubated with saturating concentration (100 μ M) of enzyme substrate DEVD-7-amino-4-methylcoumarine (DEVD-AMC) in the above buffer at 37°C for one hour. The levels of released AMC were measured using an excitation wavelength of 380 nm and emission wavelength of 460 nm with a spectrofluorometer (Hitachi, Tokyo). For non-specific activity, Caspase-3 specific inhibitor Ac-DEVD-CHO was added at a concentration of 10 μ M to the cytosolic extracts 30 min prior to the addition of the substrate peptide Ac-DEVD-AMC. One unit is defined as the amount of enzyme required for the release of 0.22 pmol of AMC/min at 37°C. The final Caspase-3 protease activity was determined subtracting values obtained in the presence of the inhibitor. The rate of Caspase-3 activity was expressed as fluorescence units in the presence of inhibitor/min/mg protein.

Caspase-3 immunohistochemistry

Infected and control mice brains were perfused first with 0.9% saline solution followed by 4% paraformaldehyde in 0.1M PBS, pH 7.4 and were embedded in paraffin. Mouse brains were sectioned horizontally (5 μ m) on the automated rotary microtome (Leica) and deparaffinized in xylene. Antigen retrieval was carried out by microwaving sections in 10mM citrate buffer, pH 6.0, for 1 min at full power followed by 9 min at medium power. Blocking was carried out in 10% normal goat serum in PBS for 1 h at room temperature in a humidified chamber. Primary antibody (rabbit monoclonal antibody against Caspase-3) was diluted 1:100 in blocking solution and incubated for overnight at 4°C. Peroxidase conjugated anti-rabbit secondary antibody (1:250) was used for incubation time of 1 h at room temperature followed by PBS washes (3 x 5 min each). DAB (R&D systems, Minneapolis, USA) at a final concentration of 0.25 mg/ml with 0.05% H₂O₂ in PBS was used for developing till the section turns brown (typically for 3-5 min at RT). Sections were counter stained with methyl green for 10 sec. Sections were then washed with dH₂O followed by dehydration in graded ethanol and xylene and coverslipped with DPX mount.

Preparation of tissue for Transmission Electron Microscopy

In brief, the cortical and cerebellar tissue blocks were cut into 400 μ m thick section with a Vibratome. Slices were washed in cold 0.1M sodium cacodylate buffer and kept in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer until further processing. When processing resumed, slices were washed in cold cacodylate buffer and were then post fixed in 1% osmium tetroxide in cold cacodylate buffer for 1 h.

After osmium tetroxide step, 400 µm thick coronal slices were washed in 0.1M sodium cacodylate buffer. The cortex and cerebellar regions were identified with a dissection microscope, and 2 x 2 mm sections were cut out from tissue slices, dehydrated in a graded series of ethanol and embedded in Spurr Epon. Blocks were trimmed, and semi thin 0.5 µm thick sections were cut with an ultramicrotome, stained with toluidine blue and examined by LM for an overall view. Ultrathin 70-90 nm thick sections were then cut, picked up on 200 mesh copper grids, double stained with uranyl acetate and lead citrate, and scanned in Joel 100CX electron microscope.

Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP nick-end-labelling (TUNEL) assay

In situ DNA nick-end-labeling (TUNEL staining) was performed as per the instructions given in the users manual (ApoAlert DNA fragmentation Assay Kit; BD Biosciences). In brief, coronal sections were deparaffinised, washed in 100, 95, 85, 70 and 50% ethanol, 0.85% NaCl, phosphate buffered saline (PBS), 4% formaldehyde/PBS for 15 min, PBS twice and incubated for 15 min at room temperature (RT) with 20 µg/ml of proteinase K and washed in PBS, 4% formaldehyde/PBS and PBS. Sections were then incubated in equilibration buffer (200 mM Potassium cacodylate, pH 6.6; 25 mM Tris-HCl, pH 6.6; 0.2 mM DTT; 0.25 mg/ml BSA; 2.5 mM Cobalt chloride) for 10 min, followed by TdT incubation buffer (45 µl Equilibration buffer; 5µl nucleotide mix; 1µl TdT enzyme) for 60 min at 37°C in a dark, humidified chamber. The reaction was stopped by incubating the sections with 2 x SSC (3M NaCl; 300mM Na₃Citrate.H₂O) for 15 min at RT, followed by two washes in PBS and further incubated with propidium iodide (PI) for 5-10 min. This was followed by two washes in dH₂O and visualized under Fluorescence microscope.

DNA Agarose Gel Electrophoresis

The brain samples (cerebral cortex and cerebellum) were homogenized in 10 volumes of buffer (containing 100 mmol/l Tris-HCl, pH 8; 5 mmol/l EDTA; 0.2 mol/lit NaCl; 0.4% SDS; 0.2 mg/ml proteinase K), incubated overnight at 37°C, then extracted with 1:1 phenol: chloroform, and precipitated by two volumes of ethanol. DNA extracts were electrophoresed on 1.2% Agarose gels in TBE buffer. DNA was visualized by ethidium bromide staining, observed, and photographed.

Results

Morphological changes as revealed by Haematoxylin and Eosin staining & Toluidine Blue staining

Histopathological staining of brain section with hematoxylin and eosin and Toluidine blue (Figs. 1, 2 & 3) suggested changes in the neuronal cells such as redistribution of activated microglial cells towards microvessels, retraction of ramified processes, nuclear enlargement, intensification of staining, increasingly amoeboid appearance and vacuolation. The walls of the blood vessels were disrupted with the death of endothelial cells, severe edema, and focal hemorrhage. Sequestered parasitized erythrocytes and monocytes were evident adhering to the walls of some blood vessels. Increased numbers of activated microglial cells in perivascular spaces was evident in the infected sections.

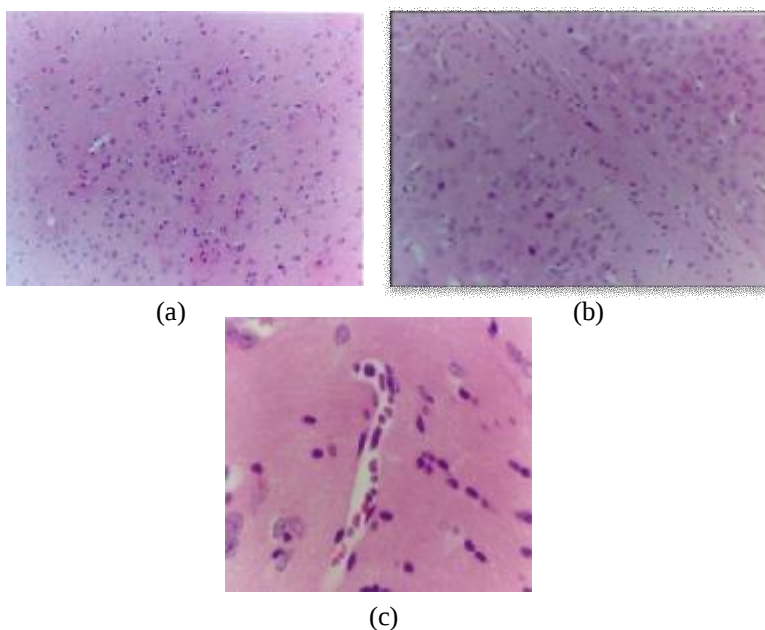


Fig. 1. Photographs of Hematoxylin-Eosin stained sections of control and infected cerebral cortex sections. (a) Control cerebral cortex. (b, c) infected cerebral cortex. Microvascular sequestration of parasitized RBC and monocytes can be seen in infected cerebral cortex. Magnification: (a, b) 100x; (c) 400x.

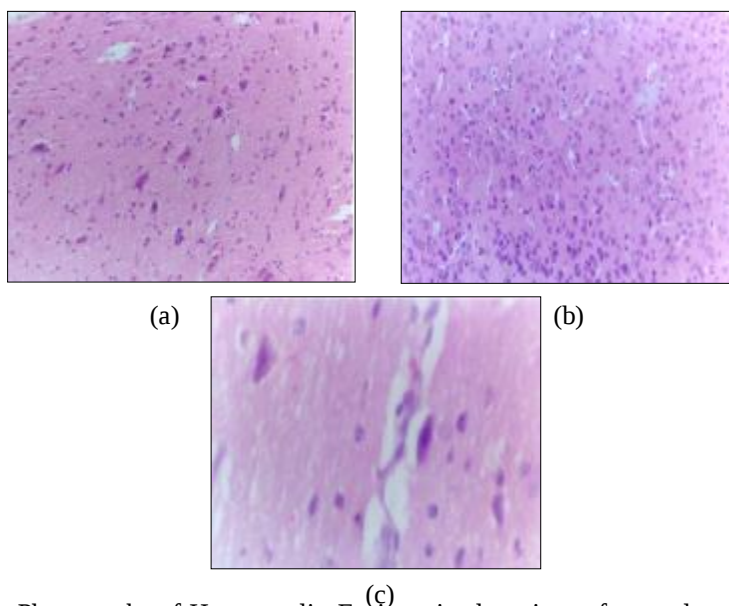


Fig. 2. Photographs of Hematoxylin-Eosin stained sections of control and infected cerebellum sections. (a) Control cerebellum. (b, c) infected cerebellum. Microvascular sequestration of parasitized RBC can be seen in infected cerebellum. Magnification: (a, b) 100x; (c) 400x.

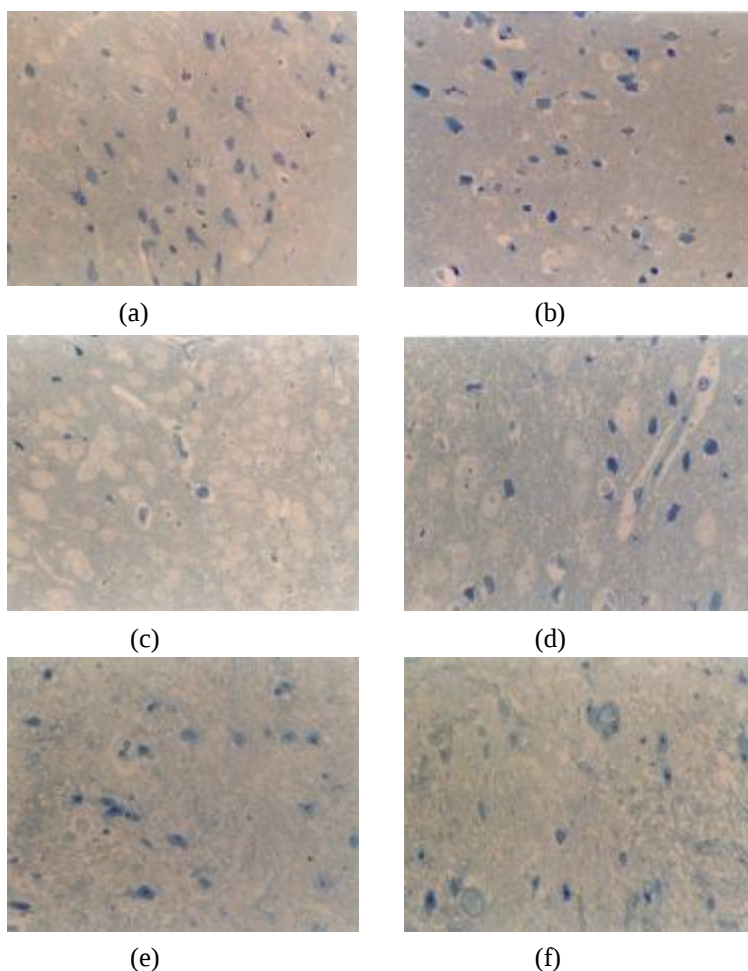


Fig. 3. Photographs of Toluidine blue staining of control and PbA-infected cerebral cortex and cerebellum sections. Control cerebral cortex (a). Infected cerebral cortex (b, c, d). Characteristics of degeneration like cell shrinkage, nuclear pyknosis can be seen in infected sections (b,c,f). Activation of reactive microglia can be seen around the microvessel in the infected cerebral cortex sections (d). Control cerebellum (e). Infected cerebellum (f). Magnification: (a, b) 100x; (c,d,e,f) 150x.

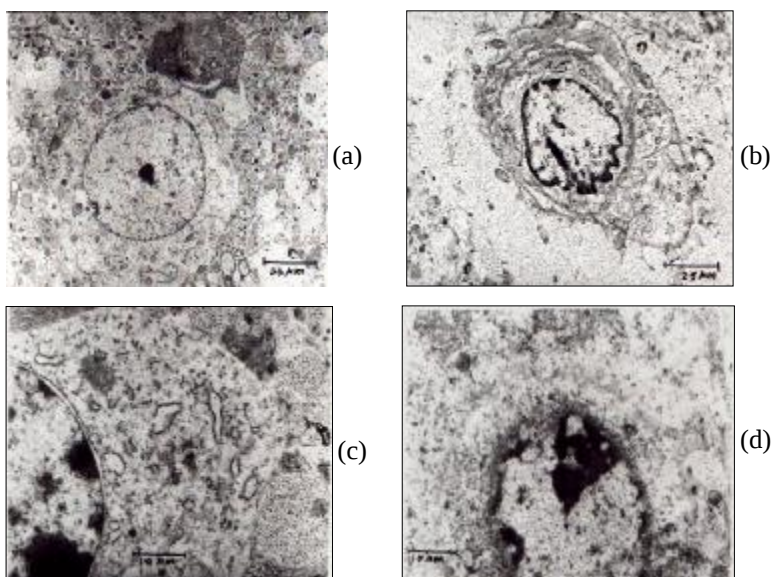


Fig. 4. Photographs of Transmission Electron Microscopic sections of control and infected cortex. (a,c) Control cerebral cortex. (b,d) Infected cerebral cortex. Characteristic features of cell death like chromatin, nuclear membrane degeneration and nuclear condensation (b,d), vacuolation and loss of cell organelles and nuclear membrane (d) can be clearly seen. Magnification: (a) 3000x; (b) 4000x; (c,d) 10,000x.

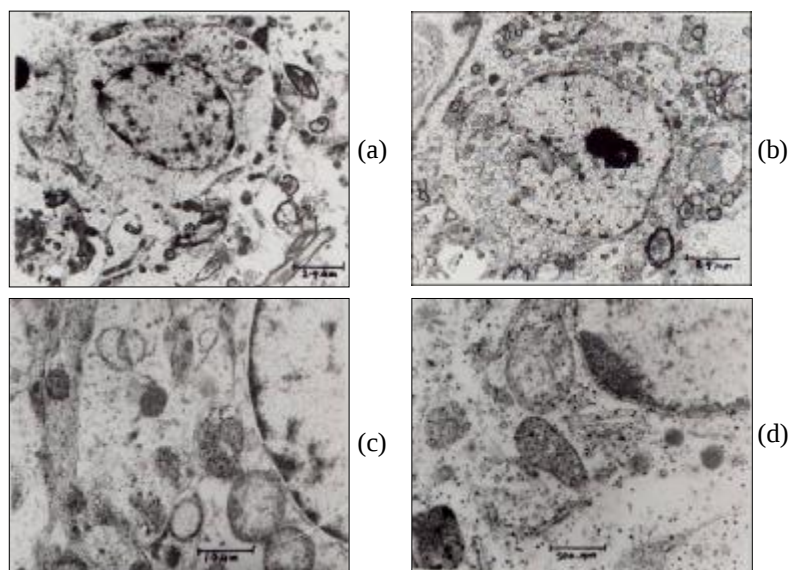


Fig. 5. Photographs of Transmission Electron Microscopic sections of control and infected cerebellum. (a,c) Control cerebellum. (b,d) Infected cerebellum. Characteristic features of cell death like chromatin and nuclear condensation (b,d), vacuolation and swelling of cell organelles (mitochondria) (d) can be clearly seen. Magnification: (a,b) 3500x; (c) 10,000x; (d) 20,000x.

Ultrastructural changes in Nucleus and Mitochondria: Implications for necrotic mode of cell death

The TEM studies of CM infected brain tissue showed changes in the cell architecture of both cerebral cortex and cerebellum of infected animals (Figs. 4b, 4d, 5b, 5d). Higher magnification of the neural elements in the vicinity of the parasite sequestered microvessels showed many of the cells with features typical of necrosis (Figs. 4b, 4d, 5b, 5d). These changes included extensive swelling and vacuolation of mitochondria with distinct loss of cristae suggestive of a late stage of mitochondrial degeneration (Fig. 5d). The structural changes associated with the mitochondrial anomalies may have a profound impact on its functional and biochemical aspects that coincides well with our earlier observation of cytochrome-c release during CM pathology that could be a probable downstream event to mitochondrial membrane depolarization (Kumar *et al.*, 2002). Complete disintegration of other cell organelles like Golgi, ER, polysomes, a compromised plasma membrane and nuclear membrane were the other features of necrosis heralded by CM pathology (Fig. 4d) as compared to controls (Fig. 4c). In spite of extensive cytoplasmic degeneration, the structural integrity of the nucleus was well preserved though it appeared swollen with condensed chromatin in some cells (Fig. 5d). Our observations clearly demonstrate that cytoplasmic changes predominate over nuclear changes during the pathology of CM and are in general agreement with overt cytolysis that follow inflammation during necrosis.

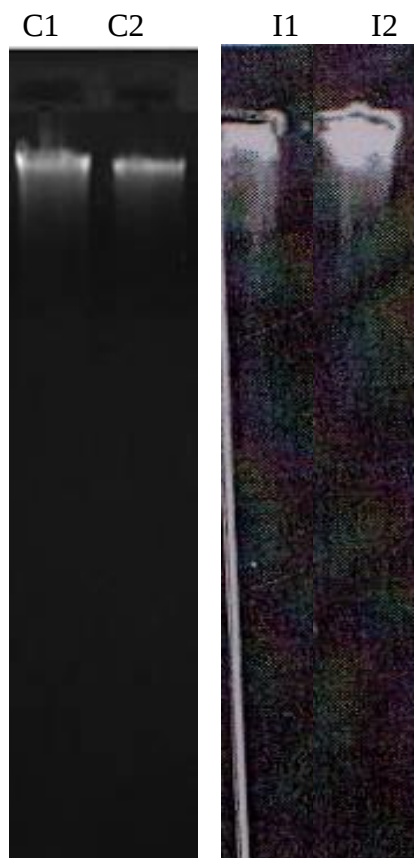


Fig. 6. DNA Agarose gel electrophoresis showing DNA laddering pattern in infected cerebral cortex and cerebellum. C1, control cerebral cortex; I1, infected cerebral cortex; C2, control cerebellum; I2, infected cerebellum.

Terminal deoxynucleotidyl Transferase (TdT) - mediated dUTP nick-end-labelling (TUNEL) assay

During apoptosis, cellular endonucleases cleave nuclear DNA between nucleosomes, producing a mixture of DNA fragments whose lengths vary in multiples of 180 to 200 bp. The ApoAlert DNA Fragmentation assay kit detects apoptosis-induced nuclear DNA fragmentation via a fluorescence assay. The assay is based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) (Gavrieli *et al.*, 1992). TdT catalyzes incorporation of fluorescein-dUTP at the 3'-

hydroxyl ends of fragmented DNA; fluorescein-labeled DNA can be detected via fluorescence microscopy. Apoptotic cells exhibited strong, nuclear green fluorescence using a standard fluorescein filter set (520+/-20nm) in the infected cerebral cortex and cerebellum (Figs. 8b,8d). Nuclei of TUNEL positive cells were lightly to moderately stained in few cells from both the cerebral cortex and cerebellum of CM infected brain (Figs. 8b, 8d).

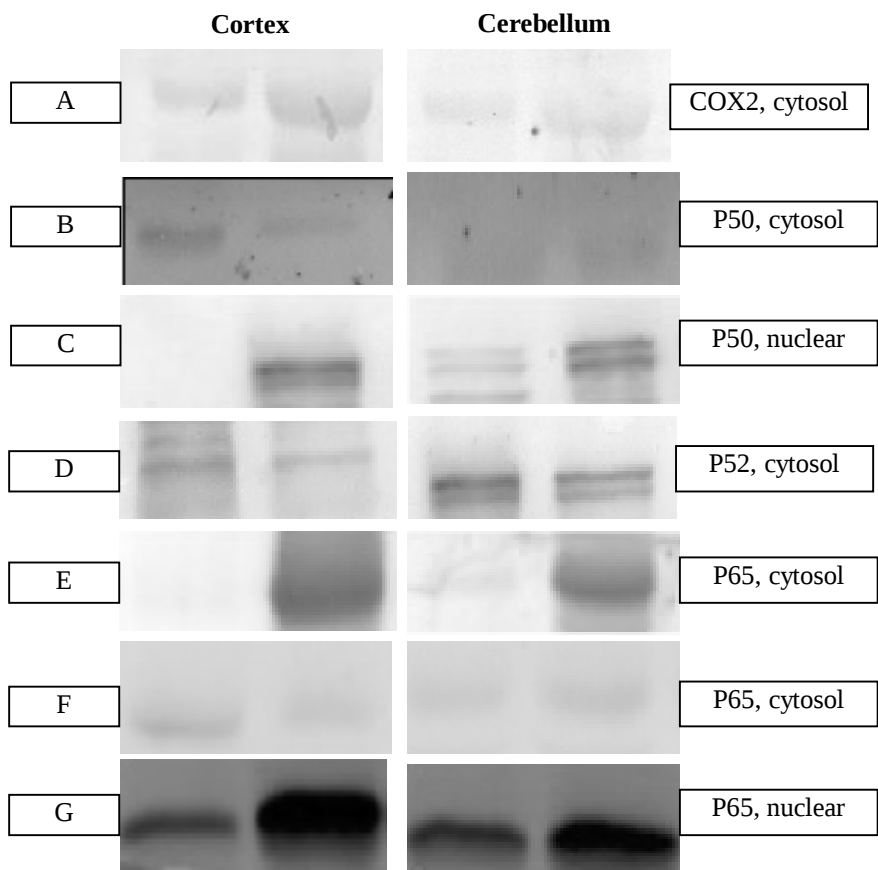


Fig. 7. Western blot analysis of COX-2, p50, p52 & p65 of cortical and cerebellar samples.

Western blot analysis of COX2 (Fig.7A), and NF-κB family of proteins p50 (Fig 7B, 7C), p52 (Fig 7D, 7E) & p65 (Fig: 7F, 7G) of cortical and cerebellar samples. Fig 7A, 7B, 7D & 7F represents cytosolic and Fig 7C, 7E & 7G represents nuclear fractions respectively. C1 and I1 represents control and infected cortical samples, whereas C2 and I2 represents control and infected cerebellar samples.

Oligonucleosomal fragmentation of DNA

The DNA isolated from both the cerebral cortex and cerebellum showed distinct oligonucleosomal fragmentation (Fig. 6).

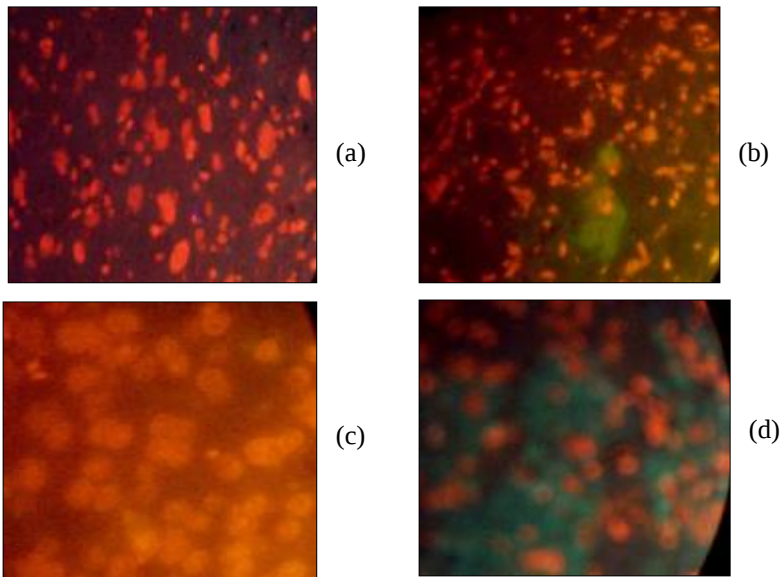


Fig. 8. TUNEL Staining of cerebral cortex and cerebellum. Photographs of paraffin sections stained for TUNEL and counter stained with propidium iodide. (a) Control cerebral cortex, red fluorescence indicate normal cells. (b) Infected cerebral cortex with DNA nicks indicated by green fluorescence. (c) Control cerebellum. (d) Infected cerebellum with numerous cells having DNA nick. Magnification: (a,b,c,d) 400x.

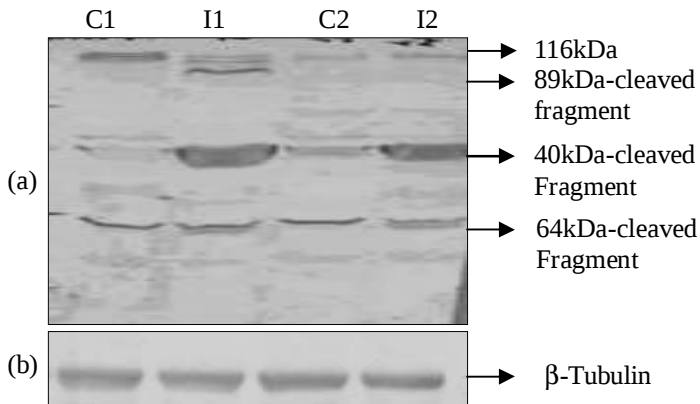


Fig. 9. Immunoblot analysis of PARP. Immunoblot analysis of PARP in the whole tissue extracts of control and infected cerebral cortex, cerebellum. Equal amount of protein was electrophoresed by SDS-PAGE and transferred to nitrocellulose membrane and probed with primary antibody to PARP and β -tubulin (b). The result of β -tubulin was shown in the lower panel as an internal control for equal protein loading. The result is representative of four independent experiments with similar results. The lanes: C1-control cerebral cortex; I1-infected cerebral cortex; C2-control cerebellum; I2-infected cerebellum.

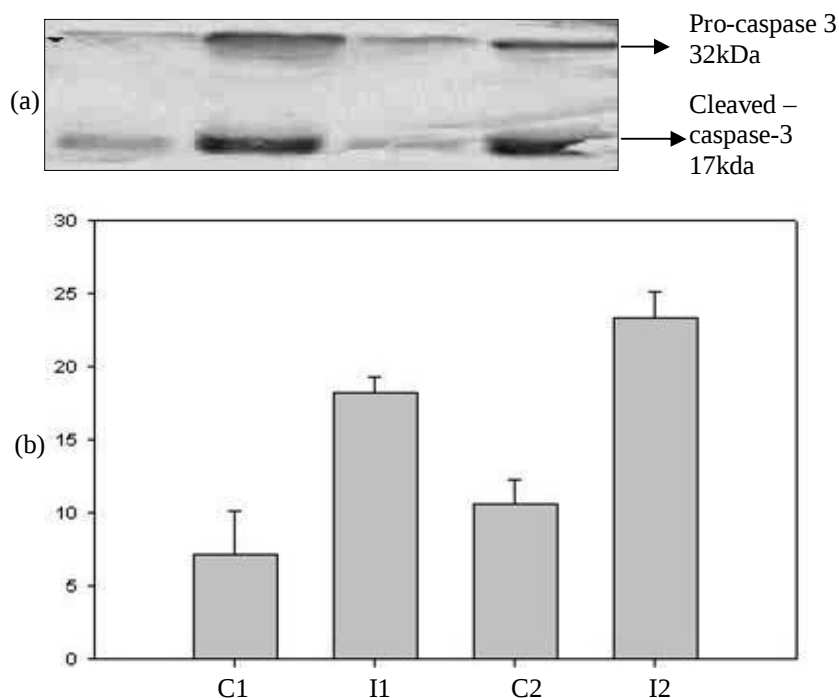


Fig. 10. Caspase-3 immunoblot and activity assay (a) Immunoblot analysis of caspase-3 in cytosolic extracts of control and infected cerebral cortex, cerebellum. Equal amount of protein were electrophoresed by SDS-PAGE and transferred onto a nitrocellulose membrane and probed with primary antibody to caspase-3. The result is representative of four independent experiments with similar results. (b) Caspase-3 activity was assayed in the cytosolic extracts from control and infected cerebral cortex and cerebellum. Activity was determined by spectrofluorometry using Ac-DEVD-AMC as the substrate and expressed in arbitrary units on the y-axis. Data are mean $\pm$ SEM (bars) values from four independent experiments. Student t-test statistical analysis (control x infected treatment) was used. Values of $**P<0.001$ and $*P<0.004$ were considered significant. On the x-axis: C1, control cerebral cortex; I1, infected cerebral cortex; C2, control cerebellum; I2, infected cerebellum.

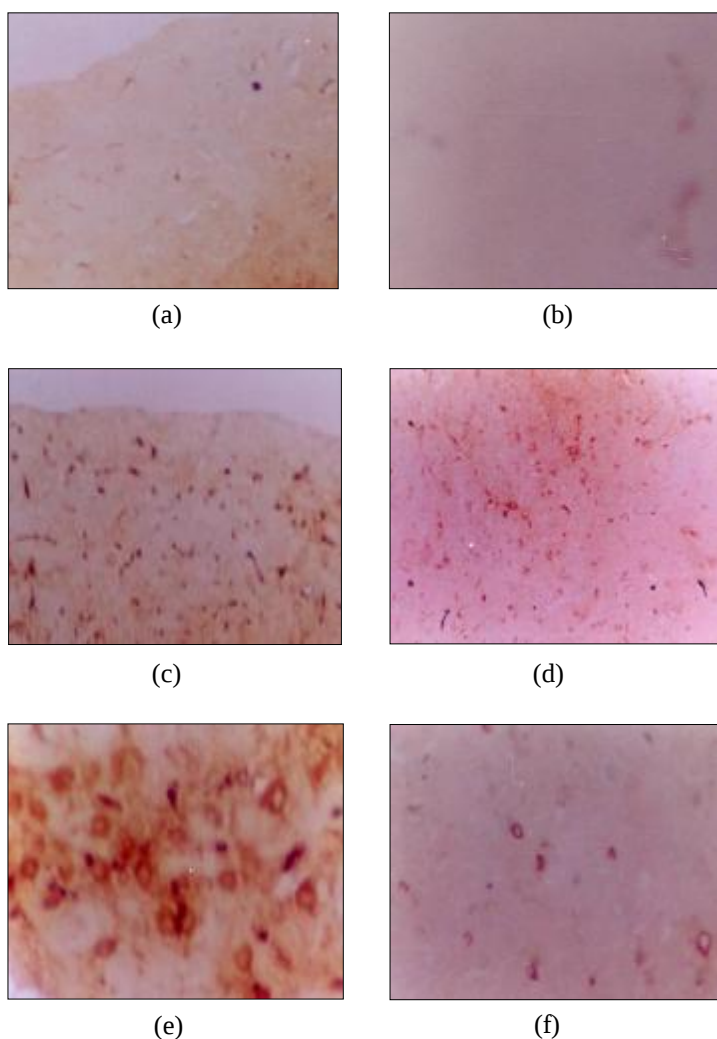


Fig. 11. Caspase-3 immunohistochemistry of control and infected cortex and cerebellum. Photographs of paraffin sections (a,b) Control brain. (c,d,e,f) Numerous caspase-3 positive brain cells were observed in infected cerebral cortical and cerebellar sections. (e) Infected cerebral cortex cells showing caspase-3 immunoreactivity in the cytosol. Magnification: (a,b,c,d) 100x; (e,f) 400x.

Degradation of PARP to 64kD and 40 kD signature fragment is a feature of necrosis

We used an antibody that could detect the cleaved forms of PARP. We could detect the 64 kD and 40 kD bands along with characteristic signature fragment of 89 kDa in the infected cerebral cortex and cerebellum (Fig. 9). This suggests that 64kD fragment of PARP cleavage might be associated with Granzyme B cleaved fragment and 40kD might be associated with calpain cleaved fragment. Cleavage of PARP might occur when the cell is no longer able to repair its DNA and could play a role in cellular disassembly that might ensure commitment to either apoptosis or necrosis. Previous studies from our lab have shown the formation of characteristic 89 kD fragment in the infected samples. Based on our observation we conclude that pathways other than apoptosis are also activated in mice brain upon *PbA* infection.

Processing and activation of Caspase-3, a key transducer and effector of apoptosis

Caspase-3 in physiological conditions exists as a 32 kDa zymogen and when activated, is cleaved into a small prodomain and two subunits of 20- and 12-kDa, respectively. The two subunits associate with another pair of subunits to form active Caspase-3 tetramer. An antibody, which recognizes both the p20 subunit as well as the proform of Caspase-3, was used. Immunoblotting with this antibody showed processing of Caspase-3 with the appearance of active p20 fragment in the cytosol of infected cerebral cortex and cerebellum (Fig. 10a). Increased levels of precursor form of Caspase-3 were also detected in the cytosolic fraction of both infected cerebral cortex as well as cerebellum.

Active Caspase-3 levels coincide with the enhanced Caspase-3 activity

Several indices were used to measure the activation of Caspase-3 induced upon *P. berghei* ANKA infection. To complement the observations of western analysis, Caspase-3 activity was measured by assessing the cleavage of fluorogenic synthetic substrate Ac-DEVD-AMC in cytosolic extracts prepared from control and infected samples. *P. berghei* ANKA infection resulted in increased Caspase-3 activity in cerebral cortex and cerebellum. To ascertain that the increased activity is indeed due to caspase-3, specific inhibitor of caspase-3, Ac-DEVD-CHO was also tested in the assay (Fig. 10b). The inhibitor abrogated the increased activity in infected samples suggesting that caspase-3 was indeed activated upon *PbA* infected in cerebral cortex and cerebellum.

Caspase-3 immunohistochemistry

Caspase-3 immunoreactivity using rabbit anti-human/mouse Caspase-3 active, revealed more immunoreactive active Caspase-3 cells in the infected cerebral cortex and cerebellum, which indicates degenerating cells undergoing apoptosis (Figs. 11c, d). Though some Caspase-3 immunoreactivity was also found in the control brain, which can be accounted for the basal levels of Caspase-3 in the normal brain (Figs. 11a, b). Moreover, Caspase-3 staining was clearly localized in the cytosol of the neuronal cells in the infected brain (Figs. 11e, f).

Increased levels of COX2 during cerebral malaria in cerebral cortex and cerebellum

COX2 was evinced by western blot analysis (Fig. 7a) of the cytosolic fraction obtained from control and infected mice. COX2 was observed to be increased in infected mice in cerebral cortex, and cerebellum. The greater induction of COX2 in CM could be due to the higher levels of cytokines (IFN- γ , TNF- α , and IL-1) found in the brain.

Induction of NF- κ B/Rel family proteins p50, p52 and p65 during Cerebral Malaria

NF- κ B family proteins (members of Rel transcription factor family) are ubiquitously expressed and are inducible transcription factors that regulate the statement of genes involved in processes such as immunity inflammation, growth, development, viral gene transcription and cell death regulation. Nuclear translocation of NF- κ B family proteins p50 was evinced by Western blot analysis of the cytosolic fraction (Fig. 7 b) and nuclear fraction (Fig.7C) obtained from control, and infected brain (from cortex and cerebellum). There was a decrease in the levels of these proteins in the cytosolic fraction whereas it was found to be increased in nuclear fraction.

Western blot analysis of p52 was done from cytosolic fraction (Fig. 7d) and nuclear fraction (Fig. 7e) obtained from control and infected and aspirin treated (from cortex and cerebellum). The levels of p52 were found to be decreased in cytosolic fraction of cerebral cortex while the levels were found to be increased in nuclear fraction of infected mice.

Levels of p65 were evinced by Western blot analysis of the cytosolic fraction (Fig. 7f) and nuclear fraction (Fig. 7g) obtained from control, infected brain (from cortex and cerebellum). The levels of p65 was found to be increased in nuclear fraction in infected mice of cerebral cortex and cerebellum NF- κ B proteins are normally sequestered in the cytoplasm in an inactive state by the I κ B family of inhibitory proteins.

In general, activation of NF- κ B is the result of I κ B phosphorylation by the multi-subunit I κ B kinase (IKK) complex, which leads to I κ B degradation. Various physiological and pathological stimuli promote I κ B phosphorylation, leading to its ubiquitin mediated-proteasomal degradation, allowing the release and nuclear translocation of an active form of the NF- κ B complex. In the present study, the increased levels of NF- κ B p65 and p50 subunits in the nuclear fraction suggest the possibility of I κ B degradation in the cytosol, thereby relieving the NF- κ B subunits to translocate into the nucleus. Capillary blockade due to microvascular sequestration of parasite-infected red blood cells (pRBC) during cerebral malaria may result in insufficient blood flow and reduced oxygen carrying capacity leading to a situation akin to hypoxia. Therefore, it is plausible to envisage a role for hypoxic conditions also in the activation of NF- κ B during cerebral malaria, since NF κ B activation has been earlier reported during hypoxia/reoxygenation.

Discussion

CM, the most severe complication of the CNS, is mediated in a graded manner by cytokine over-production, Th2 response, cytokine- induced phenotypic changes of brain microvascular endothelial cells and recruitment of immune cells into the damaged tissue following parasite sequestration in microvessels of brain. Moreover, role of proinflammatory cytokines has been implicated in apoptosis (Licinio, 1997). In a recent study an absolute requirement of the TNFR2 for the expression of CM in *PbA* infected mice was confirmed (Piguet *et al.*, 2002). Indeed TNF- α induces profound alterations in vascular endothelial cells, with redistribution of cell junction protein, production of inflammatory cytokines and apoptosis (Piguet *et al.*, 2002; Grell *et al.*, 1999). The molecular signals that regulate these cellular responses may also influence the morphological aspects of the cell types subjected to insult. In a bid to analyze such changes associated with CM pathology, we employed a combination of morphological and biochemical techniques to reveal the nature of damage associated with the neural elements during infection. TNF- α induces apoptosis by forming a complex called the DISC (death inducing signalling complex) with intracellular proteins. FADD, an adaptor protein binds to Fas and activate caspase-8 in response to Fas signalling and binds the adaptor proteins TRADD to activate caspase-8 in response to TNF- α (Thomas *et al.*, 2002). Nuclear karyorrhexis or fragmentation was apparent in some cells where TUNEL reaction was positive at series of sites where DNA damage was induced. Other cells that were TUNEL positive showed condensed nuclear material suggestive of an early stage of apoptosis. However, many cells that were TUNEL positive could not show a clear cellular boundary reflecting that DNA damage is probably an event secondary to organellar or membrane degeneration which fits well with our TEM results which revealed an extensive organellar and plasma membrane disintegration, though the nuclei were intact. Though TUNEL assay is an excellent marker for dying cells, it shows a positive reaction for both apoptotic and necrotic modes of cell death. This implies that TUNEL can signify DNA damage but not essentially the mode of cell death operational during any paradigm. The significance of TUNEL assay in the present study was to use it as a biochemical marker to monitor DNA strand breaks and to further strengthen our observation that PARP activation during CM pathology is an event associated with DNA damage. The condensed nuclear material evident from the TEM studies and TUNEL assay are suggestive of early stages of apoptosis that highlight the biochemical changes associated with chromatin which may eventually lead to DNA fragmentation.

The fragmentation of DNA could be associated with caspase like activity and a probable downstream event to the activation of Ca²⁺ and Mg²⁺ dependent endonucleases. To confirm this, in the present study we have clearly demonstrated expression of caspase-3 in the infected cerebral cortex and cerebellum, suggestive of an apoptotic mode of cell death. Our immunoblot analysis showing the upregulation of caspase-3 was well correlated with the biochemical caspase 3 activity assay and histochemical localization of caspase 3 in infected neural tissue as compared to control mouse brains. An alternative effect of the proinflammatory cytokines is the modulation on the production of eicosanoids (prostaglandins and leukotrienes), which are important in the maintenance of the structure and function of blood vessels.

The role of eicosanoids is well established during the pathology of cerebral malaria (Xiao *et al.*, 1999). A recent study by Ball *et al.* showed the upregulation of Cyclooxygenases (COX1 & COX2) in the brain upon infection of cerebral malaria (Ball *et al.*, 2004). The up-regulation in the expression of phospholipase A2, COX1, and COX2 enzymes is likely to be the result of increased production of proinflammatory cytokines such as TNF- α , IFN- γ , and IL-1b in malaria. It is well established that the production of these cytokines is increased in murine cerebral malaria (de Kossodo and Grau, 1993). The increase in eicosanoid enzyme activities may play a role in the pathogenesis of cerebral malaria. The increased expression of eicosanoid enzymes at day 15 of the infection coincided with the peak occurrence of cerebral malaria. This increased production of phospholipase A2 and release of 5-lipoxygenase upon immune activation by malaria infection can result in the increased synthesis of leukotrienes and other hydroxy acids. Because the latter cause leukocyte adhesion and activation, increased vascular permeability and production of proinflammatory cytokines (Gerritsen, 1996), it is likely that they will promote the pathogenesis of cerebral malaria. On the other hand, the increased release of arachidonic acid after immune activation can lead to the increased production of prostaglandins under the reaction of COX, which cause immunosuppression, reduced leukocyte adhesion, decreased cytokine excretion, and vasoprotection. The latter in turn can prevent much of the hyper immune responsiveness associated with cerebral malaria. The upregulation of COX in turn is dependent on Nuclear Transcription factors, the NF- κ B family upon the release of various cytokines in the vicinity during the pathology of cerebral malaria. Moreover, COX2 in particular is up regulated in the brain and as an inflammatory mediator is responsible for both apoptotic and necrotic modes of cell death. With this background in mind, we have checked for the expression levels of COX2, NF- κ B p50, p52 and p65 subunits. Our results clearly demonstrate that the levels of COX2 is increased in both cortex and cerebellum of infected mice compared to controls, and the nuclear translocation of various NF- κ B correlated well with the upregulation of COX2.

PARP is a nuclear enzyme that is activated both during necrosis and apoptosis. However, the pattern of cleavage is variable in both modes of cell death with characteristic signature fragments of 89kD in apoptosis, 43kD and 29kD in necrosis. We could detect the 64kD and 40kD bands along with characteristic signature fragment of 89kDa in the infected cerebral cortex and cerebellum (Fig. 9). This suggests that 64kD fragment of PARP cleavage might be associated with Granzyme B cleaved fragment and 40kD might be associated with calpain cleaved fragment. Cleavage of PARP might occur when the cell is no longer able to repair its DNA and could play a role in cellular disassembly that might ensure commitment to either apoptosis or necrosis. Previous studies from our lab have shown the formation of characteristic 89kD fragment in the infected samples. Based on our observation we conclude that pathways other than apoptosis are also activated in mice brain upon *PbA* infection.

In conclusion, we found widespread activation of Caspase-3 during murine CM. The alterations in the brain tissue were more pronounced in terminal stages of the disease and focused on neurons. We have also found increased expression of COX2, NF- κ B p50, p52 and p65 subunits and characteristic signature fragment cleavage of PARP implying necrotic mode of cell death. Apoptotic and necrotic phenotype of neurons, was confirmed by TEM analyses. Hence, apoptotic cell death

in the brain could be not only a potential reason for CM-related morbidity and mortality but also an important mechanism for the induction of the cerebral complication of *Plasmodium berghei* infection.

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Application of recombinant proteins in molecular detection of *Toxoplasma gondii*

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Abstract

The common serological tests employed in the diagnosis of *Toxoplasma gondii* toxoplasmosis include, dye test (DT), indirect haemagglutination test (IHA), complement fixation test (CFT), modified agglutination test (MAT), latex agglutination test (LAT) and direct agglutination test (DAT). However, the sensitivity of these tests is regarded to be low and they are also considered difficult to be used for screening of large number of sera as far as cost-benefit ratio is concerned. The paper deals with the application of recombinant proteins in molecular detection of *T. gondii*.

Keywords

Toxoplasma gondii; Serological tests; Molecular diagnosis; Recombinant proteins

Introduction

Toxoplasma gondii, the tissue cyst forming coccidium is one of the most polyxenous parasites known to date, which has a facultative heteroxenous life cycle and can infect probably all warm-blooded animals ranging from birds to mammals including human beings. From the zoonotic point of view, *T. gondii* is one of the most important protozoan parasites causing abortion or a congenital disease in the intermediate hosts. The heteroxenous life cycle of *T. gondii* was elucidated in the late 1930s when it was found that the faeces of cats might contain an infectious stage of *T. gondii*, which can induce infection when ingested by intermediate hosts (Hutchison, 1965). In 1970, knowledge of the coccidian life cycle of *T. gondii* was completed by the discovery of sexual stages in the small intestine of cats (Dubey and Beattie, 1988; Jackson and Hutchison, 1989; Dubey, 1993, 1998). It was further attributed that *T. gondii* is a tissue cyst forming coccidium with a heteroxenous life cycle in which an asexual phase of development in various tissues of herbivorous or omnivorous intermediate hosts is linked to a sexual phase of development in the intestine of carnivorous definitive host (Tenter *et al.*, 2000). Tachyzoites, bradyzoites and sporozoites are the three infectious stages of *T. gondii*. The oocysts are excreted

in faeces of the definitive host (Felidae) after 10 days of ingestion of cysts (bradyzoites), 18 days following ingestion of sporulated oocysts and 15 days after ingesting tachyzoites. Of these, the bradyzoites induced cycle is the most efficient because nearly all cats fed tissue cysts shed oocysts whereas, less than 30% of cats fed tachyzoites or oocysts shed oocysts. Unlike many other coccidian, oocysts of *T. gondii* are less infective and less pathogenic to definitive host (cats), as compared with intermediate hosts (mice, pigs, humans). The oocyst-induced infection is considered more severe in humans and animals than tissue cyst induced infection irrespective of the number of organisms affected and has a great epidemiological relevance (Dubey, 1998).

Prevalence

T. gondii is prevalent in many parts of the world. The reports on occurrence of toxoplasmosis vary from 42-92% in domesticated animals depending upon the cultural and food habits of different countries, the host species involved, strain of the organism involved, etc. Information on sero-epidemiology of toxoplasmosis is scanty in India. According to available literature, seroprevalence of *T. gondii* ranges from 7.9 to 80% in various species of domesticated animals studied with highest incidence in goats. *Toxoplasma* specific antibodies were detected in 23% of sheep and 12.5% of goats from Palampur (H.P.). In Kumaon region, 81.2% of Pashmina goats and 90% of the local goats were found positive (Dubey *et al.*, 1993). In meat-producing animals, tissue cysts of *T. gondii* were observed in tissues of infected pigs, sheep, goat most frequently, and less frequently in infected poultry, dog, and horse. Tissue cysts are found only rarely in beef or buffalo meat, although antibodies were detected in 92% of cattle and 20% of buffaloes studied serologically (Tenter *et al.*, 2000).

Human toxoplasmosis

Although infection with *T. gondii* in humans is very common, clinical disease is largely confined to few risk groups. Most cases of *T. gondii* infections in immunocompetent human are asymptomatic. The AIDS epidemic has rejuvenated research on toxoplasmosis. Up to 50% of AIDS patients die due to toxoplasmosis encephalitis (Luft and Remington, 1992). Encephalitis is the predominant clinical presentation of toxoplasmosis in immunosuppressed chronic infection. Severe manifestation, such as encephalitis, sepsis syndrome and shock, myocarditis, or hepatitis may occur, but are very rare in immunocompetent human (Ho-Yen, 1992). *Toxoplasma* is also an important cause of abortions and stillbirths after primary infection in pregnant women. The organism can cross the placenta and infect the fetus. The symptoms of congenital toxoplasmosis include abnormal changes in head size (hydrocephaly or microcephaly), intracranial calcifications, deafness, seizures, cerebral palsy, damage to the retina, and mental retardation. Some sequelae of congenital toxoplasmosis are not apparent at birth and may not become apparent until the second or third decade of life. Some cases of acute toxoplasmosis in adults are associated with psychiatric symptoms such as delusions and hallucinations (Torrey and Yolken, 2003).

Since the early 1950s, infection with *T. gondii* has also been recognized as an important cause of retinochoroiditis. Ocular toxoplasmosis may be the result of a prenatal infection or an infection that might have been acquired postnatally.

Incidence of disease varies from 0-100% between countries depending upon the animal host species involved. A recent study estimates that more than one third of the world human population is presumed to be harbouring the organism (Tenter *et al.*, 2000). In the USA 50% of adult population are reported to be seroreactive to the organism (Dubey and Beattie, 1998), whereas 20-40% of the population in Great Britain and 80-90% in France are reported to be sero-positive to *T. gondii* (Saava *et al.*, 1990). Congenital toxoplasmosis has been reported in every 1 to 8 live births out of 1000 reported in USA annually (Dubey, 1990).

In addition to blood and milk, tachyzoites have been detected in other body fluids including saliva, sputum, urine, tears and semen (Dubey and Beattie, 1988, Evans, 1992), but there is no evidence available as for horizontal transmission of *T. gondii* in human via any of these routes (Tenter *et al.*, 2000). Singh and Nautiyal (1993) reported 77% seroprevalence of toxoplasmosis in females and 37% in males from Kumaon region. An increasing trend in the incidence of toxoplasmosis particularly among women has been further reported (Sharma *et al.*, 1997). A nationwide survey for *Toxoplasma* specific antibodies in humans indicates an overall prevalence rate of ~20% among Indians. The status of toxoplasmosis among humans in India has been reviewed (Singh *et al.*, 1997; Singh and Pandit, 2004). A rSAG1-based ELISA revealed 25.2% CSF positive for presence of *Toxoplasma* specific antibodies among HIV-AIDS infected patients (Tewari *et al.*, unpublished data). The specificity and sensitivity of the result have been compared with other serological tests.

Genomic organization of *Toxoplasma gondii*

The genome of *T. gondii* consists of three DNA components, located in the nucleus, apicoplast and mitochondrion. The nuclear genome is haploid for most of the parasite's life cycle, except for a brief diploid phase in the cat intestine before meiosis (Pfefferkorn and Pfefferkorn, 1980). It consists of 11 chromosomes and the haploid DNA content is estimated to be 87 Mb. The 11 chromosomes are designated by Roman numerals Ib, Ia, II to X, and range in size from approximately 1.8 Mb to >10 Mb (Sibley and Boothroyd, 1992).

Diagnosis

Various laboratory techniques are used in the diagnosis of toxoplasmosis. The common serological tests employed for detecting *T. gondii* specific antibodies are the dye test (DT) (Sabin and Feldman, 1948), indirect haemagglutination test (IHA) (Jacob's and Lunde, 1957), complement fixation test (CFT), modified agglutination test (MAT) (Desmonts and Remington, 1980), latex agglutination test (LAT), and direct agglutination test (DAT) (Dubey and Desmonts, 1987; Devada *et al.*, 1998; Gamble *et al.*, 2005). The sensitivity of the tests described above is low and are difficult to use for screening of large number of sera as far as cost-benefit ratio is concerned. Although, dye test is reported to be highly specific and the sensitivity levels of the test correspond to IFAT, it is rarely used in large scale because of the potential risk factor associated with the use of live tachyzoites.

Application of recombinant SAG1 and GRA7 in serodiagnosis of toxoplasmosis

The recombinant proteins which are presumed to be the important candidates for diagnosis as well as subunit vaccine development against *T. gondii* are SAG1, SAG2, MIC3, ROP2, GRA1 and GRA7 (Tewari, 2007; Tewari and Rao, 2007). Surface antigen 1 (SAG1) of Chennai and Izatnagar isolates of *T. gondii* were cloned using pGEM-T cloning vector and subsequently custom sequenced for nucleotides. SAG1 and GRA7 nucleotide sequences (GenBank Accession Numbers DQ872517 and DQ872520) of Izatnagar isolate showed 98.8% and 97.5% homology and with RH strain, respectively. Similarly, deduced amino acid sequence analysis revealed 96.7% and 95.6% homology with reference RH strain. The sequence analysis revealed 99.9% homology between the isolates of *T. gondii*. SAG1 and GRA7 were expressed using pET- 32 series vector and BL 21(DE3) pLysS host cells. The 3D conformation of the rSAG1 generated using modular version 7 software, revealed 98% conformational similarity to RH strain. Recombinant proteins were purified to homogeneity using metal affinity chromatography. The purified SAG1 protein was subjected to refolding process using glutathione (oxidized and reduced) to assess its biological activity. The immunoreactivity of the recombinant protein was confirmed by Western blot analysis which revealed specific immunoreactivity at 47 kDa and 43 kDa region, respectively. Further studies on immunoreactivity of the expressed recombinant SAG1 and GRA7 was done by rGRA7 ELISA and cocktail ELISAs using hyperimmune sera against whole tachyzoites. The recombinant SAG1 and GRA7, expressed as thioredoxin fusion protein, were standardized for sensitive serodetection of toxoplasmosis in goats with a specificity of 88.4% and sensitivity of 83.3% and 80% respectively (Velmurugan *et al.*, 2008). A cocktail of the recombinant proteins showed greater diagnostic efficiency with 88.4% specificity and 86.6% sensitivity.

Conclusion

It may be concluded that the serological assays may be used as powerful tools for epidemiological studies on toxoplasmosis. However, the specificity and sensitivity of the serological assays are compromised when native proteins are used as diagnostic agent. This may be attributed to the presentation of a large number of epitopes with inherent molecular structural similarities spanning across the species barrier which can be negotiated by the use of stage-specific immunodominant antigens. The epidemiological data thus generated will be invaluable in timely intervention for the control of infection.

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Animal trypanosomosis: *Trypanosoma evansi* - An emerging threat to human

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Abstract

T. evansi is a heterogenous haemoprotozoan parasite causing 'surra' in different animals like cattle, buffalo, horse, camel, swine, deer etc. This parasite is widely prevalent in India, resulting in considerable losses in productivity in livestock including abortion and death. The parasite is transmitted by biting flies such as *Tabanus*, *Stomoxys*, *Haematopota*, *Lyporsia*, *Hippobosca*, soft tick and other blood sucking arthropods. Previous reports from India and Malaysia have identified trypanosomes similar to *T. lewisi* in peripheral blood of three patients with short febrile episodes. In all the three patients, infection was only transient and the patients recovered without treatment. Very recently, *T. evansi* infection from human beings has also been reported from Maharashtra, India, which is very much significant in respect of change in the character of host adaptability of the parasite and its future zoonotic significance. This parasite was confirmed using a range of parasitological, immunological, and molecular diagnostic tests. Hence, it is a challenge for practitioners to combat the newly emerging zoonotic haemoprotozoan infection, which requires further exploration and confirmation.

Keywords

Protozoa; *Trypanosoma evansi*; Zoonosis; Newly Emerging Diseases

Introduction

Trypanosoma belongs to the Class Kinetoplastida, a monophyletic group of unicellular parasitic protozoa (Hamilton, 2004). These parasites infect a variety of hosts and cause morbidity and mortality in animals and human beings. Human trypanosomiasis is endemic in Africa and South America. In Africa, the disease is known as human African trypanosomiasis (HAT) or sleeping sickness, is caused by *Trypanosoma brucei gambiense* (chronic form) or *T. b. rhodesiense* (acute form), whereas the American trypanosomiasis, known as Chagas' disease, is caused by *T. cruzi*. In addition to human infectious trypanosomes, a variety of other *Trypanosome*

species cause animal trypanosomiasis with a wide geographic distribution. Nagana is caused by *T. b. brucei* in Africa and affects cattle; *T. congolense* and *T. vivax* infect domestic and small animals; and surra is caused by *T. (Trypanozoon) evansi* and infects mainly camels, cattle, and buffalos and other wild animals on all continents (Hoare, 1972). *T. b. brucei* and *T. congolense* are very much similar to *T. b. gambiense* and *T. b. rhodesiense* and transmitted by tsetse fly bites, while *T. evansi* is spread by mechanical transmission of infected blood through hematophagous insects such as tabanid flies. *T. evansi* is closely related to other *Trypanozoon* species, including *T. b. gambiense* and *T. b. rhodesiense*, at the genetic level. However, there is no cyclical transformation of *T. evansi* within the flies and this parasite remains monomorphic throughout its life cycle, while *T. brucei* subspecies are pleomorphic, presenting a range of forms at different points in the life cycle.

Human infection by animal species of *Trypanosoma* is usually uncommon because of a trypanolytic factor in human serum. However, it has been demonstrated that *T. congolense* and *T. evansi* are capable of resisting human plasma in certain circumstances (Hawking, 1978) and at least one report describes a patient in Côte d'Ivoire harboring both *T. brucei* and *T. congolense* with associated clinical signs (Truc *et al.*, 1998). The degree to which trypanosome species that are not usually associated with human infection might actually infect humans is difficult to ascertain since such infections may be short-lived and pass undiagnosed. For example, in two sporadic cases of *T. lewisi* (a common parasite of rats) infections in man in India and Malaysia the three infected patients recovered without treatment (Shrivastava and Shrivastava, 1974; Johnson, 1933).

Griffith Evans (1880), a Veterinary Officer serving in India, was the first to discover *T. evansi*, in the blood of equines and camels in Dera Ismail Khan District of Punjab (presently in Pakistan). *T. evansi* cause 'surra' in animals like cattle, buffalo, horse, camel, swine, deer etc having 30% morbidity and 1-3% mortality. This parasite is widely prevalent in India resulting in considerable losses in productivity in livestock including abortion and death. Other species encountered from India include *T. theileri*, which affects mainly bovines is usually non-pathogenic and may become pathogenic under stress conditions. Reports are also there regarding sporadic occurrence of *T. equiperdum*, which causes dourine in equines. The parasite infects various host species and is transmitted by biting flies such as *Tabanus* and *Stomoxys* species, as well as by vampire bats including *Desmodus rotundus* (Hoare, 1972). Camels and horses are very susceptible to the infection and death can occur within weeks or few months. *T. evansi* causes heavy economic losses to the livestock owners as it leads to progressive emaciation anemia, edema, pyrexia, reduced weight gains, lowered milk and meat yields (Singh, 1985; 1992). The office International des Epizooties mentions the *Surra* disease under list B diseases of significance (OIE, 2004). *Surra* is endemic in China, the Indian subcontinent, Southeast Asia, Northern Africa, the Middle East, South America, the Philippines, Bulgaria, parts of the former USSR and parts of Indonesia. In India the disease is prevalent in the states of Haryana, Punjab, Uttar Pradesh and Jammu and Kashmir in Northern India; Rajasthan, Gujarat and Maharashtra towards west; Andhra Pradesh, Karnataka, Tamil Nadu towards south and Bihar and West Bengal in eastern India.

Recently *T. evansi* infection from human beings has been reported from Maharashtra, India (Joshi *et al.*, 2005, 2006; Powar *et al.*, 2006), which is very much significant in respect of changes in the character of host adaptability of the parasite and its future zoonotic significance. After discovery of the first recorded case of human infection with *T. evansi*, serological screening of 1806 persons from the village of origin of the patient in India was performed by Shegokar *et al.* (2006), using the card agglutination test for trypanosomiasis and *T. evansi*. A total of 410 (22.7%) people were found positive by whole blood, but only 81 were confirmed positive by serum. However, no trypanosomes were detected in the blood of 60 people who were positive at a high serum dilution. The results probably indicate frequent exposure of the human population to *T. evansi* in the study area, which suggests frequent vector transmission of parasites to humans. Although *T. evansi* is not infective for humans, a follow-up of seropositive persons is required to observe the evaluation of human infection with this parasite.

Diagnosis of *T. evansi* infection usually starts with clinical symptoms or the detection of antibodies to *T. evansi*. Conclusive evidence of *T. evansi* infection, however, relies on detection of the parasite in the blood or tissue fluids of infected animals and human. Unfortunately, parasitological techniques cannot always detect ongoing infections as the level of parasitemia is often low and fluctuating, particularly during the chronic stage of the disease (Nantulya, 1990). Molecular detection of *T. evansi* using the polymerase chain reaction is a highly sensitive and specific alternative to parasitological tests.

Conclusion

Previous reports from India and from Malaysia have identified trypanosomes similar to *T. lewisi* in peripheral blood of three patients with short febrile episodes (Shrivastava and Shrivastava, 1974; Johnson, 1933). In all three patients, infection was only transient and the patients recovered without treatment. The cases of *T. evansi* reported from Maharashtra, India are extraordinary in that these are normally non-human infectious parasites survived and proliferated for at least five months in a human. It is now a priority to determine whether other people in the area and beyond harbor *T. evansi* infections and to investigate as to how this parasite, hitherto believed to be exclusively a pathogen of animals, is transmitted to man. The reported human cases of *T. evansi*, is an event of far reaching implications and puts *T. evansi* on a new and different perspective as a emerging zoonotic parasite of animal trypanosomosis.

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Development of vaccines against parasites: Current status and future directions

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Abstract

Parasitic diseases cause huge economic losses to the livestock industry. Increasing reports of anthelmintic and acaricide resistance, sky rocketing costs of developing new drugs and fears of drug residues in food chain and environment have compelled to look for alternative approaches to controlling parasitic diseases in animals viz. biological control, pasture management, exploitation of host genetic resistance and vaccines. At present, very few anti-parasite vaccines are commercially available. Several approaches can be adopted to develop vaccines against parasites like attenuation (irradiation, tissue culture, precociousness), sub-unit vaccines, recombinant vaccines, DNA based vaccines, use of synthetic antisense oligonucleotides and inhibition of parasite induced apoptosis. But there are innumerable constraints in the development of vaccines, like the ability of the parasite to deflect the host immune response, polymorphism of surface proteins of parasites, their host-specificity and several developmental stages in their life-cycle. Still vaccination has been successful against *Boophilus microplus* (TickGARD), *Theileria annulata* (Rakshavac-T), *Toxoplasma gondii* (Toxovax), poultry coccidiosis (Immucox, Livacox, Coccivac and Paracox), lungworms (Dictol, Difil, and Huskvac), *Taenia ovis* (45W) and *Echinococcus* (EG95). ‘Genomics’ and ‘Proteomics’ can be utilized in identifying novel targets of parasites *in silico* using available genome sequences in data banks as can be the gene knockout RNA interference (RNAi) technique.

Keywords

Parasite; Vaccine; Attenuation; Recombinant technology; Immunodominant antigen

Introduction

Parasitic diseases cause huge economic losses to the livestock industry. Several broad spectrum anthelmintics and acaricides are available to combat parasite menace in animals. Despite the fact that these drugs are safe and cheap, the search for alternative methods to control parasites is gaining ground in the recent past, owing to increasing reports of anthelmintic resistance (Yadav and Garg, 2007) and acaricide resistance (Barre *et al.*, 2008). Besides, the cost of finding and developing new drugs

is high and the issue of drug residues in food chain and environment is being addressed. Among the alternative approaches of controlling parasites, like biological control (Khan *et al.*, 2004), pasture management and exploitation of genetic host resistance, use of vaccines would also play a key role.

At present, very few vaccines are commercially available against parasites. However, in a surge to develop sustainable parasite control and with the availability of new biotechnological tools, more vaccines will be added to the existing ones. These vaccines are safe, eco-friendly, do not leave chemical residues in food chain (consumer friendly) and several animal owners prefer prophylaxis with vaccines to treatment with chemicals. However, in reality the picture is not as rosy as the vaccines will be able to match the efficacy of anti-parasitic drugs remains to be seen or could they prevent clinical pathology in animals or reduce the number of parasites on pasture when used alone or in conjunction with biological control, grazing management (Frish, 1999; Pruett, 1999). In either case, the use of vaccines should yield monetary gains to the animal owner, if they are to become a reality (Dalton and Mulcahy, 2001).

Approaches to develop vaccines against parasites

1. Classical Approach:

(i). Killed whole parasite vaccines: Like the tick vaccine that started off with whole tick homogenate.

(ii). Attenuated parasite vaccines: The principle involved in such vaccines is that the attenuated organism follows the same migration route but fails to attain maturity. Attenuation of parasite /stages can be attained by irradiation of infective larvae (L₃) (*Dictyocaulus*, *Ancylostoma*), schistosome cercariae or sporozoite of *Plasmodium*; serial passage in tissues cultures (*Theileria* vaccine), or early passage (precocious strain of *Eimeria*). Killed/attenuated vaccines offer multiple and diverse antigens to the host and are thus better capable of protecting the host against parasite variants. However, the disadvantage is that the parasite material is to be procured in large quantities (the parasites do not divide *in vitro*) and there are chances of reversion to virulence.

2. Sub-unit vaccines: Like the peptide vaccines that consist of short synthetic sequences coinciding with the immunodominant epitopes.

3. Recombinant vaccines: They are stable, safe and cheap. The target (protective) antigen can be procured in bulk quantities and excludes the maintenance of host animals and isolation of protective antigens. TickGARD is one such commercially available recombinant vaccine against *Boophilus microplus* or EG95 and 45W against *Echinococcus* and *Taenia ovis* respectively. However, polymorphism in parasite may render the recombinant antigen ineffective against some strains.

4. DNA based vaccines (Nucleic acid vaccines): They consist of a parasite gene of interest (i.e. parasite gene coding for immunodominant protein with promoter) cloned into a plasmid. The vaccine is administered by injection (genes in muscles are taken up by antigen presenting cells-APCs) or by a gene gun which contains antigen

coated gold particles (directly targets the vaccine into APCs). Such vaccines are safe; the encoded protein is produced in its natural form and both humoral and CMI responses are produced. The expression of antigen is prolonged generating sufficient cells (so multiple vaccinations are avoided). Besides, the cost of DNA vaccines is lower as compared to recombinant vaccines. The disadvantage of nucleic acid vaccines is that they are designed against protein antigens and not against diseases caused by polysaccharide antigens. Also there are fears that plasmid DNA may integrate into host chromosome. Anti-DNA antibodies might be produced resulting in autoimmune disorders. However, this approach against parasites is still in its infancy due to lack of complete knowledge regarding mechanisms of their action.

5. Edible vaccines: These are based on transgenic plants expressing the protective parasite antigens. These can be especially used in preventing gastrointestinal (GI) parasitic infections.

Among the other approaches, use of antisense oligonucleotides and inhibition of parasite induced apoptosis are gaining momentum. Antisense oligonucleotides inhibit mRNA function and hence gene expression sequence. Scientists are working towards developing synthetic antisense oligonucleotides that inhibit sequential expression of genes responsible for change in variant surface glycoproteins in *Trypanosoma*. As a result the parasite will lose its ability to change its surface coat antigens and thus will fail to evade immune response like the recombinant antigen against cyclophilin.

Some parasites employ strategies like inducing apoptosis of host T cells to suppress host immunity. The pro-apoptotic antigens produced by parasites can be identified and neutralized by antibodies directed against them.

Current status of parasitic vaccines

The first commercially available anti-parasitic vaccine was developed by Jarret in 1960 against bovine lungworm *Dictyocaulus viviparus* and was based on irradiated third stage larvae (Dictol, Difil, and Huskvac). The larvae are produced by *in-vivo* passage in donor calves.

A similar vaccine was developed against canine hook worm *Ancylostoma caninum* (Steves *et al.*, 1973). But it had to be withdrawn as it failed to meet commercial expectations. Also the vaccine had a short self life, resulted in respiratory side effects and failed to impart sterile immunity.

Recombinant vaccines namely 45W and EG95 have been developed against *Taenia ovis* and *Echinococcus*, respectively i.e. cysticercosis and hydatidosis. The antigen is present on the surface of the oncosphere which when adheres to the gut wall; vaccination produces antibodies directed against them and interferes with successful attachment as the cyst is destroyed by antibody-complement mediated lysis (Lightowlers *et al.*, 2000). Vaccination can be used to prevent infections in man indirectly (vaccinating intermediate host) or directly (vaccinating man).

Cathepsin L₁ and cathepsin L₂ proteases molecules secreted by *Fasciola* sp. are being extensively explored to offer protective immunity in cattle and sheep. A

protection level of 60% and 71-81% reduction in faecal egg counts in sheep (Piacenza *et al.*, 1999) and 42-69% reduction in fluke burdens and 60% reduction in viability of eggs released by parasite in cattle has been observed following vaccination with these two molecules. The surviving flukes in cattle exhibited stunted growth and also produced less severe liver damage (Dalton *et al.*, 1996). The vaccine exerted anti-embryonation effect on the fluke eggs (98% eggs recovered from bile ducts following vaccination did not embryonate) thus affecting transmission of fasciolosis (Dalton and Mulcahy, 2001). Recombinant glutathione – S- transferase (γ GST) induces a protection level of 19-69% against *Fasciola* in cattle and 57% in sheep (Spithill, 1999). Another molecule Fatty Acid Binding Protein (FABP) offered 55% protection in cattle following *Fasciola* infection. Leucyl aminopeptidases (LAP) from parasites are gaining attention as potential candidates for vaccine development. Acosta *et al.* (2008) conducted vaccination trials using γ FhLAP (family of LAP) for rabbit immunization and observed a strong IgG response and significant protection following challenge with *F. hepatica* metacercariae. Marcilla *et al.* (2008) have identified LAP and phosphoenolpyruvate carboxykinase as immunodominant antigens using proteomic approach and immunoblotting techniques from *Fasciola* infected patients.

In the recent past, the search for vaccines against GI nematodes including *Haemonchus*, *Oesophagostomum* and *Trichostrongylus* has hastened. The concealed antigen H₁₁ (a 110 KDa integral membrane protein having aminopeptidase A and M activity) has been employed to induce protection against haemonchosis. H₁₁ is expressed in nematode microvilli and enables blood meal digestion. Following vaccination, antibodies are produced that bind to this surface molecules and disrupt the feeding activity of fourth stage larvae and older worms. Vaccination of lambs offers more than 90% protection against the wireworm (Andrews *et al.*, 1997). Egg production reduced by 98% in vaccinated pregnant (third trimester) ewes when challenged with *H. contortus*. Although the antigen H₁₁ is not expressed under natural conditions, vaccination of lambs and ewes may certainly serve to block the transmission of infection to the pasture. Another gut associated antigen H-gal-GP has been shown to reduce worm load by 72% and EPG by 93% (Knox and Smith, 2001). Prasad *et al.* (2007) identified two immunodominant polypeptides (60 and 120 kDa) in somatic antigen of adult *H. contortus* by western blotting and immunoprecipitation which can be utilized for immunodiagnosis or immunoprophylactic purposes in the wireworm infections.

Most of the vaccines against protozoa are live vaccines. But the parasite strain used have been selected for truncated life-cycles e.g. ‘Toxovax’ against *Toxoplasma gondii* (S48 strain that does not produce tissue cyst) or complete but shortened life-cycles (i.e. precociousness e.g. Paracox against *Eimeria*) or repeated passage of the parasite through splenectomized calves (e.g. *Babesia bovis* and *B. bigemina* strain) or *in vitro* cultures (‘Rakshavac-T’ against *Theileria annulata*). Anti-*Eimeria* vaccines can be live e.g. ‘Coccivac’ and ‘Immucox’ or live attenuated e.g. Livacox and Paracox. In the former, attenuation has been attained by adapting the parasite to grow in developing chicken embryo (*E. acervulina* and *E. maxima*) and by precociousness in the latter. Recombinant anti-coccidial vaccines are in the process of development. A recombinant sporozoite refractile body protein has been observed to provide partial protection and significant reduction in intestinal lesions against *E. tenella*, *E. acervulina*, *E. maxima* and *E. necatrix* challenge (Crane *et al.*,

1991). Maternal immunization with gametocyte antigens of 230, 82 and 56 kDa (transmission blocking immunity) to chicks via antibodies in eggs resulted in 60-80% reduction in oocyst output when chicks were challenged with *E. maxima* (Wallach *et al.*, 1995). Padmavathi and Kumar (2003) tested the safety and potency of Immucox (containing *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella*) in broiler chicken against experimental coccidiosis and found it to be effective and safe. The vaccinated birds showed more average weight gain and no prophylactic anticoccidial medication was required.

‘Toxovax’ protects ewes intended for breeding and checks embryonic death and abortion. It is contraindicated in pregnant animals and those less than 6 weeks of age. Vaccinated animals should not be slaughtered until 42 days post-vaccination (Buxton, 1993). Nasal delivery of DNA to protect against toxoplasmosis (Velge-Roussel *et al.*, 2000) has been tried in experimental studies.

Rakshavac-T- a schizont vaccine against *T. annulata* (ODE strain at 150 passages) is used at the dose rate of 5×10^6 schizont infected lymphoblasts. It is administered in calves above 2 months of age, is safe in pregnant animals and offers protection for a period of 36 months. The c-DNA approach utilizing merozoite surface antigens of *T. annulata* (Tams1) (Parbhani strain) is underway. The genes coding the antigens have been cloned (Rajendran and Ray, 2007).

Initially vaccination against babesiosis was done by preparing vaccine donors i.e. infecting splenectomized calves using avirulent strains of *B. bovis* and *B. bigemina*. The method is recommended by FAO in areas where babesiosis is enzootic. But there are risks of transmitting other infections from the donor besides the problems of severe reactions and production of anti-erythrocytic antibodies.

Vaccination protocols consisting of culture derived soluble exoantigens released by *Babesia* in blood of animals or into supernatant medium of *in vitro* cultures are being developed. Based on these lines, a vaccine named ‘Pirodog’ is being marketed in France for *B. canis*. It contains concentrated antigens adjuvanted with saponin and its use has shown encouraging results in controlling clinical disease in dogs (Moreau, 1987).

‘Neogard’ vaccine is based on killed tachyzoites of *Neospora caninum* and is being used to reduce abortions in cattle. ‘Giardia Vax’ against giardiasis in dogs and cats is based on extracts of axenically cultured *G. duodenalis* trophozoites. The vaccinated animals have showed reduced period of cyst shedding as well as oocyst output (Olson *et al.*, 2000). ‘Anaplaz’ is a live attenuated vaccine against *Anaplasma marginale* and the antigen employed is Major Surface Protein (MSP) complex. Cryptosporidiosis is an upcoming protozoan infection in young ones. Various delivery systems like nasal delivery of DNA or via colostrum (by vaccinating dams) are being explored for the purpose of inducing immunity (De Graf *et al.*, 1999).

Very recently Leishmune® vaccine has been registered in Brazil against canine visceral leishmaniasis. The vaccine consists of a purified fraction (fucose mannose ligand-FML) isolated from *Leishmania donovani* (Dantes-Torres, 2006). Additionally the vaccine can be used as a transmission blocking vaccine thus enabling control of zoonotic visceral leishmaniasis.

The first commercially available recombinant vaccine against parasites is TickGARD against *B. microplus* tick. It is based on concealed antigen Bm86 expressed in *E.coli*. Vaccination induces production of antibodies directed against Bm86 molecule present on the midgut cells of the parasite. The antibodies bind to this molecule and interfere with blood feeding activity of the one-host tick (Willadsen, 1995). Although vaccination produces a tick control of just 20-30%, the reproductive capacity of the female tick is drastically hampered. A similar recombinant vaccine Gavac expressed in yeast *Pichia pastoris* has been developed and commercialized in Cuba (Garcia-Garcia *et al.*, 2000). A second generation *B. microplus* vaccine namely TickGARD Plus has been developed that contains an additional Bm95 antigen.

Recently, Peconick *et al.* (2008) have demonstrated that the epitope sequences of synthetic vaccine SBm 7462 [based on immunogenic epitopes 4822, 4823 and 4824 contained within protein Bm86 derived from the Australian Yeerongpolly strain of *Rhipicephalus (Boophilus) microplus*]] are conserved in South American populations of this tick. This conservation plays a key role in generating similar immunological response of different populations of the tick in cattle.

A recombinant vaccine directed against hypodermin A of *Hypoderma lineatum* has been developed. Besides, vaccines are being developed for *Haematobia irritans*, *Lucilia cuprina*, *Pediculus humanus* (Pruett, 1999).

Constraints in development of vaccines against parasites

- Parasites employ strategies to misguide the host immune response viz. coating with host antigens (so are not recognized as foreign), downregulate cellular response by producing cytokine mimics, intracellular localization or continually producing novel surface proteins.
- Parasites may exhibit protein polymorphism of surface proteins. As a result antigens associated with protective immunity are not essentially conserved among species or among isolates of the same species (Maizel and Kurniawan-Atmadja, 2002). This parasite genetic diversity further compounds the development of effective vaccine.
- Parasites are highly host specific. Most of the time they are reared in rodent models whose immune response pathways may vary as compared to the natural host.
- A parasite is made up of numerous complex antigens which are difficult to identify and characterize. The parasite has several developmental stages and undergoes several life-cycle changes inside the host presenting to it a different subset of antigens. Thus, rendering the task of identifying the protective molecule even more challenging (Sonstegard and Gasbarre, 2001; Knox and Redmon, 2007).

It is well said that *prevention is better than cure* and vaccines are one such tool used for prophylaxis. Rapid identification of protective parasite antigens can speed up the development of parasite vaccines. Traditionally parasite antigens are fractionated and antigens identified by Western Blotting. But most of them are immunogenic and very few protective and that too are not exposed in natural infections. To overcome this predicament, 'Genomics' and 'Proteomics' can be utilized in identifying such novel targets *in silico* using available genome sequences in the data banks i.e. without isolating equivalent native proteins first. Likewise,

gene knockouts RNA interference (RNAi) techniques can be employed to identify target molecules for vaccine production (Knox *et al.*, 2007). These approaches though attractive as compared to the traditional ones are still far away from being translated into a commercial reality. The delivery of the final recombinant molecule is also critical in inducing protective immunity (Vercrysse *et al.*, 2004) as is the choice of adjuvants. That day is not far off when vaccines will offer an alternative to chemotherapy of parasites.

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Observation on one new species of the genus *Myxobolus* (Myxozoa: Myxosporea: Bivalvulida) and redescription of *Myxobolus magauddi* (Bajpai, 1981) Landsberg and Lom, 1991 recorded from freshwater fishes of Kanjali Wetland of Punjab (India)

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Abstract

The present communication describes one new species of *Myxobolus* (Myxozoa: Myxosporea: Bivalvulida), *M. naini* sp. n. from gills of *Cirrhina mrigala* (Ham.) with redescription of *M. magauddi* from gills of *Catla catla* (Ham.), freshwater fishes of Kanjali Wetland of Punjab (India), respectively.

Keywords

India; Kanjali; *Myxobolus*; Myxozoa; *Myxobolus naini*; Freshwater fish

Introduction

The genus *Myxobolus* was established by Butschli in the year 1882. Since then 744 species of *Myxobolus* have been recorded from freshwater and marine fishes all over the world (Landsberg and Lom, 1991). During our study of myxozoan parasites of freshwater fishes of Kanjali Wetland of Punjab, a new species of this genus was detected in the gill lamellae of *Cirrhina mrigala* (Ham.). The present communication deals with description of the species in accordance with the guidelines of Lom and Arthur (1989).

Materials and methods

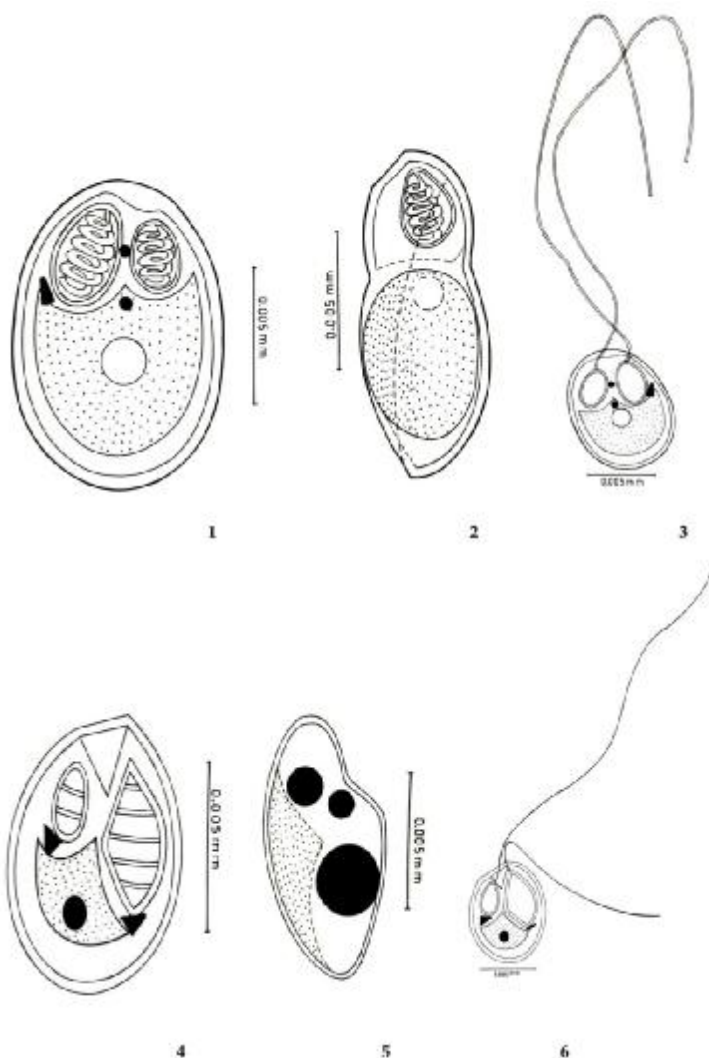
Fishes collected from Kanjali Wetland were brought to the laboratory and examined for the presence of plasmodia. 20 fishes belonging to *Cirrhina mrigala* and 15 fishes of *Catla catla* were examined. Fresh smears were treated with 8% KOH solution for the extrusion of polar filaments. For permanent preparations, air-dried smears were stained with Ziehl-Neelsen and Iron-haematoxylin. Drawings were made from stained material with the help of camera lucida. Measurement of spores was taken with the aid of a calibrated oculomicrometer. All measurements are presented in μm as mean values.

Results

Myxobolus naini sp. n. (Figs. 1-3, 7-8)

Plasmodia

Small, microscopic, present in mucous membrane of gill lamellae, 4-6 spores present per plasmodium.

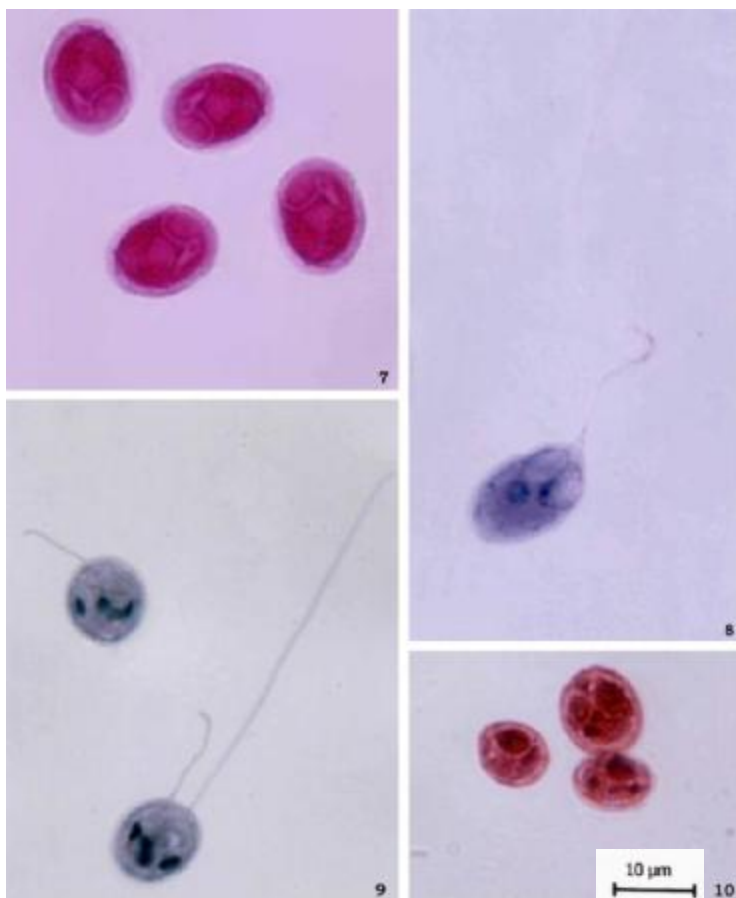


Figs. 1-3. Camera lucida drawings of the spore of *M. naini* sp.n. 1. Mature spore stained in Ziehl-Neelsen (valvular view); 2. Spore in sutural view; 3. Spore with extruded polar filaments.

Figs. 4-6. Spores of *M. magauddi* 4. Valvular view of spore stained in Ziehl-Neelsen 5. Spores in sutural view; 6. Spores with extruded polar filaments. Scale bar = 0.005 mm.

Spores (measurements based on 15 spores in frontal view)

Histozoic, oblong to oval in shape with anterior end and posterior ends bluntly rounded and measuring $12.9 \times 8.2 \mu\text{m}$ in size. Two shell valves smooth, symmetrically thick and measuring $0.67 \mu\text{m}$ in thickness (Fig. 7). Sutural line indistinct, sutural ridge moderately protruding. Two anteriorly situated polar capsules, more spherical in shape only slightly unequal in size converging towards anterior end of spore. Larger polar capsule $4.9 \times 3.1 \mu\text{m}$ in size, smaller one $3.33 \times 1.63 \mu\text{m}$ (Fig. 1). Both polar capsules occupy more than $1/4^{\text{th}}$ part of spore body. Polar filaments make 6-7 coils in larger and 4-5 coils in smaller polar capsule. Polar filaments slightly unequal when extruded, larger one $58.3 \mu\text{m}$, smaller one $33.3 \mu\text{m}$ in length. A small intercapsular appendix present. Sporoplasm agranular, homogenous occupies whole of extra capsular space behind polar capsules (Fig. 8). Iodinophilous vacuole present, $2.8 \mu\text{m}$ in diameter. One sporoplasmic nucleus, $0.33 \mu\text{m}$ in diameter, also present.



Figs.7-8. Photomicrographs of spore of *Myxobolus naini* stained with Ziehl-Neelsen (unextruded) and Iron-haematoxylin (extruded polar filaments).

Figs. 9-10. Photomicrographs of spores of *Myxobolus magauddi* stained with Ziehl-Neelsen (unextruded) and Iron-haematoxylin (extruded polar filaments).

Discussion

The shape of the spore of the present species resembles those of *M. diversus* (Nie and Li, 1973) Molnar and Szekely, 2003; *M. tilapiae* Abolarin, 1974, *M. polycentropsi* Fomena *et al.* 1985 (syn. *M. microcapsularis* Sakiti *et al.*, 1991); *M. heterosporus* Baker, 1963 and *M. synodonti* Fomena *et al.* 1985 in having anterior and posterior ends that are both bluntly rounded. The new species differs from *M. diversus*, which has two slightly unequal polar capsules, whereas in the former the spores possess two very unequal polar capsules, smaller being fairly rudimentary. The present species possesses more spherical polar capsules as compared to the more pyriform ones in *M. heterosporus* and *M. polycentropsi*. The shape of the polar capsules is similar to those of *M. tilapiae*, which are almost spherical. Spores of the present species differ from *M. tilapiae*, *M. polycentropsi* and *M. nokoueensis* (Sakiti *et al.*, 1991) in having the length of the polar capsules more than 1/4th of the length of spore. However, in this respect they are close to *M. distichodi* Kostoingue and Toguebaye, 1994 and *M. stenosis* Paperna, 1973 in having the length of polar capsule less than half of the spore length but differ in the shape of the spores, which are more tapered anteriorly in *M. distichodi* and pyriform in *M. stenosis*.

In view of these differences, the myxozoan under the present study should be considered a new species. Hence, it is named as *Myxobolus naini* sp.n. after the vernacular name of the fish, i.e. 'nain'.

Taxonomic Summary

Host: *Cirrhitina mrigala*

Site of infection: Gills

Locality: Kanjali Wetland, Punjab, India

Type specimen: Paratypes are spores stained in Ziehl-Neelsen and Iron-haematoxylin on slide no. CM/K /ZN/ 10/29.06.08 and CM/K /IH/ 11/29.06.08

Prevalence of infection: 02/20 (10.0%)

Myxobolus magauddi (Bajpai, 1981) Landsberg and Lom, 1991 (Figs. 4-6, 9, 10)

Plasmodia

Small, microscopic, occur in mucous membrane around gill lamellae. 10-15 spores present per plasmodium.

Spores (measurements based on 20 spores in frontal view)

Spores sub-spherical in outline, 10.0 x 8.7 µm in size, 5.0 µm in thickness (Fig. 9), boat shaped in lateral view. Sutural line indistinct. Shell valve relatively thin, symmetrical, smooth, without any parietal folds. Anterior end somewhat flat between openings of two polar capsules. Two polar capsules highly unequal, larger 3.83 x 3.20 µm smaller, 2.46 x 1.93 µm in size, slightly tapering toward anterior end of spore. Polar filaments situated perpendicular to longitudinal axis of polar capsules, making 4-5 coils in larger polar capsules, 2-3 in smaller. A very large intercapsular appendix present at anterior end. Two capsulogenic nuclei, triangular in shape present beneath each polar capsule. Sporoplasm composed of homogenous material, contains one sporoplasmic nucleus.

Remarks

Present observation on *Myxobolus magauddi* (Bajpai, 1981) Landsberg and Lom, 1991 are in conformity with the original description except for some minor variations in size of the spore and length of polar filament when extruded. Earlier, the parasite was recorded from the gills of *Trichogaster fasciatus* in West Bengal (India). A new locality, Kanjali Wetland and a new host *Catla catla* are recorded for this parasite in the present study.

Taxonomic Summary

Host: *Catla catla*

Site of infection: Gills

Locality: Kanjali Wetland, Punjab, India

Percentage of infection: 10/15 (66.6%)

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Studies on infection and cross-infection of microsporidian spores of mulberry, tasar, eri and muga silkworm on economic parameters of silk to mulberry silkworm, *Bombyx mori* L.

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Abstract

Pebrine is a deadly disease of mulberry silkworm, *Bombyx mori* L., caused by the pathogen *Nosema bombycis* (Microsporidia: Nosematidae). Economic parameters of silk viz., cocoon weight, shell weight, length and circumference of cocoon, shell percentage and filament length of cocoon during microsporidian infection and cross-infection with *Nosema bombycis* and *N. mylitta* in mulberry silkworm, *Bombyx mori* L. in three different seasons are studied. Generally all the economic characters of silks are deteriorated in infected and cross-infected cocoons over healthy control. The study indicates that economic parameters of silk are directly related to the microsporidian infection and cross-infection in mulberry silkworm.

Keywords

Bombyx mori; Cross-infection; Economic parameters; Microsporidian; *Nosema bombycis*; Silk Ratio percentage

Introduction

The natural silk is produced in very few countries of the world. China and India are the major producers of silk among all the silk producing countries. While China contributes nearly 85% (130000 MT) of the total silk production (153942 MT) in the world, India produces approximately 13% of silk. As per a recent report, India produced 16525 MT of mulberry silk in the year 2006-07 (Anonymous, 2008). The production of silk becomes comparatively low due to various reasons, of which disease is noteworthy. Pebrine is the deadly disease in mulberry silkworm, *Bombyx mori* caused by the pathogen, *Nosema bombycis* (Microsporidia: Nosematidae). Several studies report that the deterioration of quality of cocoon and silk occurs due to pathogens (Jamenson, 1922; Ghosh, 1944; Steinhaus, 1949; Nomani *et al.*, 1971; Chandra *et al.*, 1995). But no detailed information is available on the influence of different concentrations of pathogens on economic parameters of silk in different seasons. The present study is an attempt to observe the influence of infection and

cross-infection of different inoculum concentrations of microsporidian pathogens on economic parameters of silk viz., cocoon weight, shell weight, shell percentage, length and circumference of mulberry cocoons and filament length in three different seasons of rearing.

Materials and methods

Collection of mulberry silkworm eggs and preparation of host

Five disease free layings of *Bombyx mori* L. (Race-Nistari, Multivoltine) were collected from Central Sericultural Research and Training Institute, Berhampore, West Bengal, India and brushed on the following day of collection. In all these cases 98% hatching and 367 average fecundity (number of eggs per laying) were recorded. Larvae of *B. mori* L. were reared on a diet of fresh mulberry leaves (*Morus indica*, Variety S1). Later, the larvae were allowed to grow till 4th moult. After resuming from 4th moult, 5th instar at '0' h larvae were considered for experiment. A batch of selected larvae in three replications was reared as healthy control.

Inoculation of microsporidia of mulberry and tasar to mulberry silkworm

Multivoltine mulberry silkworm, *Bombyx mori* L. (Race- Nistari) were reared indoors under laboratory conditions on a diet of fresh mulberry leaves at 25-28°C and 65-72% relative humidity (RH) and 12 L +12 D photoperiodic condition. Larvae were fed on fresh mulberry leaves smeared with *Nosema bombycis* and *N. mylitta* of mulberry and tasar silkworm. Briefly, the procedure involves dipping a leaf dish (28.27 cm²) in 200 µl of spore suspension, drying and then allowing the larvae to feed on the diseased leaves for 6 h. Ten leaf dishes for a batch of 60 larvae were fed to the silkworm. The mulberry leaves smeared with distilled water, were fed to the mulberry silkworms of healthy control group.

Recording of economic characters of silk

After the cocoon formation, the healthy cocoon weight, the infected cocoon weight and the shell weight (cocoons without the pupa) were taken in a digital balance to assess the effect of pathogen on cocoon and shell. Silk Ratio (SR) percentage was calculated using the following formula:

$$\text{Silk Ratio (SR) \%} = [\text{Shell weight (g)} / \text{Cocoon weight (g)}] \times 100$$

The length and circumference of cocoon were taken with slide calipers and the filament length of cocoon was measured with hand epprouvete.

Second season rearing

For the next season, procedure for inoculation, purification etc. involved was same as in case of previous rearing. The eggs were hatched during February-March and rearing was conducted at temperature between 28.5-34.5°C and 55-81% RH.

Third season rearing

For the next season, procedure for inoculation, purification etc. was involved as in case of previous rearing. The eggs were hatched during May-June and rearing conducted at 20-40.5°C and 64-90.5% RH.

Statistical analysis

All the data were statistically analyzed using ANOVA.

Results

The study revealed that, in general, the cocoon weight of control batches (1.166 g) was higher than that of infected batches, when *Nosema bombycis* infected the mulberry silkworm. But, cocoon weight of infected batches of all inoculum concentrations with *N. mylitta* were found slight higher (1.224-1.394 g) than control batches (1.220 g) only during May-June (Season 3). Further, the cocoon weight gradually increased in mulberry silkworm, when infected with higher to lower inoculum concentrations (1.52×10^8 to 1.52×10^7 spores/ml) of *N. mylitta* in successive three seasons i.e., during December-January (Season 1), March-April (Season 2) and May-June (Season 3) (Fig. 1).

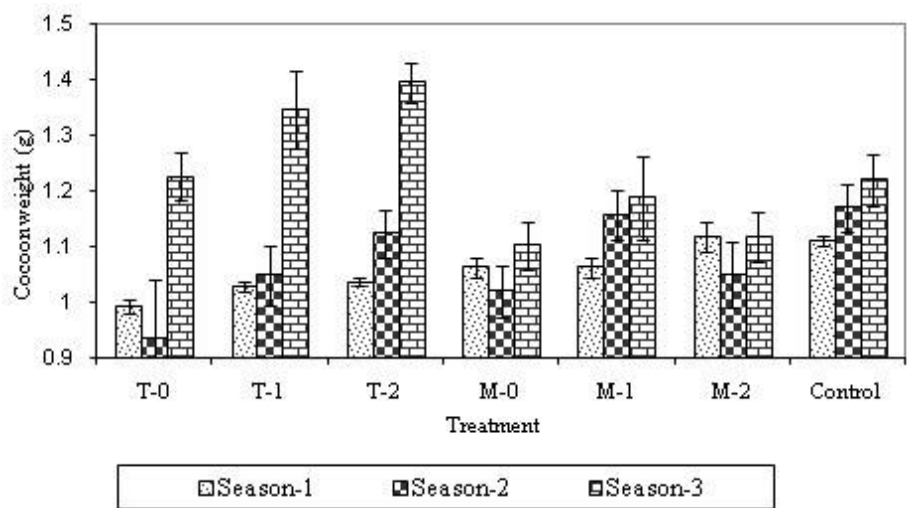


Fig. 1. Effect of pathogens on cocoon weight of silkworm, *B. mori* (M = *Nosema bombycis*, T = *N. mylitta*, S1, S2 and S3 = Season 1, 2 and 3., Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment). Bars represent standard error of mean.

There were significant ($P<0.01$) differences in the cocoon weight with respect to treatments, seasons and interactions between seasons and treatments. Generally, the mean cocoon weight of control batches (1.166 g) was higher than infected batches (1.050-1.184 g). A significant difference ($P<0.01$) of cocoon weight between T-0 (1.050 g) and T-1 (1.140 g) treated groups was observed. However, the treatments T-1 (1.140) and T-2 (1.184 g); T-0 and M-0 (1.060 g); M-0 and M-1 (1.134 g) as well as M-1 and M-2 (1.093 g) were at par. A significant ($P<0.01$) difference between season 1 (1.057 g) and season 3 (1.227 g) as well as season 2 (1.071 g) and season 3 was evident but, the results of season 3 and 1 were at par (Table 1).

The shell weight was always higher in control batches and highest (0.187 g), during May-June (Season 3) then gradually declined during March-April (Season 2) (0.168 g) and December-January (Season 1). The increasing trend of shell weight was observed with the decreased concentration of pathogens (T-0 to T-1 to T-2) in both the cases, when *Nosema bombycis* and *N. mylitta* were inoculated into mulberry silkworms. *N. mylitta* infection was more effective in decreasing the shell weight in the silkworm, compared to *N. bombycis* infection. Higher dosage of inoculum concentration (1.52×10^8 spores/ml) was effective to decrease the maximum shell weight (0.137-0.141 g) in both the cases of treatments (Fig. 2).

Table 1. Effect of pathogens on cocoon weight of silkworm, *B. mori*

	Season 1	Season 2	Season 3	Mean
T-0	0.99044	0.934938	1.2242	1.04985
T-1	1.02708	1.046838	1.3457	1.13987
T-2	1.03538	1.121825	1.3935	1.18358
M-0	1.06113	1.018388	1.1008	1.06009
M-1	1.06125	1.154900	1.1865	1.13420
M-2	1.11538	1.048563	1.1162	1.09337
Control	1.10925	1.168288	1.2195	1.16568
Mean	1.05713	1.070534	1.2266	

Analysis of variance

Source	DF	SS	MSS	VR(F)		CD at 5%
Treatment	6	0.48	0.08	3.59	**	0.075858031
Season	2	1.24	0.62	28.01	**	0.049660739
T x S	12	0.77	0.06	2.91	**	0.131389964
Error	147	3.26	0.02			
Total	167	5.76				

(M = *Nosema bombycis*, T = *N. mylitta*, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment; ** $p > 0.01$ (significant at 1% level).

There was significant ($P < 0.01$) difference in shell weight among treatments, seasons and interactions between seasons and treatments. Generally, the mean shell weight of control batches (0.176 g) was higher than infected batches (0.137-0.164 g). The significant difference ($P < 0.01$) between the treatments was T-0 (0.137 g) and M-0 (0.141 g); T-0 and T-1 (0.147g); T-1 and T-2 (0.157 g) as well as M0 and M1 (0.161 g). However, the results were at par between M-1 and M-2 (0.164 g). A significant difference between season 1 (0.149 g) and season 2 (0.155 g) was observed, but the results were at par between season 2 (0.155 g) and season 3 (0.159 g). A significant ($P < 0.01$) difference between interaction of seasons and treatments was also noticed (Table 2).

The SR% decreased with the increase of inoculum concentrations in both the cases, when *N. bombycis*, and *N. mylitta* were cross-infected to mulberry silkworm. However, December - January (Season 1) and May-June (Season 3) were effective to decrease the SR%, when *N. mylitta* infected to mulberry silkworm although the seasonal effect was not prominent in March-April (Season 2) (Fig. 3).

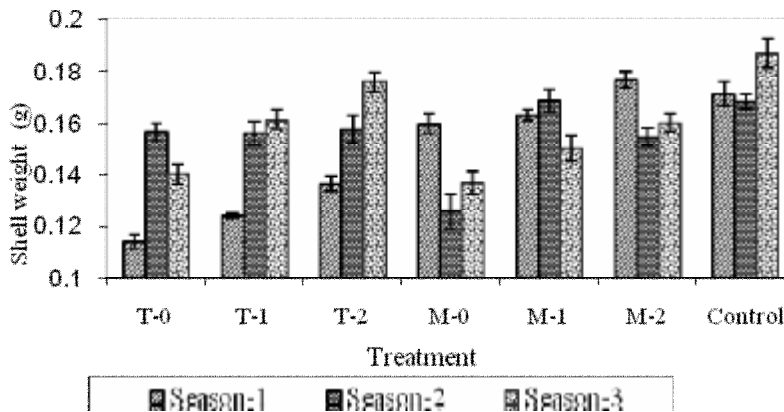


Fig. 2. Effect of pathogens on shell weight of silkworm, *B. mori* (M = *Nosema bombycis*, T = *N. mylitta*, S1, S2 and S3 = Season 1, 2 and 3, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment). Bars represent standard error of mean.

Table 2. Effect of pathogens on shell weight of silkworm, *B. mori*

	Season-1	Season-2	Season-3	Mean
T-0	0.114	0.157	0.140	0.137
T-1	0.124	0.156	0.161	0.147
T-2	0.137	0.158	0.176	0.157
M-0	0.160	0.126	0.137	0.141
M-1	0.163	0.169	0.150	0.161
M-2	0.177	0.155	0.160	0.164
Control	0.171	0.168	0.187	0.176
Mean	0.149	0.155	0.159	

Analysis of variance

Source	DF	SS	MSS	VR(F)		CD at 5%
Treatment	6	0.03	0.01	34.11	**	0.006527706
Season	2	0.00	0.00	9.67	**	0.004273386
T x S	12	0.03	0.00	17.57	**	0.011306318
Error	147	0.02	0.00			
Total	167	0.10				

(M = *Nosema bombycis*, T = *N. mylitta*, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment; ** $p > 0.01$ (significant at 1% level).

A significant ($P < 0.01$) difference in SR% among treatments, seasons and interactions between seasons and treatments was observed. Generally, the mean shell weight of control batches (15.409%) was higher than infected batches (12.706-15.168 %). There was a significant ($P < 0.01$) difference of SR% among M-0 (13.273%) and M-1 (14.42%); T-1 (13.16%) and M-1 (14.42%); T-2 (13.338%) and M-2 (15.168%). The results were at par between the treatments T-0 (12.706%) and T-

1; T-1 and T-2 as well as M-1 and M-2. There was a significant difference between season 1 and season 3 as well as season 2 and season 3, but results were at par between season 2 and season 1. A significant difference ($P<0.01$) between the interaction of seasons and treatments was prominent (Table 3).

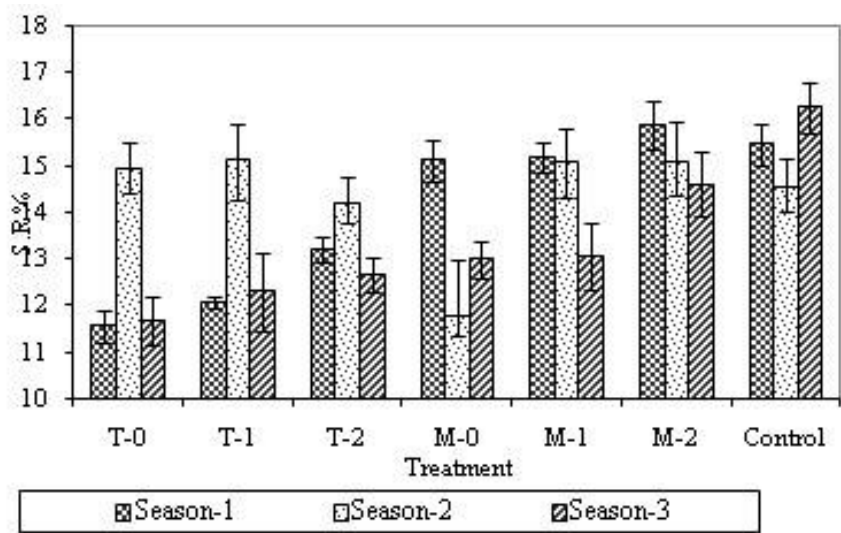


Fig. 3. Effect of pathogens on shell ratio % of silkworm, *B. mori* (M = *Nosema bombycis*, T = *N. mylitta*, S1, S2 and S3 = Season 1, 2 and 3, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment). Bars represent standard error of mean.

Table 3. Effect of pathogens on shell ratio% of silkworm, *B. mori*

	Season-1	Season-2	Season-3	Mean
T-0	11.536	14.914	11.668	12.706
T-1	12.073	15.121	12.288	13.160
T-2	13.190	14.156	12.668	13.338
M-0	15.095	11.740	12.983	13.273
M-1	15.170	15.040	13.049	14.420
M-2	15.856	15.059	14.590	15.168
Control	15.449	14.540	16.239	15.409
Mean	14.053	14.367	13.355	

Analysis of variance

Source	DF	SS	MSS	VR(F)		CD at 5%
Treatment	6	205.04	34.17	8.91	**	0.997668
Season	2	37.600	18.80	4.90	**	0.653127
T x S	12	213.39	17.78	4.63	**	1.728012
Error	147	564.03	3.840			
Total	167	1020.05				

(M = *Nosema bombycis*, T = *N. mylitta* , Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment; ** $p > 0.01$ (significant at 1% level).

Generally, the length of the cocoon was higher in control batches (3.370 - 3.720 cm) than any infected batches (3.100 - 3.550 cm). The length of cocoon was found declining gradually higher to lower during December-January (Season 1), March-April (Season 2) and May-June (Season 3). It decreased very much when highest dose of *N. mylitta* (T-0) (3.320 cm) and lowest dosage of *N. bombycis* (M-2) (3.100 cm) were given to mulberry silkworm. Season 1 was the suitable season for other treatments as well as control batches. In general, the length of cocoon decreased with the increase of inoculum concentrations and decreased gradually from seasons 1, 2, and 3, successively (Fig. 4).

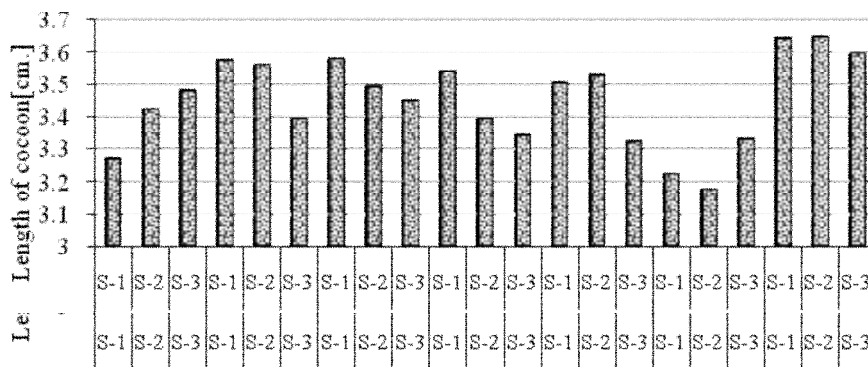


Fig. 4. Effect of pathogens on length of cocoons, *B. mori* (M = *Nosema bombycis*, T = *N. mylitta*, S1, S2 and S3 = Season 1, 2 and 3, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment). Bars represent standard error of mean.

There was a significant ($P < 0.01$) difference in length of cocoon in relation to treatments, seasons and interactions between seasons and treatments. Generally, highest mean length of cocoon was observed in control batches (3.629 cm) and lowest in M-2 (3.243 cm). A significant difference between T-0 (3.39 cm) and T-1 (3.508 cm) as well as M-2 (3.243 cm) and T-0 was observed, while the results were at par between the treatment T-1 and T-2 (3.507 cm) as well as M-0 (3.425 cm) and M-1 (3.452 cm) (Table 4).

The circumference of cocoon (4.80-4.10 cm) decreased gradually from seasons - 1 to 2 to 3 in control batches. However, the best performance (4.80 cm) was found during season 1. This observation was also noticed in both the treatments, when *N. bombycis* and *N. mylitta* were given to mulberry silkworm. The effect of infection was clearly observed in both the cases in all the seasons, particularly when inoculum concentrations (1.52×10^7 spores/ml) were lower (M-1 and T-1) and lowest (1.52×10^6 spores/ml) (T-2 and M-2). However, higher inoculum concentrations (1.52×10^8 spores/ml) in both treatments (T-0 and M-0) caused maximum effect to decrease the circumference of cocoon (3.60-4.10 cm.), particularly during March-April (season 2) (Fig. 5).

Table 4. Effect of pathogens on length of the cocoons of *B. mori*

	Season 1	Season 2	Season 3	Mean
T-0	3.270	3.420	3.480	3.39
T-1	3.574	3.560	3.392	3.50867
T-2	3.578	3.492	3.450	3.50667
M-0	3.538	3.394	3.342	3.42467
M-1	3.506	3.528	3.322	3.45200
M-2	3.224	3.176	3.330	3.24333
Control	3.644	3.648	3.596	3.62933
Mean	3.476	3.460	3.416	

Analysis of variance						
Source	DF	SS	MSS	VR(F)		CD at 5%
Treatment	6	1.27	0.21	9.43	**	0.051413
Season	2	0.07	0.03	1.49		NS
T x S	12	0.49	0.04	1.82		NS
Error	84	1.89	0.02			
Total	104	3.72				

(M = *Nosema bombycis*, T = *N. mylitta*, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment; ** $p > 0.01$ (significant at 1% level; NS = not significant).

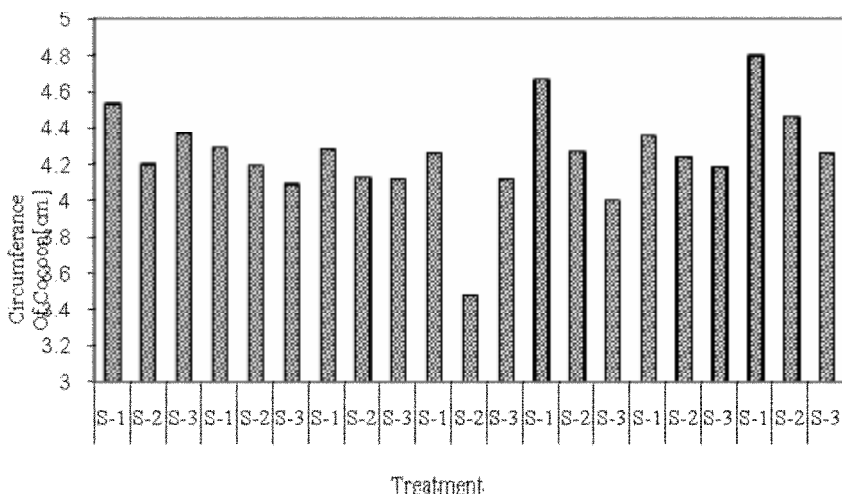


Fig. 5. Effect of pathogens on circumference of cocoons of *B. mori* (M = *Nosema bombycis*, T = *N. mylitta*, S1, S2 and S3 = Season 1, 2 and 3, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment). Bars represent standard error of mean.

There were significant ($P<0.01$) differences among the treatments, seasons as well as interaction of treatments and seasons. Generally, highest mean circumference of cocoon was observed in control batches (4.367 cm) and lowest in M-0 (3.953 cm). There was a significant ($P<0.01$) difference between the treatments T-0 (4.316 cm) and M-0 (4.393 cm); M-0 and M-1 (4.283 cm) as well as M-1 and M-

2 (4.183 cm). The results were at par between T-1 (4.15 cm) and T-2 (4.15 cm). A significant ($P<0.01$) difference between seasons December-January (4.442 cm), March-April (4.107 cm) and May-June (4.042 cm) was noticed, of which the season December-January showed the best results. There was a significant ($P<0.01$) difference between the interaction of treatments and seasons also (Table 5).

Table 5. Effect of pathogens on circumference of the cocoons of *B. mori*

	Season 1	Season 2	Season 3	Mean
T-0	4.60	4.10	4.25	4.31667
T-1	4.25	4.15	4.05	4.15000
T-2	4.30	4.15	4.00	4.15000
M-0	4.20	3.60	4.00	3.93333
M-1	4.60	4.35	3.90	4.28333
M-2	4.35	4.20	4.00	4.18333
Control	4.80	4.20	4.10	4.36667
Mean	4.442	4.10	4.04	

Analysis of variance

Source	DF	SS	MSS	VR(F)		CD at 5%
Treatment	6	1.85	0.31	10.45	**	0.058884
Season	2	3.20	1.60	54.15	**	0.066839
T x S	12	1.67	0.14	4.71	**	0.160661
Error	84	2.48	0.03			
Total	104	9.20				

(M = *Nosema bombycis*, T = *N. mylitta*, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment; ** $p > 0.01$ (significant at 1% level).

The filament length of control batches (390-415 m) was higher than treated batches (225-390 m). The same trend was also found with respect to higher to lower dosage of inoculum concentrations (T-0, T-1 and T-2 as well as M-0, M-1 and M-2) and from season 1, season 2 and season 3 gradually, when *N. bombycis* and *N. mylitta* were infected to mulberry silkworm. Dosage of inoculum concentrations was a major factor to reduce the filament length and maximum decrease (221.35-304 m) in filament length was observed in highest dosage of inoculum concentrations (1.52×10^8 spores/ml) of *N. bombycis* infected mulberry silkworm (Fig. 6).

There was a significant ($P<0.01$) difference of filament length among the treatments, seasons as well as interaction of treatments and seasons. Generally, mean filament length in control batches (399.9 m) was found higher than infected batches (271.55 - 374.33 m). A significant ($P<0.01$) difference between the treatments T-1 (337.97 m) and T-2 (368.25 mt); M-0 (271.55 m) and M-1 (333.2 m); M-1 and M-2 (374.33 m) as well as T-0 (325.58 m) and M-0 (271.55 m) was observed. However, the results were at par between the treatments T-0 and T-1; T-1 and M-1 as well as T-2 and M-2. Similarly, significant ($P<0.01$) differences among season 1 (329.44 m), season 2 (345.36 m) and season 3 (358.39 m) were also noticed, of which season 3 showed the best results. A significant ($P<0.01$) difference between the interaction of seasons and treatments was also evident (Table 6).

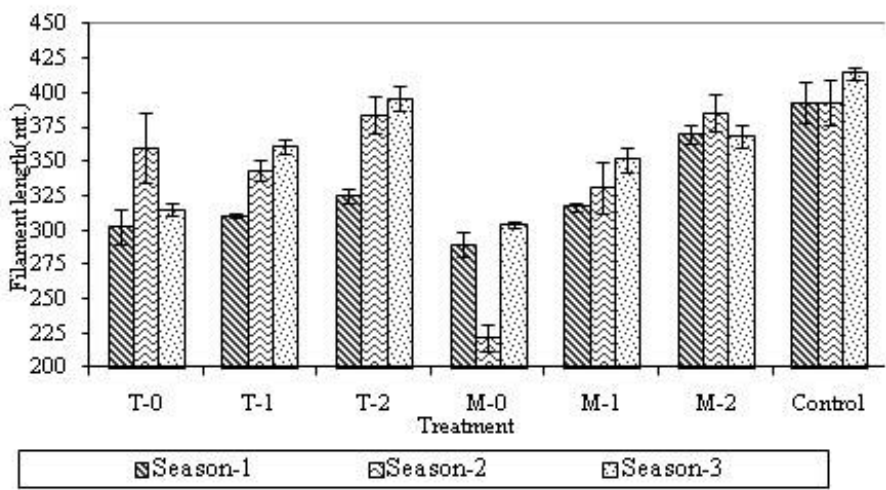


Fig. 6. Effect of pathogens on filament length of cocoons, *B. mori* (M = *Nosema bombycis*, T = *N. mylitta*, S1, S2 and S3 = Season 1, 2 and 3, Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment). Bars represent standard error of mean.

Table 6. Effect of pathogens on filament length of the cocoons of *B. mori*

	Season 1	Season 2	Season 3	Mean
T-0	302.3	359.8	314.6	325.5
T-1	310.0	343.2	360.7	337.9
T-2	325.0	384.0	395.7	368.2
M-0	289.3	221.3	304.0	271.5
M-1	317.1	331.1	351.4	333.2
M-2	369.5	385.4	368.1	374.3
Control	392.9	392.6	414.2	399.9
Mean	329.4	345.3	358.3	

Analysis of variance						
Source	DF	SS	MSS	VR(F)		CD at 5%
Treatment	6	311192.82	51865.47	41.40	**	18.02778862
Season	2	29417.23	14708.62	11.74	**	11.80195799
T x S	12	80696.92	6724.74	5.37	**	31.22504584
Error	189	236789.58	1252.85			
Total	209	658096.55				

(M = *Nosema bombycis*, T = *N. mylitta*; Dose 0, 1 and 2 = 1.52×10^8 , 1.52×10^7 and 1.52×10^6 spores/ml, Control = No treatment;***p* > 0.01(significant at 1% level).

Discussion

The present study on cross-infection of microsporidian spores of mulberry, tasar, eri and muga silkworm on economic parameters of silk to mulberry silkworm, *Bombyx mori* L. revealed that several economic parameters namely, cocoon weight, shell weight, shell ratio%, length and circumference of cocoons and filament length of cocoons are decreased with the infection and cross-infection of microsporidia. Pebrine-infected cocoons are flimsy in nature and therefore unfit for reeling. These findings are in agreement with the observation of various workers (Jamenson, 1922; Ghosh, 1944; Steinhaus, 1949; Geetha Bai and Mahadevappa, 1995; Chandra *et al.*, 1995).

Jamenson (1922) and Ghosh (1944) reported that pebrine infected worms spin flimsy and poor cocoons. Steinhaus (1949) mentioned that the silk from the cocoons of infected larva usually is much inferior in strength and uniformity of thickness than that silk obtained from healthy worms. The diseased silkworms spun flimsy and irregular cocoons that are unfit for reeling (Geetha Bai and Mahadevappa, 1995). The economic parameters such as shell weight, cocoon weight and Shell Ratio % are decreased in the *N. bombycis* infected cocoons during winter and summer seasons (Chandra *et al.*, 1995). But Nomani *et al.* (1971) made contradictory statement and stated that there is no significance difference between pebrinized and healthy cocoons though healthy cocoons gave significantly better yields of filament (silk) and also of denier and renditta as compared to the diseased ones. But the authors concluded that pebrine disease adversely affects some of the important cocoon characters. Further, a positive correlation was found between ingestion of food and larval weight and cocoon weight and shell weight (Magadum *et al.*, 1996). Studies on cross-infectivity and the pathogens responsible for cross-infectivity have been taken up recently (Chakrabarti and Manna, 2006, 2008a, b, c; Madana *et al.*, 2006). Usually healthy cocoons possess higher filament length than infected one, which is correlated with consumption of leaves. However, there is a positive correlation between the amount of mulberry leaves eaten by the silkworm and the silk protein in the silk gland and the eggs laid by the silk moth (Fukuda *et al.*, 1963).

The present study thus concludes that the infection and cross-infection of microsporidian spores deteriorate the economic characters of cocoons, resulting into loss of production and quality of silk.

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Detection of “Tim” gene of sheep *Giardia* by primers of “Tim” gene of *Giardia* of human origin

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Abstract

Giardiasis is an important human parasitic disease. *Giardia* is a genus composed of binuclear flagellate protozoa. *Giardia duodenalis* is a parasitic species for a wide range of vertebrates, including humans. Heterogeneity in *G. duodenalis* has been shown by serologic, biochemical, and molecular analysis. In the present study, possible genetic similarity between sheep and human *Giardia* isolates and its probable zoonosis was investigated. Direct examination and formalin ether concentration technique were performed on the contents and tissue of intestines of sheep. Gradient sucrose method was applied for collection and purification of cysts, and DNA extraction was performed by phenol-chloroform method. Cysts DNA could hardly be extracted after repeated freezing and thawing and suspension with lysis buffer. At final stage polymerase chain reaction (PCR) was performed for DNA amplification by primers which were designed for *Giardia* with human origin. In this study, "triose phosphate isomerase" ('tim' or 'tpi') was selected as a molecular marker. Two sets of primers (PM290, PM924) were considered. We examined 308 samples in our study, including 21 positive samples. We could not succeed in culture. Three sheep isolates were determined by 290 base pair (bp) that were similar to some of human type.

The similarity of genomic characters of sheep and human *Giardia* implies a possibility of these protozoa being a zoonosis.

Keywords

PCR; Triose phosphate isomerase (tim) gene; Giardiasis; *Giardia duodenalis*; Sheep; *Giardia*; Ovine *Giardia*

Introduction

Giardia is a flagellate binuclear protozoan parasite for many vertebrates including human beings (Mayrhofer *et al.*, 1995). This parasite has two phases in its life cycle, in which the flagellate trophozoite is the active form and responsible for clinical manifestations. Infectious cyst is the other form that is excreted in association with stool and persists in the environment for weeks (Slavin *et al.*, 2002). *Giardia* infections occur via contaminated food and water. Therefore, it is considered the most important causative agent of childhood diarrhea, traveler's diarrhea and diarrhea in homosexuals (Lujan *et al.*, 1998). Giardiasis accounts for an important parasitic disease of human and extensive researches on different aspects of this disease have been conducted so far.

It is estimated that about 200 millions cases worldwide are infected by this parasite and nearly 500000 new cases are added yearly (Thompson and Lymbery, 1990). A precise and systematic understanding of *Giardia* would be of immense help to understand the disease pattern recognition, drug sensitivity monitoring and treatment, virulence and infectivity, zoonotic position and disease control. However, there are still major defects in our knowledge about biological characteristics of this organism and its relevant disease.

One of the most important not-responded problems in this field is the role of animals as carriers and an ambiguity, whether or not the animal-specific species are similar to those of human origin. Is giardiasis a zoonotic disease and whether animals have any role in its transmission? Recently, attention has been focused on the determination of species (Thompson and Lymbery, 1990). At the moment, the determination of the genus based on genetic approaches is difficult and identification of the species is based only on morphological criterion (Thompson and Lymbery, 1990). In 1952, based on morphological characters Filice confirmed only three species within the genus:

- 1) *G. agilis* in cold-blooded animals (with a club like median body parallel to longitudinal axis).
- 2) *G. muris* in rodents (with two small globular median bodies).
- 3) *G. duodenalis* in mammals (with one or two transversal hammer like median body).

Recently, two more new species of bird parasites, including *G. psittaci* in budgerigar and *G. ardeae* in great blue heron have been detected. *G. duodenalis* has been found in mammals and some birds, and is of medico-veterinary importance (Thompson and Reynoldson, 1993). Genetic diversity has been demonstrated in *G. duodenalis* by means of serological, biochemical, and molecular genetic analysis (Baruch *et al.*, 1996). Methods based on genetic diversity, including RFLP and sequence analysis, have categorized *G. duodenalis* to genotypes 1, 2, and 3 (Nash, 1992). Homan *et al.* (1992) classified *G. duodenalis* to "Belgian" and "Polish" isolates by means of isoenzyme electrophoresis and DNA probes (Homan *et al.*, 1992). On this basis, Mayrhofer *et al.* (1995) categorized *G. duodenalis* isolates to two major assemblages including four genetic groups (Mayrhofer *et al.*, 1995). Based on the last reports, *G. duodenalis* has been classified into three groups: group

1 (WB), group 2 (JH), and group 3 (GS) (Siqi *et al.*, 2002). The influence of zoonotic pathogens on human health and well-being as a direct or indirect cause of human enteric illness is examined.

The recent application of molecular characterization procedures based on PCR has made an enormous contribution to an understanding of the genetic structure of *Giardia* populations. The aim of the present pilot study was to recognize if isolates of *Giardia* recovered from sheep are similar or not to 'tim' gene of *Giardia* of human origin.

Materials and methods

For sampling, small intestine contents as well as duodenal tissue of slaughtered sheep were collected from slaughterhouse to obtain trophozoites and cysts. In addition, contents of distal large intestine were also collected to obtain the cyst forms of parasite. Microscopic examination was done immediately after sampling in order to ensure viability of the parasites. At first, duodenal tissue was scraped to increase the probability of trophozoites observation. Then a direct smear was prepared using a drop of ringer and lugol's solution and immediately examined. Negative samples in direct examination were tested using formalin-ether concentration method. Parasite viability determination was performed using 1% eosin (vital stain). Samples with 10-15 cysts in each field of 40 x magnification and high rate of viable cysts were considered suitable for culture. Cysts collection and purification was done by sucrose gradient method. To inhibit bacterial contamination, antibiotic was added and then the samples were washed twice daily during a 48 h period (Meyer, 1976). Subsequently, a few slides of all isolates were made using trichrome stain. Majority of the samples which were not suitable for culture (those with few cysts or unviable parasites) were collected in micro tubes and kept in -70°C after washing phase. For culturing, samples were incubated in 0.1N HCL (pH = 2) in 1/9 ratio of trypsin for some cases 60 min in 37°C and 5 min in 56 °C to prepare cysts for excystation. After washing, cysts were added to TYI-S-33 culture media in sterile conditions and after two hr examined under inverted microscope for excystation; daily examination was continued up to two weeks (Phillips *et al.*, 1984). In case of parasite proliferation the tubes containing parasite were placed in ice in order to detach trophozoites from the tube walls. Samples were washed, and kept in -70°C for further molecular analysis.

In molecular phase, first of all we extracted trophozoites by means of phenol-chloroform method (Sambook *et al.*, 1989). For cysts, DNA extraction was accomplished by repeated freeze and thaw and suspension preparation with lysis buffer. In order to be sure about DNA extraction, some of the samples were run on agarose gels. In this investigation, for PCR 'tim' gene was considered. One specific primer PM290 was used, in order to amplify 290 bp amplicon respectively (Table 1). In all cases human *Giardia* was used as positive control. Gradient thermal cycler (Corbet) was used to do PCR. PCR program for these primers is shown in Table 2 (Zare Bavani, 2002). In order to be sure about PCR completion, PCR products were run on agarose gel, stained with ethidium bromide and finally assessed by UV transilluminator.

Table 1. Sequence of primers (PM290, PM924)

Primers	Forward (F)/Reverse (R)	Sequence (5' à 3')
PM290	F	GCC ATT GCT GCC CAC AAG AT
PM290	R	GTC ATC CCC TTT TCT AGA GT
PM924	F	TCA TGC ACC GTG ATT TGG AC
PM924	R	AGT TGC TTC CAT TGG CCG AT

Phase	Temperature/°C	Time/ second
1. Primary denaturing	94	240
2. PCR cycles (35 cycles)		
• Denaturing	94	30
• Annealing		
– PM290	52.5	30
– PM924	58.5	30
Extension	72	30
Terminal extension	72	300

Results

We examined 308 samples in our study, including 21 positive samples and 3 cases suspicious for *Giardia* infection. Thick walled cysts were seen by trichrome staining. Excystation occurred only in one case (OC-8; Ovine Cyst- 8th positive sample) and even this case failed to establish in the culture medium. However, viability of cultured cysts was 90-100% but excystation did not occur. DNA extraction from trophozoites directly collected from intestinal contents was done easily and then, PCR was accomplished and we were able to observe a 290 bp band, the same as human positive control in Ovine Trophozoite-11 (OT-11; Ovine Trophozoite- 11th positive sample).

From some cyst samples, DNA was hardly extracted. OC-13 and OC-5 isolates had almost similar bands to positive control in 290 bp band. However, for most cysts either very little DNA was extracted or extraction could not be carried out at all; in some instances PCR with relevant primers could not be done.

Discussion

Although *G. duodenalis* morphologically indistinguishable (Buret and Den Hollander, 1990), in our study the slides prepared by means of trichrome staining showed *Giardia* organisms with more thick walls and larger sizes in sheep isolates in comparison to human isolates. Although sheep *Giardia* cultures have been reported to be successful in some studies (Buret and Den Hollander, 1990), there are limited reports of successful *in vitro* culture of *Giardia* of sheep origin. In this study we did not succeed in culturing the parasite. We need to make suitable changes in modified-TYI-S-33 media, which is used for culturing *Giardia* of human origin. After DNA extraction, we ran sample on gel agarose to manage the DNA extraction, but in most cases DNA was either little or absent. In circumstances like this, the causative agent can be of low number in sheep isolates (infection in sheep is much milder than

human cases). The DNA isolation became possible with repeated freeze and thaw and using lysis buffer. But DNA extraction from trophozoites was performed easily similar to the human isolates. Some of the cyst samples used for PCR had very low number of cysts, about one per each field (x 40). Some samples had many cysts (3 or more cysts in each field; x 40) but we could not extract DNA (OC-2, 4, 16, 20) as the fecal material would have contained nucleic acid inhibitors, which interfering with PCR success.

DNA extractions in one isolate (OT-11), in which trophozoites had been collected from intestinal contents directly, could be accomplished rather easily but in the first phase high DNA level prevented PCR and we had to decrease the template DNA in order to succeed in doing the PCR, because of inhibitor existence in direct samples of sheep *Giardia*. For those isolates which in the first PCR had weak bands (OC-5), we could also obtain *Giardia* amplicon by means of double PCR. All cases of successful PCR in isolates were amplified with PM290, and the PCR product was 290 bp amplicon. Based on studies on human giardiasis (Zare Bavani, 2002), among 78 human cyst isolates 46 cases did not amplify by primers (probably because of DNA extraction insufficiency or inhibition), 16 isolates amplified by both primers, 8 cases amplified only with PM924 primer, 8 cases amplified only with PM290 primer but most of the latter cases amplified only one time and their second amplification was not visible (Zare Bavani, 2002). On the basis of this information, in our study molecular evaluation on 15 isolates was also done and 3 cases amplified only with PM290 prime, 12 cases did not amplify and no amplification was seen by PM 924. Therefore, noted sheep isolates amplified with PM290 primer, based on studies on human isolates are placed in assemblage A with accession numbers L02120 and U57897.

This pilot study thus indicates that “tim” gene of sheep *Giardia* is partially similar to human *Giardia*, and to show to which assemblage it belongs, RFLP based genetic testing of DNA is required. This study needs to be performed at a larger scale and culturing seems crucial to obtain more *Giardia* for DNA extraction. Additionally, since clinical symptoms of sheep and human giardiasis are the same (Kiorpes *et al.*, 1987) and because of finding *Giardia* of human origin repeatedly in domestic animals (diagnosed with molecular methods), we can propound the transmission possibility of this parasite from animals to human and vice versa, which can manifest its zoonotic probability (van Keulen *et al.*, 2002).

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RAPD-PCR assay for differentiation of *Sarcocystis bovicanis* and *S. bovifelis* in cattle

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Abstract

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was carried out using four arbitrarily designed primers to differentiate the two commonly occurring species viz., *Sarcocystis bovicanis* and *Sarcocystis bovifelis* of cattle. All the four primers directed the amplification of at least a single DNA fragment using genomic DNA as the template. The primer 1 amplified 1.3, 1.2 and 1.1, 1.0, 0.85, 0.75 kb for *S. bovicanis* and *S. bovifelis* respectively. The primer 2 amplified 0.4, 0.6, 0.7 kb and 1.2, 1.3, 1.5 kb distinct DNA fragments in each *Sarcocystis* species, which clearly differentiated the species. The primer 3 directed the synthesis of 0.8 and 0.5 kb for *S. bovicanis*. In the case of *S. bovifelis* two fragments of size 1.0 and 1.1 kb were obtained. The DNA fragments of 0.5 kb and 0.7, 0.9, 1.0 were amplified for primer 4. None of the DNA fragments were amplified with host genomic DNA in all the four primers.

Keywords

Genomic DNA; RAPD-PCR; *Sarcocystis bovicanis*; *Sarcocystis bovifelis*

Introduction

Sarcocystis, an apicomplexan protozoan once regarded as a non-pathogenic parasite has been associated with disease conditions in both animals and man (Dubey *et al.*, 1989). *S. bovicanis* and *S. bovifelis* are the two important species in cattle causing acute and chronic disease conditions. Acute sarcocystosis is characterized by reduced growth, poor feed efficiency, and abortion and has negative effects on growth rate. In chronic infections there is decreased milk production, cachexia and downgrading or degradation of the carcass infected with the sarcocystis. Diagnosis of acute sarcocystosis is not possible since the clinical signs are non-specific and parasitemia during the acute phase of the disease may be brief or too low for easy detection (Fayer and Dubey, 1986). Sarcocystosis being an occult infection cannot be

diagnosed by using conventional parasitological techniques. Though morphology remains practical, inexpensive and the mainstay for accurate identification of many parasites, in the case of *Sarcocystis* species, the morphological identification is said to be unreliable (Obendorf and Munday, 1987). Further the morphological features of the species may vary during cyst development with the type, location and degenerative state of host cell parasitized as well as with the methods used for examination or fixation (Dubey *et al.*, 1989). Similarly, high cross reactivity among different *Sarcocystis* species infecting the same intermediate host has hampered the development of species specific immunological methods for *Sarcocystis* species. These tests failed to overcome the cross reactivity between the species or closely related genus.

However, the differentiation of *Sarcocystis* species under natural conditions requires a test system that is applicable to examine a broad range of different species. Consequently, SSU rRNA is not a stable target and is degraded by enzymatic digestion. In addition, standardization of RNA hybridization assays is difficult and not suitable for epidemiological studies (Gajadhar *et al.*, 1992; MacPherson and Gajadhar, 1993). The microscopic or macroscopic examination of *Sarcocystis* is possible only at postmortem and serological tests are not species specific. Currently it is not possible to differentiate between pathogenic and non-pathogenic *Sarcocystis* species in the intermediate hosts. Therefore, RAPD-PCR which does not require specific primers was performed using genomic DNA for differentiation of *Sarcocystis* species in cattle.

Materials and methods

Isolation of cystozoites

The cystozoites were isolated (Dubey *et al.*, 1989; Shi and Yaun, 1987) from microcysts of *S. bovicanis* and macrocysts of *S. bovifelis* respectively with slight modifications. The purified cystozoites stored in phosphate buffered (PBS) saline at -20°C were used for extraction of DNA.

Extraction of DNA

The purified cystozoites stored in PBS (pH 7.2) at -20°C were used for extraction of DNA (Macpherson and Gajadhar, 1994) with slight modifications. The cystozoites of *S. bovicanis* and *S. bovifelis* were washed thrice in PBS. Three cycles of freezing and thawing were carried out at -20°C and -60°C, respectively. To one volume of packed bradyzoites 10 volumes of cold lysis buffer (pH 8.0) was added. Proteinase K was added (100 µg/ml) and 5µl of 1% aqueous solution of sodium do-decyl sulphate (SDS) were added and incubated for 5 h at 56°C with frequent movements. The lysate was extracted three times with Tris saturated phenol: chloroform (1:1). The DNA present in aqueous supernatant was precipitated by addition of 1/10th volume of 2M sodium chloride and twice the volume of chilled absolute ethanol. The mix was kept at -70°C for one hour. DNA was pelleted at 12000 rpm at 4°C for 15 min and the supernatant was carefully discarded. Then it was overlaid with 80% ethanol and centrifuged at 12000 rpm at 4°C for 10 min to remove sodium chloride. The supernatant was then discarded and the washing procedure repeated. The DNA was suspended in 50µl of Tris EDTA buffer (TE) with pH 7.4 to each tube after drying at

room temperature. The tubes were tapped gently and DNA was stored at 4°C until use. Host DNA was isolated (Sambrook *et al.*, 1989). The purity and concentration of the extracted genomic DNA was estimated in the UV-VIS Spectrophotometer (Beckman).

Polymerase chain reaction

Four random primers with nucleotide sequence of :

3¹AGACCGAAAGTCAACGCGAC5¹ and 3¹GCACGAACGCGCCACAAA5¹;
3¹CCGGGGAAAGAGCGAT5¹ and 3¹AGCCAGGAAAGATCAACGCGAC5¹

were used, which had been procured commercially (Bangalore Genei). The last two primers were newly designed and were evaluated to be used as specific primers in differentiation of *Sarcocystis* species of cattle. The amplification reaction was carried out in 0.5 ml microcentrifuge tubes using a programmable thermal cycler (BioRad) (Guclu *et al.*, 2004). Template genomic DNA (100ng) of *S. bovicanis* and *S. bovifelis* were denatured initially at 95°C for five min and then snap cooled in crushed ice pack for 10 min. Each 25 µl reaction mixture consisted of primer (10 pm), 0.5 U Taq DNA polymerase, dNTP mix (10 mM), 10x PCR buffer and sterile filtered milli Q water to a final concentration of 25 µl was added to each tube. The initial denaturation was done at 94°C for 1 min followed by annealing at 36°C for 1 min and extension for 2 min at 72°C up to 45 cycles. The final extension was carried out for 10 min at 72°C and cooling for 3min at 4°C. After completion of PCR reaction 25 µl amplified products were subjected to electrophoresis along with 100 bp DNA ladder in 1.2% agarose at 8 v/cm. The banding pattern on the gel was visualized under UV light and the image was taken with a video camera in the gel documentation system (BioRad Co.). The amplicon sizes were calculated from the standard curve plotted between log values of standard DNA ladder against their mobility. Similarity co-efficient between the isolate was calculated using the formula: $S_{XY} = 2N_{XY} / (N_X + N_Y)$ where $N_X + N_Y$ were the numbers of DNA segments amplified in species 'X' and 'Y' respectively. N_{XY} was the number of segments shared by 'X' and 'Y'. The bands were scored depending upon its intensity.

Results

Four primers of nucleotide length 20, 18, 16 and 22 bp with GC content more than 50% were used in PCR assays where the optimal annealing temperature was 36°C. All the four primers directed the amplification of at least a single DNA fragment using both *S. bovicanis* and *S. bovifelis* genomic DNA as the template. Primer 1 amplified two fragments of size 1.3 and 1.2 kb for *S. bovicanis* where as for *S. bovifelis* four fragments 1.1, 1.0, 0.85 and 0.75 kb were amplified and none of the fragments were observed in host DNA (Fig. 1). A second primer with 19 nucleotides in length produced DNA fragments of 0.4, 0.6 and 0.7 kb for *S. bovicanis* (Fig. 2). The fragments of 1.2, 1.3 and 1.5 kb were amplified for *S. bovifelis*. No bands were visible in host DNA after gel electrophoresis. The Primer 3 amplified two fragments of 0.8 and 0.5 kb of *S. bovicanis* (Fig. 3). In the case of *S. bovifelis* two fragments of size 1.0 and 1.1 kb were obtained. There were no DNA fragments present in the host DNA. In the present study, *S. bovicanis* DNA produced only single fragment of size 0.5 kb whereas *S. bovifelis* DNA amplified 0.7, 0.9 and 1.0 kb (Fig. 4) for primer 4. In host DNA none of the fragments were visible. The similarity co-efficient between the two species by RAPD-PCR was found to be 0.3.

(MacPherson and Gajadhar, 1993). Consequently, a different rationale was used to develop a PCR based diagnostic test for species-specific detection and differentiation of the *Sarcocystis* species (Frederick *et al.*, 1991; Joachim *et al.*, 1996; Ndiritu *et al.*, 1996). A series of species specific oligonucleotides was generated that can be used as primers for specific amplification of SSU rRNA gene fragments from genomic DNA templates of the homologous parasite species by PCR.

Later, the identification of hypervariable regions in the 18s rDNA of *Sarcocystis* species made possible the development of species-specific and genus specific DNA probes (Holmdahl *et al.*, 1993; Ellis *et al.*, 1995; Jeffries *et al.*, 1997). The major disadvantage with the PCR is the requirement of nucleotide sequence information from which primers are designed. Hence, Welsh and McClelland (1990) and Williams *et al.* (1990) independently introduced a novel PCR assay which did not require nucleotide sequence information from which to design primers. Therefore, to overcome cross reactions and to clearly differentiate the pathogenic and non-pathogenic *Sarcocystis* species of cattle RAPD-PCR assay was performed during this study.

During this study, all the four primers directed the synthesis of more than one fragment and each primer revealed unique DNA banding patterns for both *S. bovicanis* and *S. bovifelis*. RAPD-PCR in this study revealed a clear cut difference in the fragment pattern between *S. bovicanis* and *S. bovifelis*. In the case of host DNA none of the bands/fragments could be detected, thereby indicating that all the four primers did not direct the synthesis of host DNA fragments.

Similar findings were reported (MacPherson and Gajadhar 1994) and differentiated *S. cruzi* DNA combined with bovine DNA using RAPD-PCR. Five primers of various length and sequences were used and multiple DNA fragments ranging from 2.2 to 0.5 kb were found. An intense band with an electrophoretic mobility corresponding to approximately 0.8 kb was shown to be specific for *S. cruzi* and was absent in *T. gondii* or *S. campestris*. Guclu *et al.* (2004) used RAPD technique to amplify short regions of cattle *Sarcocystis* species and observed separate clusters for *S. bovicanis*, *S. bovi hominis* and *S. bovifelis*. They observed only one fragment of 0.3 kb for OSA-04 primer in *S. bovi hominis*, where as OSA-06 showed amplification of 0.3 and 0.25 kb fragment for *S. bovi hominis* and *S. bovifelis*, respectively. In primers OSA-06, OSA-07 and OSA-08 more than two DNA fragments were shown to be amplified for *S. bovicanis*, *S. bovifelis* and *S. bovi hominis*.

However, analysis of seven *Eimeria* species by RAPD-PCR showed that DNA fragments ranging from 200 to 2200 bp were synthesized in the different reactions (Macpherson and Gajadhar, 1993) and the percent GC content of primers and the number of fragments amplified were correlated. Granstrom *et al.* (1994) identified a 550 bp DNA fragment unique to *S. neurona* that was not found in *S. cruzi*, *T. gondii*, three *Eimeria* species and bovine DNA. In this study no cross reactivity between the isolate and other coccidian species by hybridization could be seen. A RAPD-PCR derived marker was shown to be useful in differentiation of pathogenic and non-pathogenic *Sarcocystis* species of sheep where a fragment of about 1280 bp from genomic DNA of *S. tenella* was synthesized but not from genomic DNA of *S. gigantea* or *T. gondii* (Joachim *et al.*, 1996). However, Marsh *et*

al. (1996) distinguished *S. neurona* from other closely related protozoal parasites using *S. neurona* specific DNA sequences in the nuclear small subunit ribosomal RNA (nss-rRNA).

In RAPD assays unique DNA banding patterns were observed that could be exploited to identify different species (MacPherson and Gajadhar, 1993). In general, those primers that had the highest GC content were responsible for multiple DNA fragments after PCR. During this study, the primer B with 61 per cent GC and 18 nucleotides in length produced six DNA fragments. In contrast, the primer A with 20 nucleotides (45% GC) also amplified six fragments. However, primers that were 16 and 22 nucleotides in length (C and D respectively) with more than 50 per cent GC produced fewest DNA fragments. The above finding was in concurrence with MacPherson and Gajadhar (1993) and Guclu *et al.* (2004) who recorded similar results with seven *Eimeria* species and *Sarcocystis* of cattle respectively.

The fingerprints generated during this study showed variation in the band intensity, wherein with each primer at least one DNA fragment was amplified more efficiently than others in the same reaction. Similarly, variation in the intensity of DNA fragments was observed (MacPherson and Gajadhar, 1993). The more intense bands could be probably due to priming within repeated regions of the *Sarcocystis* genome which would result in more copies being produced during PCR. The shorter primers consistently produced DNA fragment patterns containing at least one DNA fragment. This suggested that shorter oligonucleotides may generate more characteristic DNA fingerprints.

Further, the two newly designed primers viz. primer C and primer D resulted in amplification of *S. bovicanis* and *S. bovis* genomic DNA. Hence, these results suggest that the primers used in the present study can be used as diagnostic probes for *S. bovicanis* and *S. bovis*. The RAPD-PCR assay of this study may be particularly useful to study the taxonomy and epidemiology of members of the family Sarcocystidae. Instead of relying on ultrastructural or transmission studies for the identification of tissue coccidia in cattle, investigators may use DNA assays to accurately determine the presence of *Sarcocystis* species in cattle.

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Prevalence of *Plasmodium falciparum* in Jodhpur (Rajasthan), India

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Abstract

A study on the epidemiology of *Plasmodium falciparum* (*P.f.*) in Jodhpur(Rajasthan), India made during November, 2007 to February, 2008, revealed that out of a total of 7418 subjects examined, 116 (1.56%) were positive for *P.f.* Of the infected cases, 75 (1.01%) were males and 41 (0.55%) were females. Highest parasitic prevalence was in the age-group of 21-30 years and lowest in 81-90 years. The infection percentage was more in urban (81.89%) and literate (90.10%) than in non-urban (18.10%) and illiterate (09.48%). The infection rate was 18.10% in those who were on frequent travel. Mixed infection with *P. vivax* was found in 19.82% subjects and re-infected cases were 02.58%. Degree of infection was found to be mild in 45 (38.79%), moderate in 29 (25.0%) and high in 08 (06.89%).

Keywords

Epidemiology; Jodhpur; Malignant malaria; Pathogenic; *Plasmodium falciparum*; Protozoa

Introduction

Malignant tertian malaria, caused by *Plasmodium falciparum* is a pathogenic and deadly communicable parasitic disease. It is believed that of all the malarial cases, roughly 50% infections are caused by *P. falciparum* (Roberts and Janovy Jr., 2005). According to a World Health Organization (WHO) report there were an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years (WHO, 2008). WHO classifies India as among one of the 109 countries that are endemic to or were previously endemic with malaria (WHO, 2008). It is therefore necessary to recognize the risk probabilities of malaria in prone regions like India. Keeping this in mind, the present study was undertaken to study the prevalence of *P. falciparum* malaria with respect to factors like, age, sex and educational status of subjects, etc. in Jodhpur city of Rajasthan, India.

Materials and methods

The survey was carried out in various hospitals and disease diagnostic centres in Jodhpur, Rajasthan during November, 2007 to February, 2008. The parasitic examination was done by preparation of thick and thin film (smear preparation) with the blood collected from the subjects by finger prick. The malarial parasite Quantitative Buffy Coat (Q.B.C.) test was employed for identifying the malarial parasite in the peripheral blood. The age, sex, locality, educational status, drugs given, body temperature and other related symptoms of the patients were recorded at the time of sample collection.

Results

Of the 7418 subjects examined 116 (1.56%) were positive for *Pf*. Of the infected cases, 75 (1.01%) were males and 41 (0.55%) females. The infection rate was at its peaks during the month of November (2.06%) (Table 1). Highest prevalence was observed in the age-group of 21 to 30 years (2.24%) and lowest in 81-90 years (Table 2). The infection was more prevalent in urban (81.89%) and literate (90.51%). The percentage of *P. falciparum* infection was also comparatively high 18.10% in those who undertake frequent journeys for their livelihood. Mixed infections with *P. vivax* were recorded in 19.82% subjects. The prevalence of re-infected cases was 02.58%. The examination of peripheral blood film and Q.B.C. showed a more prevalence of trophozoite stage (49.13%) than gametocyte stage (02.58%). The degree of infection, i.e., mild, moderate and high, were found to be 38.79%, 25.0% and 06.89%, respectively for those patients whose examination was done by either by Q.B.C. or with smear preparation (Table 3). No cases of *P. falciparum* infection were found in pregnant woman. The common drugs used against *Pf* malaria were chloroquine, quinine, artemisinin-derivatives, sulphadoxine-pyrimethamine, doxycycline and primaquine.

Table 1. Prevalence of *Plasmodium falciparum* in Jodhpur (Rajasthan), India

Months	No. of hosts examined			No. of hosts infected (%)		
	Male	Female	Total	Male	Female	Total
November	1428	704	2132	27 (1.27)	17 (0.80)	44 (2.06)
December	1147	568	1715	25 (1.46)	08 (0.47)	33 (1.92)
January	1136	608	1744	11 (0.63)	11 (0.63)	22 (1.26)
February	1135	692	1827	12 (0.66)	05 (0.27)	17 (0.93)
Total	4846	2572	7418	75 (1.01)	41 (0.55)	116 (1.56)

Discussion

In this study, the prevalence of falciparum malaria was found to be more common in the individuals belonging to urban settings than non-urban settings. This finding is in agreement with Kumar (1997), who attributed the phenomenon of higher prevalence of malaria in urban settings due to their rapid expansion and population movement. This study showed that children of 0-10 years had 1.51% infection, and subjects of 21-30 years age-group had highest rate (2.24%) of infection, whereas lowest infection rate was observed in 81-90 years of age-group. However, in another study

Table 2. Sex and age-wise prevalence of *Plasmodium falciparum* in Jodhpur (Rajasthan), India

Age groups	Number of hosts examined			Number of hosts infected			Percentage of infection		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
0-10	415	313	728	03	08	11	0.72	2.56	1.51
11-20	1039	535	1574	12	05	17	1.15	0.93	1.08
21-30	1150	590	1740	25	14	39	2.17	2.37	2.24
31-40	881	480	1361	20	07	27	2.27	1.45	1.98
41-50	722	328	1050	06	01	07	0.83	0.30	0.67
51-60	285	134	419	04	01	05	1.40	0.75	1.19
61-70	219	119	338	02	05	07	0.91	4.20	2.07
71-80	86	45	131	02	—	02	2.32	—	1.53
81-90	36	18	54	01	—	01	2.78	—	1.85
91-Above	13	10	23	—	—	—	—	—	—
Total	4846	2572	7418	75	41	116	1.55	1.59	1.56

Table 3. Prevalence of *Plasmodium falciparum* in Jodhpur (Rajasthan), India with respect to different variables

Status	Male No. infected (%)	Female No. infected (%)	Total No. infected (%)
Urban	59 (50.86)	36 (31.03)	95 (81.89)
Non-urban	14 (12.07)	07 (06.03)	21(18.10)
Literate	70 (60.34)	35 (30.17)	105 (90.51)
Illiterate	05 (04.31)	06 (05.17)	11 (09.48)
Travelers	21(18.10)	—	21(18.10)
Mixed infection	11 (09.48)	12 (10.34)	23 (19.82)
Re-infected cases	02 (01.72)	01(00.86)	03(02.58)
Trophozoite	37(31.90)	20 (17.24)	57(49.13)
Trophozoite with gametocyte	17 (14.66)	06 (05.17)	23(19.82)
Gametocyte	01(0.86)	02 (01.72)	03(02.58)
Mild	28 (24.14)	17 (14.66)	45 (38.79)
Moderate	20 (17.24)	09 (07.76)	29 (25.00)
High	07 (06.03)	01 (0.86)	08 (06.89)
Smear	27 (23.28)	10 (08.62)	37 (31.90)
Card	14 (12.07)	08 (06.90)	22 (18.97)
Q.B.C.	27(23.28)	17 (14.66)	44 (37.93)
Strip	06 (05.17)	05 (04.31)	11 (09.48)

by Munyekenye *et al.* (2005), children from 1 to 4 years of age were found to have a prevalence rate of 38.80 to 62.8% compared to adults where prevalence varied between 2.90-24.10%. Of the all the positive cases, 37.93% were detected by Q.B.C. test with acridine orange and 31.90% were diagnosed by smear test (thick and thin films of blood) with Leishman stain. Mandiratta *et al.* (2006) in their study also found Q.B.C. to be a more reliable test for *P.f.* detection. It was reported by Joshi *et al.* (2006) that importation of malarial cases was the major cause of seasonal

introduction of malaria in the desert which correlates with our study as well where 18.10% travelers contributed to infection in the area. Mohammad and Juma (2008) found the prevalence rate of *P.f.* to be 52.87%, however in the present study the prevalence of *P.f.* was noted to be quite low (1.56%). This could be due to various interventions undertaken in the recent past to control the disease in area. It is worth mentioning here that in a three year period a demonstration site saw a reduction of 86.2% in cases caused by *P. falciparum* due to community cleaning that reduces the vector density in the area (Anonymous, 2009).

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Platyhelminth parasite spectrum in edible freshwater fishes of Meghalaya

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Abstract

The helminth parasite spectrum of edible freshwater fishes in Meghalaya, North-east India was studied. The different piscine hosts examined included several species of *Labeo*, *Cyprinus carpio*, *Cirrhinus reba*, *C. mrigala*, *Danio* sp., *Puntius sarana*, *Garra* sp. (Cypriniformes), *Channa* spp (Channiformes) and *Clarias batrachus*, *C. gariepinus*, *Heteropneustes fossilis*, *Rita rita*, *Monopterus albus*, *Ompok* sp., *Bagarius bagarius* and *Mystus tengara* (Siluriformes).

The parasites recovered included two Monogenea (*Diplozoon* sp. and *Bifurcophaptor indicus*), five Digenea (metacercaria of *Euclinostomum heterostomum* and *Clinostomum complanatum*, adults of *Genarchopsis punctati*, *Neopodocotyle* sp. and *Phyllodistomum* sp.), several cestodes (*Senga* sp., metacestode of Trypanorhyncha, *Lytocestus* spp and *Djombangia penetrans*), and one acanthocephalan species (*Pallisentis* sp.). The occurrence and distribution pattern of the parasites were also studied regarding the prevalence, mean intensity and abundance of infections. Of all the fish hosts examined, *Channa* spp and catfishes showed a higher intensity of infection compared to cyprinid fishes. However, the overall prevalence of helminth infection in the piscine hosts in the region was found to be quite low.

Keywords

Helminth; Monogenea; Trematoda; Cestoda; Trypanorhyncha; Caryophyllidea; Acanthocephala; Freshwater fishes

Introduction

A great diversity of parasitic organisms viz. protozoans, arthropods and helminths, besides microbes are important in fishes all over the world (Hoffman, 1967). Of these, helminths emerge as a predominant group of parasites thriving on these hosts. Freshwater fishes can serve as a source of medically important parasitic zoonoses that include trematodiasis, cestodiasis and nematodiasis. In recent years diseases transmitted by fish have probably become more widely distributed and have greater economical and medical impacts than recognized earlier (Ko, 1995).

The number of species of helminth parasites of piscine hosts is measured in thousands, many of which are known to be serious menace to their hosts. Several workers studied the helminth fauna of fish hosts describing many newer species or records from India and made further advancements in this field (Mehra, 1980; Srivastava, 1982; Soota, 1983; Malik and Sing, 1992; Gopalakrishna and Jahageerdar, 1996; Chakrabarti and Dutta, 2006). However, the spectrum of helminth parasites of piscine hosts has been scantily studied and there is limited information available on this aspect with regard to Northeast India in general and Meghalaya in particular. Soota and Ghosh (1977) conducted a sporadic faunistic survey of parasites in fishes of Meghalaya and reported the occurrence of 13 species of trematodes in them. In another study, a rich diversity of caryophyllidean cestode parasites was reported to be occurring in siluroid fishes of the region (Chakravarty and Tandon, 1988; Tandon *et al.*, 2005). The present study aimed to make an exhaustive survey of the parasite spectrum of the common food fishes in Meghalaya, with particular reference to cypriniform, channiform, symbranchiform and siluriform fishes.

Materials and methods

Several species of the fish genera *Labeo*, *Cyprinus*, *Cirrhinus*, *Puntius*, *Garra* and *Catla* (Cypriniformes); *Channa spp* (Channiformes); *Monopterusuchia* (Symbranchiformes); and species of *Clarias*, *Heteropneustes*, *Rita*, *Bagarius* and *Mystus* (Siluriformes) were surveyed from different locations in the state of Meghalaya for their platyhelminth parasite spectrum and load (Fig. 1, Table 1).

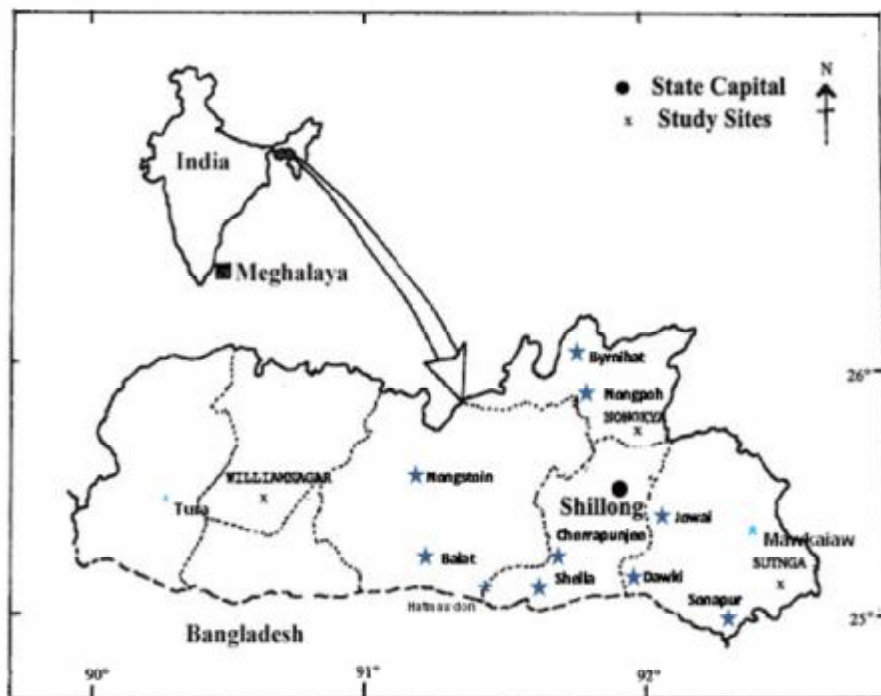


Fig. 1. Map of Meghalaya, showing the different surveyed areas.

Table 1. Piscine hosts examined from different localities in Meghalaya

Locality Name of Host	Dawki	Shella	Balat	Cherrapunjee	Byrnihat	Nongpoh	Ri- bhoi	Nongstoin	Jowai	Sonapur	Tura	Muktapur	Mawkaiaw
Cyprinidae :													
<i>Labeo pangusia</i>	+	+	+	-	-	-	-	-	-	-	-	+	-
<i>Labeo rohita</i>	-	-	-	+	+	+	-	+	-	-	+	-	-
<i>Labeo calbasu</i>	+	+	-	-	-	-	-	-	-	-	+	-	-
<i>Labeo gonius</i>	+	-	-	+	+	-	-	-	-	-	-	-	-
<i>Labeo boga</i>	+	-	+	-	+	+	-	+	-	-	+	+	-
<i>Neolissocheilus</i>	+	-	-	-	-	-	-	-	-	-	-	-	-
<i>hexagonolepis</i>													
<i>Cirrhinus reba</i>	+	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cirrhinus mrigala</i>	-	-	-	-	-	-	-	-	-	-	+	-	-
<i>Puntius sarana</i>	+	-	-	-	-	-	-	-	-	-	+	-	-
<i>Botio Dario</i>	+	-	-	-	-	-	-	-	-	-	-	-	-
<i>Garra sp.</i>	+	-	+	-	-	-	-	-	-	-	-	-	-
<i>Cyprinus carpio</i>	-	-	-	+	-	-	-	-	-	-	-	-	-
<i>Catla catla</i>	-	-	-	+	-	-	-	-	-	-	-	-	-
Channidae :													
<i>Channa striatus</i>	+	-	-	-	+	+	-	-	+	+	-	-	-
<i>Channa orientalis</i>	-	-	-	+	+	+	-	+	+	-	-	-	+
<i>Channa gachua</i>	-	-	-	-	-	-	-	-	+	-	-	-	-
<i>Channa punctatus</i>	+	-	+	+	-	-	-	-	-	-	+	-	-
Siluridae :													
<i>Clarias batrachus</i>	+	-	-	+	+	-	+	-	-	+	+	-	-
<i>Clarias gariepinus</i>	+	-	-	+	-	-	+	-	-	-	-	-	-
<i>Heteropneustes fossilis</i>	+	-	-	+	+	+	+	-	+	+	+	-	-
<i>Mystus tengara</i>	+	-	-	+	-	-	-	-	-	+	-	-	-
<i>Rita rita</i>	-	-	-	-	+	-	-	-	-	-	-	-	-
<i>Chaca chaca</i>	+	-	-	-	-	-	-	-	-	-	-	-	-
<i>Monopterus cuchia</i>	+	-	-	-	-	-	-	-	-	-	+	-	-
<i>Bagarius bagarius</i>	-	-	-	-	-	-	-	-	-	-	-	+	-
<i>Ompak sp.</i>	+	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Host-wise prevalence, mean intensity and abundance of the various platyhelminth parasites of piscine hosts in Meghalaya

Sl.No	Name of Parasite	Name of Host	No. of Hosts examined	No. of Hosts infected	Prevalence (%)	Mean Intensity (MI)	Abundance
1.	Monogenea						
	<i>Diplozoon sp.</i>	<i>Labeo pangusia</i>	78	40	51.2	1.05	0.53
		<i>L. boga</i>	48	6	12.5	1.33	0.16
	<i>Bifurcohaptor indicus</i>	<i>Mystus tengara</i>	65	6	9.23	4.16	0.38
2.	Digenea						
	<i>Genarchopsis punctati</i>	<i>Channa punctatus</i>	134	20	14.9	1.5	0.22
		<i>C. striatus</i>	69	9	13	2.2	0.28
	<i>Clinostomum complanatum</i> (metacercariae)	<i>C. punctatus</i>	134	1	0.74	1	0.007
		<i>C. striatus</i>	69	2	2.89	15.5	0.44
		<i>C. orientalis</i>	84	1	1.19	7	0.08
		<i>Heteropneustes fossilis</i>	138	1	0.72	26	0.18
	<i>Euclinostomum heterostomum</i> (metacercariae)	<i>C. striatus</i>	69	1	1.44	3	0.04
		<i>C. punctatus</i>	134	1	0.74	1	0.007
	<i>Phyllodistomum sp.</i>						
	<i>Neopodocotyle sp.</i>	<i>C. punctatus</i>	134	3	2.23	3	0.06
		<i>L. rohita</i>	41	1	2.43	1	0.02
		<i>L. boga</i>	48	3	6.25	5.3	0.33
3.	Cestoda						
	Pseudophyllidea:						
	<i>Senga sp.</i>	<i>C. punctatus</i>	134	4	2.98	2	0.05
	<i>Trypanorhyncha</i> (metacestode)	<i>Clarias batrachus</i>	72	1	1.38	3	0.04
	Caryophyllidea:						
	<i>Lytocestus spp.</i>						
	<i>Djombangia penetrans</i>	<i>C. batrachus</i>	72	68	94.4	14.5	13.6
		<i>Heteropneustes fossilis</i>	138	6	4.34	8.6	0.37
4.	Acanthocephala						
	<i>Pallisentis sp.</i>	<i>C. orientalis</i>	84	1	1.19	1	0.01
		<i>C. striatus</i>	69	7	10.1	5.57	0.56
		<i>C. punctatus</i>	134	12	8.95	3.16	0.28

Table 3. Host-wise prevalence of newly reported helminths from the region

Sl. No.	Name of parasite	Number of hosts examined (number infected) / Prevalence (%)								
		<i>Labeo pangusia</i>	<i>L. rohita</i>	<i>L. boga</i>	<i>Channa striatus</i>	<i>C. orientalis</i>	<i>C. punctatus</i>	<i>Mystus tengara</i>	<i>Clarias batrachus</i>	<i>H. fossilis</i>
1.	Monogenea									
	<i>Diplozoon</i> sp.	78(40)/51.2	-	-	-	-	-	-	-	-
	<i>Bifurcohaptor indicus</i>	-	-	-	-	-	-	65(6)/9.23	-	-
2.	Digenea									
	<i>Euclinostomum heterostomum</i>	-	-	-	69(1)/1.44	-	134(1)/0.74	-	-	-
	<i>Phyllodistomum</i> sp.	-	-	-	-	-	134(3)/2.23	-	-	-
	<i>Neopodocotyle</i> sp.	-	41(1)/2.43	48(3)/6.25	-	-	-	-	-	-
	<i>Clinostomum complanatum</i>	-	-	-	69(2)/2.89	84(1)/1.19	134(1)/0.74	-	-	138(1)/0.72
3.	Cestoda									
	<i>Senga</i> sp.	-	-	-	-	-	134(4)/2.98	-	-	-
	Trypanorhyncha (metacestode)	-	-	-	-	-	-	-	72(1)/1.38	-
4.	Acanthocephala									
	<i>Pallisentis</i> sp.	-	-	-	69(7)/10.1	84(1)/2.27	134(12)/8.9	-	-	-

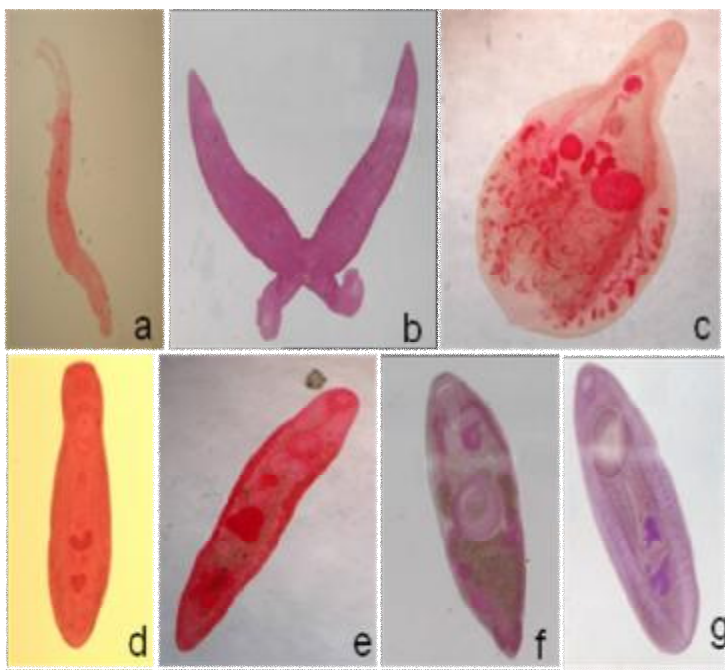


Plate 1. Photomicrographs of monogenean and digenean parasites recorded in the study.

- a. *Bifurcohaptor indicus*. b. *Diplozoon* sp.
 c. *Phyllodistomum* sp. d. *Euclinostomum heterostomum*.
 e. *Neopodocotyle* sp. f. *Genarchopsis punctati*.
 g. *Clinostomum complanatum*.

The fishes were collected from the different collection sites such as streams, rivers, and fish markets etc., brought to the laboratory. Their external body surface and organs such as gills, eyes, scales, fins, buccal cavity etc. were examined for the presence of ectoparasites; the peritoneal lining of the body cavity, the internal organs such as heart, lungs, liver, gall bladder, spleen, stomach, intestine, swim bladder, kidneys, gonads, viscera, mesenteries etc. were scanned thoroughly for the presence of endoparasites. The helminths parasites recovered were counted, fixed in 70% alcohol and processed for suitable whole mount stained preparations following standard protocols.

Their identification was done following Yamaguti (1959, 1961, 1971), Jones *et al.* (2005), Gibson *et al.* (2002) and Khalil *et al.* (1994).

The data were analysed for the following parameters (Margolis *et al.*, 1982):
 Prevalence (%) = Number of infected host x100/Total number of host examined
 Abundance = Number of parasites /Total number of host examined
 Mean intensity = Number of parasites /Total number of infected host

Results and discussion

A total of 12 platyhelminth species were identified in the parasite spectrum. These included two Monogenea (*Diplozoon* sp. and *Bifurcohaptor indicus*), five Digenea (metacercaria of *Euclinostomum heterostomum* and *Clinostomum complanatum*, adults of *Genarchopsis punctati*, *Neopodocotyle* sp. and *Phyllodistomum* sp.), four representatives of the cestode group (*Senga* sp., metacestode of Trypanorhyncha, *Lytocestus* spp. and *Djombangia penetrans*) and one acanthocephalan species (*Pallisentis* sp.) (Plates 1, 2). The distribution of platyhelminth parasites in different piscine hosts examined is shown in Table 2 and those constituting the first or new host and locality records are listed in Table 3.

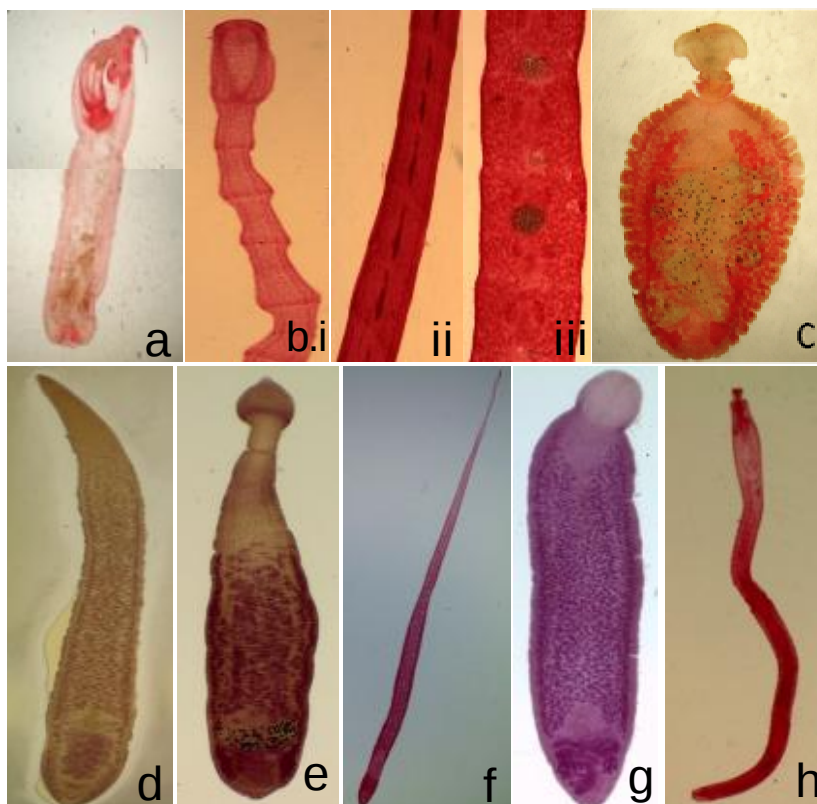


Plate 2. Photomicrographs of cestode and acanthocephalan parasites recorded in the study.

a. Metacestode of Trypanorhyncha. **b.** *Senga* sp. i. scolex with immature segments, ii. immature segments, iii. mature segments. **c.** *Djombangia penetrans*. **d.** *Lytocestus heteropneusti*. **e.** *L. birmanicus*. **f.** *L. filliformis*. **g.** *L. indicus*. **h.** *Pallisentis* sp.

The parasite spectrum among the cyprinoid fishes included one monogenean (*Diplozoon* sp.) and one digenetic trematode (*Neopodocotyle* sp.) with no record of cestodes. Exploration of monogenoidean in fishes of this region is yet to get the coverage it deserves, as only 10-15% of the host species have been screened so far. In India about 2,546 fish species, belonging to 969 genera under 254 families

and 39 orders are known (Talwar, 1991) and the monogeneoids number about 300 species, belonging to 125 genera under 30 families (Pandey and Agarwal, 2008). Various workers have reported the occurrence of *Diplozoon* spp from different parts of the Indian subcontinent. Dayal (1941) described *D. indicum* from *Barbus sarana* which was the first record of the genus from India and was followed by works of Kaw (1950), Tripathi (1957), Kulkarni (1971), Pandey (1973), Gupta and Krishna (1979), Fotedar and Parveen (1987), Agarwal and Kumar (1989), Ahmad and Chishti (1994), Chishti and Peerzada (1995). The occurrence of *Diplozoon* as reported in the present study constitutes the first ever report of the parasite from the region, with *Labeo pangusia* and *L. boga* as new host records.

The *Channa* spp showed a wide range in spectrum of parasites, which comprised the digenenean genera: metacercariae of *Euclinostomum heterostomum* and *Clinostomum complanatum* and adult flukes of *Genarchopsis punctati*, and *Phyllodistomum* sp., the cestode *Senga* sp. the acanthocephalan, *Pallisentis* sp. According to recent reports there are five species of the genus *Neopodocotyle*, all from Indian freshwater fish (Dayal, 1950; Gupta and Chakravarty, 1966; Sircar and Sinha, 1969; Rai, 1971; Agarwal and Kumar, 1986). In Meghalaya, Soota and Ghosh (1977) reported the first occurrence of *Neopodocotyle indica* in the intestine of the host fish *Tor tor* from Shillong, but in the present study *Neopodocotyle* sp. was reported from different hosts, *L. rohita* and *L. boga*. Sinha (1987) described the pathogenicity of *Genarchopsis goppo* on the stomach wall of the freshwater fish *Channa gachua*; however, *G. punctati* reported from *C. punctatus* and *C. striatus* in our study did not show any such pathogenicity to the host species. A number of *Phyllodistomum* spp have been described so far from freshwater fishes of India (Dayal, 1949; Kaw, 1950; Gupta, 1951; Jaiswal, 1957; Srivastava, 1938; Motwani and Srivastava, 1961; Rai, 1964), but the occurrence of *Phyllodistomum* sp. in our study is the first report from this region. The only cestode recovered in our study was *Senga* sp. from *C. punctatus*. This cestode has earlier been reported mostly from catfishes and from *Channa* spp (Johri, 1956; Ramadevi and Rao, 1973; Gupta and Sinha, 1980; Shinde and Deshmukh, 1980; Majid and Shinde, 1984; Gupta and Parmar, 1985; Gairola and Malhotra, 1986a, 1986b, 1986c; Malhotra, 1988; Banerjee *et al.*, 1990).

The siluriform fishes harboured the monogenean *Bifurcohaptor indicus*, metacercaria of *Clinostomum complanatum*, metacestode of the Order Trypanorhyncha and a variety of monozoic cestodes of the Order Caryophyllidea represented by several species of *Lytocetus* (Mehra, 1930; Ramadevi, 1973; Mackeiwicz, 1981; Chakravarty and Tandon, 1988; Kolpuke *et al.*, 1999; Tandon *et al.*, 2005) and *Djombangia penetrans* (Sahay and Sahay, 1976; Satpute and Agarwal, 1980; Kundu *et al.*, 1985). Thirteen species of the genus *Bifurcohaptor* have been described from catfishes of India (Jain, 1958; Kulkarni, 1969; Tripathi, 1957; Agarwal and Kumar, 1977; Gupta and Sharma 1982; Kumar and Agarwal, 1982; Agarwal and Singh, 1981; Gupta, 1983; Swarup and Jain, 1984; Pandey and Singh, 1989; Pandey *et al.*, 2002). Of these *Bifurcohaptor indicus* Jain, 1958 has been described from *Mystus vittatus*. In the present study *B. indicus* was reported from *Mystus tengara*. Trypanorhynchans are almost exclusively found in marine fish but Southwell and Prasad (1918) found cysts of a trypanorhynchan in the muscles of *Hilsa ilisha* an estuarine fish that is known to have anadromous migratory pattern.

Saxena (1980) described the occurrence of a trypanorhynchan larva in the mesentery tissues of a freshwater fish, *Silonia silondia* from a preserved collection of the material made in the year 1948. This appears to be the first report of a trypanorhynchan larva from a truly freshwater fish from India. The metacestode representing the Order Trypanorhyncha from the catfish *Clarias batrachus* is in fact the first report of the parasite from the northeastern region of the country and from a newer host.

Among the Cypriniformes fishes the overall prevalence of infection varied from 2.43 % (*L. boga*) to 51.2% (*L. pangusia*), with mean intensity of 1-5.3, and abundance of 0.02-0.53. The Channiformes showed the prevalence of infection from 0.74 to 14.9%, mean intensity 1-15.5 and abundance ranging between 0.007 and 0.56. In contrast, the siluriform fishes showed the highest prevalence of infection ranging between 0.72 to 94.4%, mean intensity of 3-26 and abundance 0.04-13.6. Among the parasites recorded, the most predominantly occurring group was the monozoic cestodes with the prevalence of 94.4% in *C. batrachus* and a mean intensity of 26 in *H. fossilis*. The next most frequent species was the monogean *Diplozoon* sp. with a prevalence of 51.2% and mean intensity of about 1.33. A rich diversity of caryophyllidean cestodes has been reported to be occurring in siluroid fishes of the region (Chakravarty and Tandon, 1988; Tandon *et al.*, 2005). The Channiformes and the catfishes showed higher prevalence and intensity of infection compared to the cypriniform fishes. The overall prevalence and intensity of infection was found to be generally quite low among the fish hosts examined.

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Occurrence of the metacestode of *Taenia* sp. and the nematode, *Capillaria hepatica* in liver of wild rodents from bamboo growing areas of Mizoram

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Abstract

Rodents are destructive pests inflicting incalculable loss to agro-based economy and aid in dissemination of many parasitic infections causing zoonoses. In Mizoram (Northeast India), rodent outbreaks are known to occur periodically with the onset of bamboo flowering causing a tremendous destruction to food grains and as per the folk belief, often resulting in famine. In view of their underlying threat as serious pests of crop plants and also as reservoir of zoonoses, a study on the parasite spectrum of rodent hosts in Mizoram was undertaken, with special references to identifying plausible infectious agents that could serve as a potential tool for biological control.

A total of 278 rodents belonging to 6 genera and 9 different species were collected from different localities and locations in Mizoram. Two worm parasites- *Capillaria hepatica* and the metacestode of *Taenia* sp. were frequently encountered. Whereas 22.3% of the rodents harboured creamy white-coloured cysts showing typical taeniid features, in 24.4% lesions associated with eggs or adult worms, consistent with *C. hepatica*, were detected. Histopathological and histochemical studies of the infected liver revealed the presence of the metacestode inside a fibrous tissue encapsulation. Presence of spindle-shaped and multinucleated cells was observed in the granulomatous lesions surrounding the eggs of *C. hepatica*. Central necrotic area and septal formations mainly characterized the lesions in the liver infected with both the parasites.

Keywords

Melocanna baccifera; Rodents; *Capillaria hepatica*; *Taenia* sp.; Histopathology; Bio-control

Introduction

Rodents, a diverse groups of mammals characterized by their chisel-shaped incisors, are one of the most successful, abundant and destructive pests inflicting incalculable losses to standing crops, harvested crops in threshing floors, stored food

grains and other commodities. Rodents also harbour a number of helminth parasites, which can be transmitted to human beings and other vertebrates; they also serve as reservoir host and aid in dissemination of these worms to domestic animals and man thus causing zoonoses.

In the state of Mizoram (Northeast India) the rodent outbreaks are intertwined with the onset of bamboo flowering, particularly of the species *Melocanna baccifera*, causing a tremendous destruction to food crops and resulting in famine. This gregarious bamboo flowering, which is known locally as 'Mautam', occurs periodically after every 48 ± 1 years and since the last confrontation with the flowering was in 1959, it was again expected during the period 2007-2009. About 31% of the total forest area of Mizoram is covered by bamboo forests, of which about 90% is covered by *M. baccifera* alone, which has been flowering since the year 2003. According to the folk belief, a tremendous rodent outbreak occurs concurrent with this event, as the rodents feed on the flowers and seeds of the dying bamboo trees, which, in turn, triggers a rapid increase in the birth rate of the rodents leading to a huge population explosion of the rats rampaging through vast agricultural fields and destroying paddy and vegetables. The rat rampage does not confine itself to agricultural fields alone but even ventures into human dwellings, attacking granaries etc and often resulting in famine. There is evidence to suggest that occurrence of famine in concurrence with the bamboo flowering phenomenon is a real happening and not merely a superstitious belief (John and Nadgauda, 2002).

Although various measures have been taken to control outbreak of rodents in Mizoram, exhaustive studies on their parasites have never been undertaken. In view of their underlying threat as serious pests of crop plants and also as reservoir of zoonoses, a study on the parasite of rodent hosts in Mizoram was undertaken. Two parasites, both of which occur in the liver - the metacestode of *Taenia* sp. and a nematode *Capillaria hepatica* were frequently encountered during the survey study.

Taenia taeniaeformis is a taeniid cestode found in the intestine of cats, other felines and carnivores as definitive hosts. Rodents serve as the intermediate host, in which the larval form or the metacestode, *Cysticercus fasciolaris*, develops in the liver and other organs as a fluid-filled bladder worm. Sarcomas of the rat liver due to the presence of *C. fasciolaris* have long been known and several experiments using this metacestode to induce malignant growth of the connective tissues in the liver of rats were successfully conducted as early as the first quarter of the twentieth century (Bullock and Curtis, 1920, 1924-1926, 1928). Liver fibrosarcoma due to the presence of *C. fasciolaris* was suggested to be an appropriate model for studying parasitic carcinogenesis and pathogenesis in wild rats (Tucek *et al.*, 1973).

C. hepatica is a nematode found in the liver of rodents and other lagomorphs that can also parasitize man (Cochrae *et al.*, 1957; Cislaghi and Radice, 1970; Berger *et al.*, 1990; Choe *et al.*, 1993) and has been the most frequently encountered species in wild and house rodents (Junker *et al.*, 1998; Seong *et al.*, 1998). A higher prevalence of this parasite in wild rats was observed in northern parts of India (Mittal, 1980; Gupta and Trivedi 1988; Somvanshi *et al.*, 1995; Chahota *et al.*, 1997). The female worms of *C. hepatica* die soon after laying eggs and disintegrate inside the liver, forming focal necro-inflammatory lesions that heal

Table 1A. Collection sites of rodents hosts in bamboo growing areas of Mizoram

S.No	Locality Hosts	Hlimen	Samtlang	Lungleng	Aizawl	Kepran	Bilkhawthlir	Lunglei
1.	<i>Rattus rattus</i>	+	+	+	-	+	+	+
2.	<i>R. nitidus</i>	+	-	+	-	+	+	-
3.	<i>R. norvegicus</i>	-	-	-	-	-	-	+
4.	<i>Mus musculus</i>	+	+	+	+	+	-	-
5.	<i>Berylmys mackenziei</i>	+	+	-	-	-	+	-
6.	<i>B. bowersi</i>	+	-	+	-	-	+	-
7.	<i>Bandicota bengalensis</i>	+	-	-	+	+	+	-
8.	<i>Niviventer fulvescens</i>	+	+	-	-	-	+	-
9.	<i>Cannomys badius</i>	+	-	-	-	-	-	-

Table 1B. Collection sites of rodents hosts in bamboo growing areas of Mizoram

S.No	Locality Hosts	Darlawn	Kolasib	Bukvannei	Khawzawl	Sawleng	Lab- maintained
1.	<i>Rattus rattus</i>	+	-	-	-	+	-
2.	<i>R. nitidus</i>	+	-	-	+	+	-
3.	<i>R. norvegicus</i>	+	-	-	-	-	+
4.	<i>Mus musculus</i>	-	-	-	-	-	+
5.	<i>Berylmys mackenziei</i>	+	+	-	-	-	-
6.	<i>B. bowersi</i>	+	-	-	-	+	-
7.	<i>Bandicota bengalensis</i>	+	-	+	-	+	-
8.	<i>Niviventer fulvescens</i>	-	-	-	-	+	-
9.	<i>Cannomys badius</i>	-	-	-	-	-	-

Table 2. Prevalence of the metacestode of *Taenia* sp. and *Capillaria hepatica* in rodents hosts in Mizoram

Parasite Type	Hosts	No. of hosts examined	No. of hosts infected (Metacestodes)	Prevalence (%)
<i>Cysticercus fasciolaris</i>	<i>Rattus rattus</i>	88	26	29.50
	<i>R. nitidus</i>	64	12	18.75
	<i>R. norvegicus</i>	8	4	50.00
	<i>Berylmys mackenziei</i>	22	9	40.90
	<i>B. bowersi</i>	12	2	16.67
	<i>Bandicota bengalensis</i>	20	6	30.00
	<i>Niviventer fulvescens</i>	15	1	6.67
	<i>Mus musculus</i>	44	2	4.54
<i>Capillaria hepatica</i>	<i>R. rattus</i>	88	26	29.54
	<i>R. nitidus</i>	64	26	40.60
	<i>B. mackenziei</i>	22	7	31.80
	<i>B. bowersi</i>	12	2	16.60
	<i>N. fulvescen</i>	15	6	40.00
	<i>M. musculus</i>	44	1	2.27

by encapsulation, calcification and resorption (Luttermoser, 1938; Ferreira and Andrade, 1993; Gotardo *et al.*, 2000). Eggs are released only when the host dies and its liver decays or are eaten by another carnivore and in such cases the egg are released with the faeces of the carnivore. Several studies have been done on the pathogenesis of *C. hepatica* and its effect on the liver tissue. In laboratory mice, the infection can reduce the reproductive output or even cause death of the host (Luttermoser, 1938; Singleton and Spratt, 1986).

In view of their frequent occurrence, the present study aimed to find out the effect of the metacestode and *C. hepatica* on the liver of the infected hosts and to ascertain their potential for biological control of rodents. For the purpose a histopathological approach was adopted.

Materials and methods

A total of 278 rodents were collected from 12 different locations, the commonly prevalent rodent species in the region being *Rattus rattus*, *R. nitidus*, *R. norvegicus*, *Bandicota bengalensis*, *Berylmys mackenziei*, *B. bowersi*, *Mus musculus*, *Niviventer fulvescens* and *Cannomys badius*. (Table 1A, B). The rats were captured alive in the field and brought to the laboratory and autopsied for recovery of the parasites from different organs such as liver, stomach, intestine, lungs and urinary bladder.

Upon necropsy cream-coloured cysts were observed on the hepatic parenchyma and on opening the cyst, a viable creamy white larva was observed. Some of the parasites were processed for morphological studies following standard procedures of fixation and stained whole mount preparation. Presence of *C. hepatica* was also observed by gross examination of the liver tissue and the nematode was easily recognized externally by the presence of irregular white or yellowish white nodules containing the eggs or adult worms and scattered all over the surface of the liver. The liver tissue containing both the parasites was separated and washed with phosphate buffered saline (PBS) and stored at -40°C . The fresh frozen tissues of the infected and control (uninfected) liver was sectioned in cryostat (Model no LEICA CM 1850) at $14\text{ }\mu\text{m}$ thickness and at -20°C and stained with hematoxylin and eosin (H&E). For histopathological studies, alteration if any, in collagen, lipids and number of eosinophils were used as the parameters, for detection of which Masson's trichrome, Sudan black and Congo red methods respectively were used following Pearse (1968).

Results and discussion

In 22.3% of the rodent hosts creamy white-coloured cysts were observed mostly in the caudal and lateral lobes of the liver. The number of cysts collected from a single host varied from 1-15. Morphological examination of the stained parasites revealed typical characteristics of taeniid cestodes with the presence of an armed rostellum with two rows of large and small hooks and four prominent suckers on the scolex (Figs. 1A & B), a long neck and pseudo segmentation of the entire body length with terminal bladder; all of these characters are in consistence with the larvae of *Taenia taeniformis*. In 24.4% of the hosts examined, granulomatous lesions associated with eggs or adult nematode worms, consistent with *Capillaria hepatica*, were detected either scattered on the liver surface or localized in a single lobe. Prevalence of the

metacestode was found to be highest in the rodent species *R. norvegicus*, whereas *R. nitidus* showed highest prevalence of *C. hepatica* (Table 2).

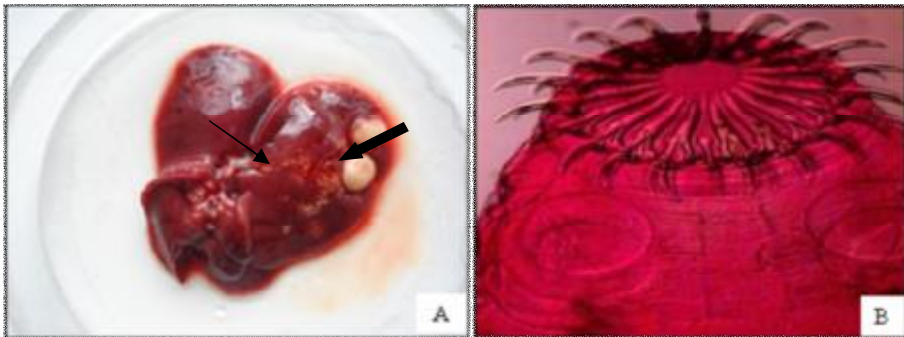


Fig. 1. A- *Taenia* sp. cysts attached to the liver lobe (—→) and irregular white streak showing the presence of *C. hepatica* (—→).
B- Scolex of metacestode of *Taenia* sp. with two rows of hooks and prominent suckers, a closer view.

Histopathological studies of the infected liver revealed distortion of the normal morphology of the liver parenchyma and inflammation due to the presence of both the parasites (Fig. 2). The presence of metacestode was revealed inside a well defined fibrous tissue capsule. The cells appeared spindle shaped and clustered together with abnormal nuclei in and around the area where the metacestode occurs (Fig. 2A); in some areas the cells seemed to be larger as compared to normal and a large number of cells were multinucleated and the normal architecture of the liver cells seemed to be altered. With Masson's trichrome stain neoplastic cells were shown having black coloured nuclei with red coloured cytoplasm and an abundant deposition of blue coloured collagen sheath (Fig. 2B). With Sudan black stain, numerous blue coloured nuclei were found in the *C. hepatica* - infected liver; in fact the presence of the lipids was found to be more on the surface of the *C. hepatica* eggs as compared to the hepatic parenchyma (Fig. 2C). Partially calcified worm debris and collections of immature and mature eggs were found in the area where the *C. hepatica* worms occurred and disintegrated. Granulomatous lesions surrounding the eggs of *C. hepatica* were detected (Fig. 2D); sometimes the lesion contained only a calcified core, besides these inflammatory lesions there also occurred septal formations within the infected liver (Fig. 2E). The clusters of *C. hepatica* egg on the liver parenchyma were clearly visible having ovoid structure with bipolar caps. Abundant eosinophilic cytoplasm was observed in the region where the metacestode of *Taenia* sp. and *C. hepatica* occurred adjacently (Fig. 2F).

The presence of the metacestode of *Taenia* sp. and *C. hepatica* altered the normal morphology of the liver of rats. Hepatic sarcoma associated with *Taenia* sp. in wild rats has been reported (Tucek *et al.*, 1973). In consistence with the present observations spindle-shaped pleomorphic neoplastic cells invading the liver parenchyma and the cells separated by collagen have been reported earlier in metacestode-infected Sprague Dawley rats (Hanes, 1995; Wohrmann and Teredesai, 2002); in addition infiltration of eosinophils, plasma cells and macrophages at the

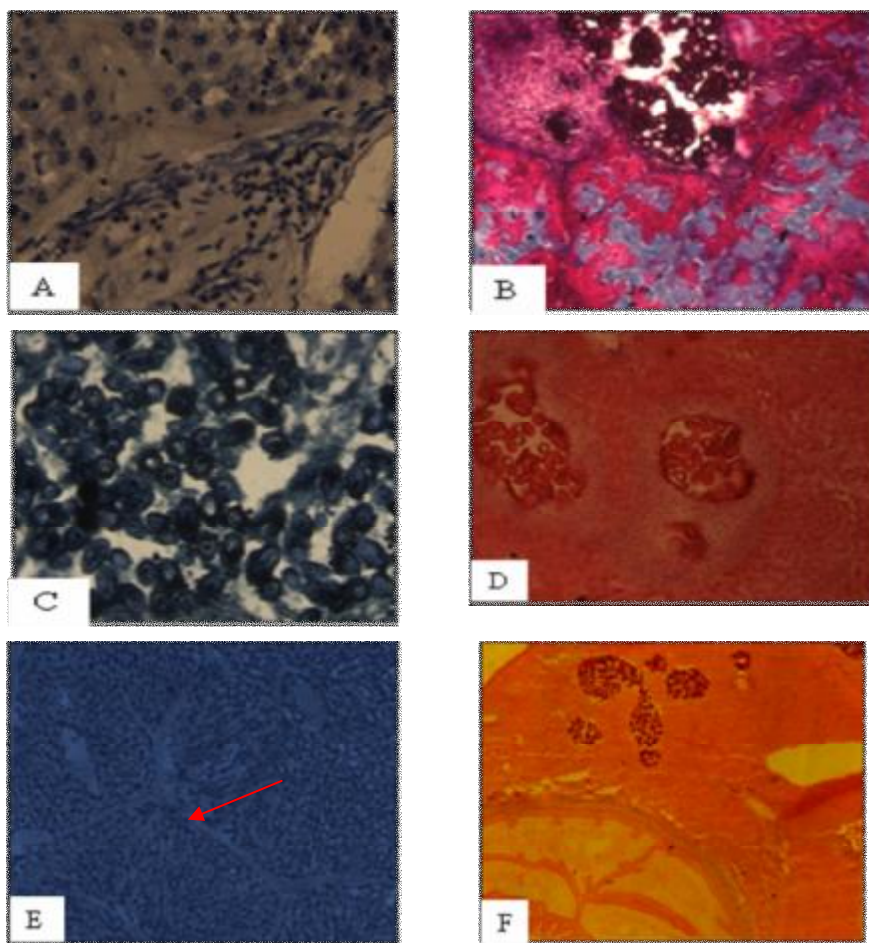


Fig. 2. Histopathology of the parasite-infected liver.

- A. Spindle shaped cells aggregating near the area where the parasite occurs.
- B. Infected liver tissue, showing nuclei (black), Cytoplasm (red) and large deposition of collagen sheath (blue); Masson's trichrome stain.
- C. Presence of lipids in the liver tissues infected with *C. hepatica*; Sudan black.
- D. Granulomatous liver lesion with clusters of *C. hepatica* eggs.
- E. Thin fibrous septa appear across the parenchyma of the infected liver. (→)
- F. Fibrous tissue encapsulation of the metacystode and abundant eosinophilic cytoplasm around the area where both the parasites occur adjacently (Haematoxylin and Eosin).

site of infection was also reported (Kumar *et al.*, 2006). Lethargy, weight loss, anoerexia and sudden death are the clinical signs that have been associated with the presence of the meatcestode in the liver of rats (Hanes, 1995; Tucek *et al.*, 1973). In the case of *C. hepatica*, focal encapsulating fibrous response caused by the dead worms, as also observed in the present study, eventually progresses to form septal fibrosis and cirrhosis has also been reported (de Souza *et al.*, 2000). Considering the pathological effect of both the parasites on the liver of the rodent hosts, it would be worthwhile to investigate further to ascertain their potential as a biological control tool.

Acknowledgement

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Molecular characterization of *Lytocestus* spp (Eucestoda: Caryophyllidea) from *Clarias batrachus* (L.) in Meghalaya

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Abstract

As many as seven species of *Lytocestus* the monozoic cestodes of the Order Caryophyllidea are reported parasitizing *Clarias batrachus* in Northeast India. In the present study, we aimed at determining the validity of these taxa and ascertaining if cryptic species occur in the *Lytocestus* populations. Two frequently encountered species, *Lytocestus indicus* and *L. birmanicus*, are distinguishable from each other on the basis of phenotypic characters viz., scolex morphology, shape of gonads, extent and position of vitellaria and excretory pore, etc. *In- toto* organization of the nervous system in the two species, as revealed by bromoindoxyl staining, exhibits a similarity in having an orthogonal plan, brain in the form of inconspicuous cerebral ganglion, and unmyelinated but ganglionated nerve fibres in the post-scolex region. To ascertain differentiation of the two species at molecular level, DNA was isolated from the adult parasites and the rDNA ITS2 regions were PCR-amplified using platyhelminth universal primers. The sequences obtained were analysed using BLAST and ClustalW bioinformatics tools and phylogenetic trees were generated using MEGA 4.1. Analysis of results is discussed and does help in resolving taxonomic issues pertaining to species of *Lytocestus*.

Keywords

Cestode; Caryophyllidea; Pseudophyllidea; Freshwater catfish; rDNA ITS2; Phylogeny; Nervous system

Introduction

Cestodes of the order Caryophyllidea (Platyhelminthes: Eucestoda) are widely distributed intestinal parasites of benthic-feeding siluriform and cypriniform freshwater fishes of the world with an almost global distribution (Mackiewicz, 1981). They are typified by a monozoic or monopleuroid body plan, i.e. their body lacks internal or external segmentation and has a single set of reproductive organs, unlike that of all other eucestodes. There are 41 genera and about 150 species within the four currently recognized families of the group, which are distributed in all of the

zoogeographical regions: **Balanotaeniidae** Mackiewicz & Blair, 1978, **Lytocestoidae** Hunter, 1927, **Caryophyllaeidae** Leuckart, 1878 and **Capingentidae** Hunter, 1930 (Mackiewicz, 1994, 2003; Oros *et al.*, 2008). There is a very high degree of endemism at the generic and species level. In North-eastern states of India, a rich diversity of caryophyllidean cestode parasites represented by 9 species under 2 genera was reported to be occurring in siluroid fishes of the region (Chakravarty and Tandon, 1988; Tandon *et al.*, 2005), out of which seven species of *Lytocestus* Cohn, 1908 are reported to be parasitizing *Clarias batrachus* alone. Two frequently encountered species, viz., *Lytocestus indicus* Moghe, 1931 and *L. birmanicus* Lynsdale, 1956, are distinguishable from each other on the basis of morphological characters viz., scolex, shape of gonads, extent and position of vitellaria and excretory pore, etc. However, the identification of closely related species, based on these conventional morphological criteria, alone can be difficult (Dick and Choudhury, 1995).

Nervous system appears to be of interest, when investigating from an evolutionary point of view. *In-toto* organization of the nervous system has been studied with histochemical demonstration of nonspecific esterases (NSE), cholinesterase (Che) and butylcholinesterase (BChe), both in adult and larval forms of cestodes (Leflore and Smith, 1976; Kotikova and Kuperman, 1977; 1978; Ramkrishna *et al.*, 1989; Farewether *et al.*, 1990).

DNA techniques utilizing genetic markers in nuclear ribosomal DNA (rDNA) and mitochondrial DNA have been employed to resolve taxonomic issues related to various helminthic parasites. Molecular techniques are increasingly being used for diagnosis, epidemiology, pathogenesis, taxonomy etc. (Vohra, 2001). The nuclear ribosomal DNA internal transcribed spacers (ITS2), have proven useful for diagnostic purposes at the level of species (Kostadinova *et al.*, 2003; Scholz *et al.*, 2004; Prasad *et al.*, 2007; Tandon *et al.*, 2007). Recently, considerable progress has been achieved in studies on the phylogeny of tapeworms (Eucestoda) and new hypotheses based on morphological, life cycle, ultrastructure and molecular data have been proposed (Hoberg *et al.*, 1997, 1999; Justine, 1998; Mariaux, 1998). To date, sequence data of only a few members of Caryophyllidea, Pseudophyllidea and Proteocephalidea are available. Thus, the phylogenetic position of these Orders still has remained problematic (Kodedova *et al.*, 2000; Oros *et al.*, 2008).

In the present study, the main objective was to authenticate species identification and describe the molecular phylogenic location of the species of *Lytocestus* collected from freshwater catfish *Clarias batrachus* (L.).

Materials and methods

Parasite material and morphology

Live specimens of *Lytocestus* were obtained from the intestine of freshly autopsied catfish *Clarias batrachus* and processed for morphological studies using standard procedures. For studying nervous system the parasite specimens, fixed in 10% neutral buffered formalin at 4°C, were processed for histochemical studies for

determining non-specific esterases (NSE) utilizing Holt and Withers', bromoindoxyl acetate method (1952). NSE activity was shown by deep indigo blue colouration.

DNA isolation, amplification, Sequencing and its Analysis

The cestodes were first immersed in digestion extraction buffer (containing 1% SDS, 25 mg Proteinase K) at 37°C overnight. DNA was then extracted by ethanol precipitation technique (Sambrook *et al.*, 1989). The rDNA ITS2 region was amplified from DNA obtained from the cestodes by PCR using the universal primers based on conserved ITS sequences of *Schistosoma* species for ITS2 following Bowles *et al.* (1995). ITS2 region- 3S (forward): 5'GGTACCGGTGGATCACTCGGCTCGTG-3' and A28 (reverse): 5'-GGGATCCTGGTTAGTTTCTTTCTCCGC-3'. The PCR amplification was performed following standard protocol (White, 1993) with minor modifications (Prasad *et al.*, 2007). For DNA sequencing, the PCR products were purified using Genei Quick PCR Purification Kit, and sequenced in both directions using PCR primers on an automated sequencer.

Bioinformatics tools used for phylogenetic analysis

Similarity search was done using BLAST (Basic Local Alignment Search Tool). For phylogentic prediction ClustalW was used for each query DNA sequence and MEGA 4.1 (Kumar *et al.*, 2008), for construction of phylogenetic trees using Neighbor-Joining distance method.

Results

The species of *Lytocestus* encountered in collections were identified as *Lytocestus indicus* and *L. birmanicus*. The general morphological characters which differentiate these species are as follows:

***L. indicus* (Fig. 1):**

Scolex unarmed, short and bluntly rounded, markedly narrower than body and provided with longitudinal furrows in some specimens; testes numerous, occupying medullary region of body, ovoid in shape, larger than vitelline follicles and extending

from base of neck to cirrus sac region posteriorly; cirrus sac prominent, opening separately before utero-vaginal pore; ovary bilobed, wing like in shape, follicular, the two lobes of ovary joined to each other by an ovarian isthmus posteriorly; vitelline follicles corticalar, in a ring around testes, no post-ovarian vitelline follicles present; eggs oval in shape, smooth, embryonated operculated.



Fig. 1. Whole mount stained preparation, LM view of *Lytocestus indicus*.



Fig. 2. Whole mount stained preparation, LM view of *Lytocestus birmanicus*.

L. birmanicus (Fig. 2): Scolex lanceolate, smooth, narrows to form the neck that gradually widens into posterior part of body; testes numerous, medullary in disposition, spherical or oval, extending a short distance from behind level of anterior vitellaria to cirrus sac posteriorly; cirrus sac oval, lined by thick muscular wall, opening slightly anterior to utero-vaginal pore; ovary bilobed, follicular, cortical, extending posteriorly behind Mehlis' gland, united by median isthmus; vitellaria transversely elongated, cortical, arranged in annular manner in between two longitudinal muscle layers, extending as far as utero-vaginal aperture, concentrated in two lateral bands on either side of body, some follicles scattered in median field, no post-ovarian vitelline follicles present; eggs smooth, oval in shape.

Nervous system of *L. birmanicus* revealed a similarity with that of *L. indicus* (Lyngdoh and Tandon, 1992). In both species the brain mass is not distinguishable in the form of cerebral ganglion but appears as a narrow portion junctioning the two main lateral nerves. A dense array of nerve fibres and cells are present posteriad to the junction and the nerve fibres, observed to be non-myelinated nerve fibres (Figs. 3, 4).

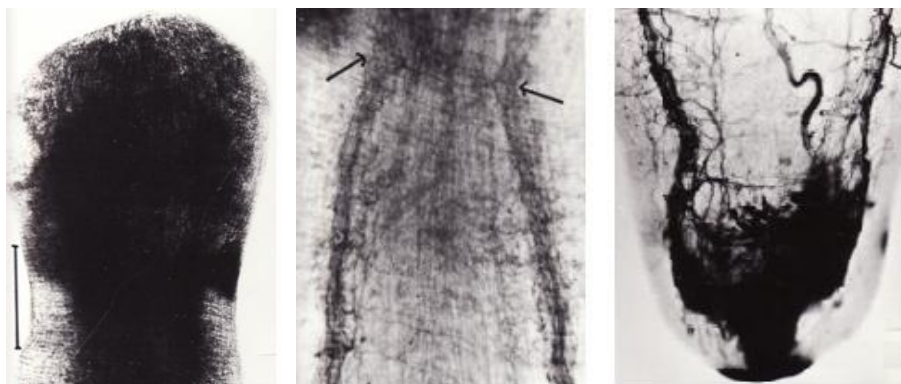


Fig. 3. Nervous system of *L. indicus*, showing scolex region, scolex-neck region and posterior end of the worm (Lyngdoh and Tandon, 1992).

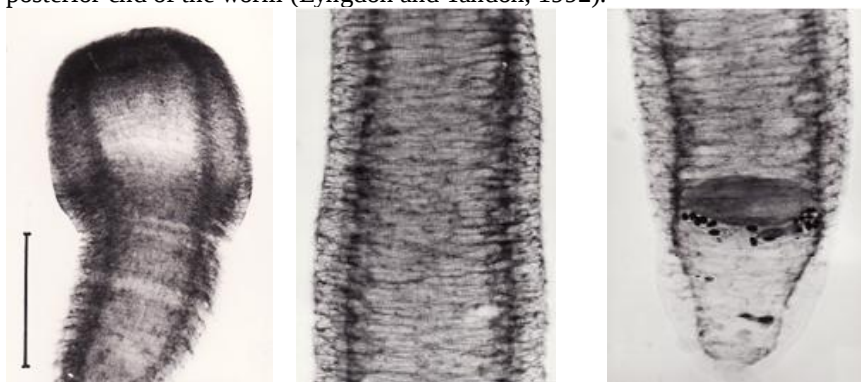


Fig. 4. Nervous system of *L. birmanicus*, showing scolex region, mid-body region and posterior end of the worm.

PCR amplification of ITS regions and its analysis

The PCR amplified products were successfully obtained using the primers as mentioned above and are depicted in Figs. 5a and 5b. Sequence analysis of ITS2 PCR product revealed that alignment of rDNA regions spanning ITS2 were 612 bp and 789 bp respectively for *L. indicus* and *L. birmanicus* (Fig. 6). The nucleotide sequences obtained were compared with available sequences of pseudophyllidean cestode species obtained from Genbank by ClustalW tree-building method (Table 1).

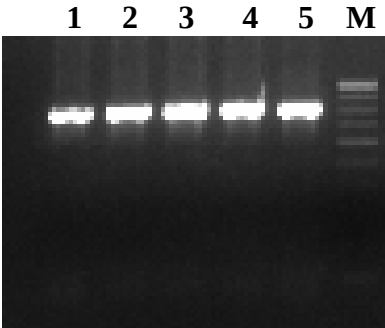


Fig. 5a. Agarose gel stained with ethidium bromide showing the PCR products of *L. birmanicus* for ITS2 (lane 1-5). M = 100 bp DNA ladder.

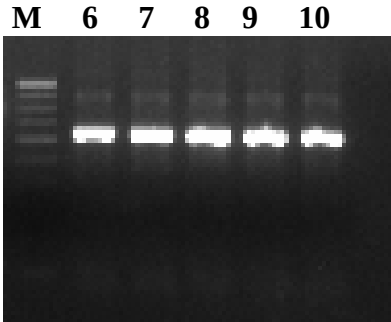


Fig. 5b. Agarose gel stained with ethidium bromide showing the PCR products of *L. indicus* for ITS2 (lane 1-5). M = 100 bp DNA ladder.

Table 1. Pseudophyllidean and Caryophyllidean species used in the study and GenBank accession numbers for corresponding ITS2 sequences

*= Sequences generated as part of this study

Species	GenBank Accession No.	Sequence length (bp)	Classification
<i>Lytocestus birmanicus</i> *	FJ957901*	789	Cestode Order: Family Caryophyllidea: Lytocestidae
<i>L. indicus</i> *	FJ957902*	612	Caryophyllidea: Lytocestidae
<i>Bothriocephalus scorpii</i>	AY340118.1	627	Pseudophyllidea: Bothriocephalidae
<i>B. acheilognathi</i>	EF417920.1	1391	Pseudophyllidea: Bothriocephalidae
<i>Eubothrium salvelini</i>	AF229029.1	1117	Pseudophyllidea: Triaenophoridae
<i>E. crassum</i>	AF229028.1	1119	Pseudophyllidea: Triaenophoridae
<i>Ligula intestinalis</i>	AY549519.1	709	Pseudophyllidea: Diphyllobothriidae
<i>Spirometra folium</i>	DQ386134.1	704	Pseudophyllidea: Diphyllobothriidae
<i>Diphyllobothrium latum</i>	DQ768176.1	1195	Pseudophyllidea: Diphyllobothriidae
<i>Didymobothrium rudolpii</i>	EF042940.1	578	Pseudophyllidea: Spathebothriidae

The BLAST hit results revealed that the query ITS2 sequences show similarity with the available sequences of various pseudophyllidean cestodes viz., *Spirometra*, *Ligula*, *Diphyllbothrium*, *Polyonchobothrium*, *Bothriocephalus*, *Didymobothrium* and *Eubothrium* species from different geographical isolates and different hosts. Intraspecific variation in length and composition of the sequence were observed in ITS2 sequences of the two species (Fig. 6).

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Lytocestus birmanicus CTGACGTGTTCTGCTGACAGGCCGCCGCCG-TAAACAAGGOCAGCCAA
Lytocestus indicus CTGATATGCTTAT-TTAGGGGTAATCAOGACTGATCCGAGGTCAGACAA
**** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus AACATACTCAGCCACGGCCGATGTACTGTACTACTGTGGC-CACCACT
Lytocestus indicus GAAGAAGAACAGCAGCAGCCT-CCTACTCCGCACAACACAACACACTGCT
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus GCTGTCCCGTCAC--CGCGCCTCTCCCAACACAAGGAGTAAACGCGAGC
Lytocestus indicus GTTATGTTGGCAGGTGCAACACATTACACACAGGGGTGTTACTCCAAC
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus AGCAGCAGTAGTAGCGCACAGACACCACGCACACCCAAACACAGCTA
Lytocestus indicus AACAAACGCTG--AGC-CAOGGACG--ATGTGCACGTGCACCCCCCACTA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus TGCGCCAAAAAAGGCCGCCAATGCTACTGGCCCTACACACACACACAC
Lytocestus indicus CACCCCAACACAGCTAC---GCGAC--GACTAAGCCACCGACCCCTCCC
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus AGTCCTACGGAOCTGCATGCACACAGGGCATCTTGAGCCACATTGA-TAA
Lytocestus indicus GGAT----GACCAOGATGC---CGCGGTGGCC--ACTCACGTGAGTAA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus GGAGCCACTGCAGTCCGAGGACACCAAGACAGCTGCTGCTGCTACTGCT
Lytocestus indicus CCCGCCACACCGCGCACCGACACACAGGTGGACTGCTGTTTGAACCAT-
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus GTCATTCTTATCTAGTCAGAGC-ATACATGGTGAGAAGAAGAAGAAC
Lytocestus indicus ---ATTAGAAGGAGCCGCTGCCGTCCATGCTC-GTCTTATGCCAATAAC
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus AACAAACCAACACCACTGCCGGGTGAOGCGCGGGGGAAOCCCTCAA
Lytocestus indicus AACACGCACACAGTACACCCACACTAC-TGAGGCGGGCAGTCTTCCAA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus GCCACGACTAATAAGGCATCGTGATAATTTATAATCAGACCCTCGGCCAG
Lytocestus indicus GCCACGACTAAGATGGCGTCGTGATATATTATACGCCACCCCTCGGCCAG
***** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus ACGTGGCCATAAGGCAAGCCTATGGCCGCAATGTGCGGTTCAAGAGGACG
Lytocestus indicus ACGTGGTCATA-GGCAAGACTATGGCCGCTATTGTG-ITCAAGACGTG
***** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus ATGATCAAAGCAGTCTGCTATTTCACATTATTTCACACAGGTGGCTGCTCT
Lytocestus indicus ATGTTCAAAGCCGTCTGCTATTTCATTTATTTCACACTGGTGGCTGGCGCT
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Lytocestus birmanicus CCTTCATCGACCCACGACCCAGGTGTCCCCCCCCGAACACAAA
Lytocestus indicus C-TTCATCGACACACGACCC-TGATG-CACCCACGACATACA--
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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Fig. 6. ClustalW pair-wise alignment of ITS2 sequence of *L. birmanicus* and *L. indicus*.

Phylogenetic trees

Phylogenetic analyses using ClustalW and multiple tree-building methods in MEGA 4.1 of the ITS2 sequences showed similar topology among trees obtained (Fig. 7), and also revealed a close relationship of *L. indicus* and *L. birmanicus*, both being in the same clade well separated from pseudophyllidean cestodes. Analysis of the query ITS2 sequences of *L. indicus*, *L. birmanicus* and available ITS2 sequences of pseudophyllidean cestodes indicates that query sequences form the basal position in phylogenetic trees obtained by ClustalW and multiple tree-building methods in MEGA 4.1.

Discussion

Taxonomy and systematics of *Lytocestus* spp have been based mainly on morphological data complemented with ecological, cytological and pathological results (Oros *et al.*, 2008). Morphological differences found on stained and mounted adult specimens have been widely used to differentiate and discriminate between platyhelminths species (Miyazaki, 1974; Mackiewicz, 2003). Both the Lytocestoid species are distinguishable from each other in general morphological appearance and diagnostic characters.

The organization of the nervous system in *L. birmanicus* is also in conformity with that of *L. indicus* in having an orthogonal plan with transverse connections in post scolex region and also unmyelinated nerves (Lyngdoh and Tandon, 1992). Thus, minor variations of *in toto* organization of the nervous system cannot differentiate between the two species.

It is not possible to distinguish between *L. indicus* and *L. birmanicus*, just on the basis of their morphology especially in case of live and unstained specimens, as both have a monozoic body plan; they look alike with just a minor differences in the scolex shape, which is short and bluntly rounded in *L. indicus* and lanceolate in case of *L. birmanicus*. The present study provides the first data on the ITS2 sequences of *L. indicus* and *L. birmanicus*, and their comparison with those of the previously studied pseudophyllidean cestodes from different geographical isolates and different hosts ranging from freshwater teleost, marine teleost, fish-eating birds, carnivores and even human. The comparison of ITS sequences of *L. indicus* and *L. birmanicus* indicates that there exist a degree of species-specific variation in the length and composition of their sequences. Analyses of the phylogenetic trees revealed that there are two major groups well separated from each other: the caryophyllidean and the pseudophyllidean, the query sequences, i.e., *L. indicus* and *L. birmanicus* comes under. Analysis of phylogenetic trees shows that the query sequences are closely related forming a well separate clade and well separated forming a basal or near basal position of pseudophyllidean cestode, which indicates that they are the much early evolved species from the evolutionary point of view. In phylogenetic analyses, as a general rule, if bootstrap value for a given interior branch of a phylogenetic tree is 70% or higher, then the topology at that branch is considered 'correct' (Prasad *et al.*, 2007). Our present results showed a bootstrap value to be greater than 70%, among the trees obtained by MEGA 4.1. Thus, ITS2 sequence could be used as species markers for identification purpose and discriminating closely related species,

which from the morphological point of view are very much similar have the same host, same geographical location and even harboring the same location within the host.

Thus PCR-based molecular data of ITS sequence supplement the morphological criteria and confirm that the two frequently encountered species of *Lytocestus*, *L. indicus* and *L. birmanicus*, are indeed two different valid species.

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Seasonal prevalence of intestinal cestodes of goats with respect to age, sex and breed in Kashmir valley, India

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Abstract

This study was carried out with an objective to assess the seasonal dynamics of intestinal cestodes in association with age, sex and breed of goat (*Capra hircus* Linn.) in Kashmir valley (India). Gastrointestinal tract (GIT) and faecal examination were the parasitological methods employed. A total of 1267 (GIT examination: 329; faecal examination: 938) goats were examined during February, 2005 to January, 2007 for intestinal cestodes. Overall, 31.5% (year 1: 31.4%; year 2: 31.6%) of the animals were found positive for infection. The parasites with their respective prevalence were *Moniezia expansa* (4.1%), *Avitellina centripunctata* (2.4%), *Stilesia globipunctata* (2.0%) and *Thysaneizia giardi* (2.1%). Infection was moderate all the year round but was highest during the autumn season with a significant relationship ($p < 0.05$) between seasons and prevalence of infection. Generally lower age groups and nomad breed showed higher infection but with insignificant difference ($p > 0.05$). The present study suggests that seasons affect the prevalence of intestinal cestodes significantly, whereas gender, age and breed of host were not significant factors in the onset of infection under temperate climatic conditions of Kashmir valley.

Keywords

Age; Breed; Goat; Intestinal cestodes; Prevalence; Season; Sex

Introduction

The temperate agro-climatic conditions, traditional animal husbandry practices, poor veterinary infrastructure, and abundance of alpine and sub-alpine pastures are natural determining factors of incidence and severity of various parasitic diseases of livestock in Kashmir valley (Tariq, 2007). Gastrointestinal parasitism is the major cause of damage and decreased productivity in the goat industry, particularly in developing countries. Soulsby (1982) mentioned that infections with tape worms in livestock are relatively less pathogenic but in heavy infections may cause reduced weight gain, diarrhoea and intestinal obstruction. In general, infection with *Moniezia* is highly pathogenic in young animals. Numerous epidemiological studies have been conducted throughout the world to arrive at the detailed information on the gastrointestinal parasites of live stock but scanty information is available on

epidemiology of intestinal cestodes of goats, in particular. The present study was therefore carried with an objective to assess the seasonal prevalence of intestinal cestodes of goats with respect to age, sex and breed in Kashmir valley, India.

Materials and methods

During this study, a total of 1267 goats (329 GIT and 938 faecal samples) were examined over two consecutive years, from February, 2005 to January, 2007. All samples belonged to goats of Bhakarwal (nomad) and Kashmiri (local) breeds. The animals were of both sexes and in the age range of less than one year to more than four years. The samples were collected on monthly basis and analyzed for seasonal prevalence of infection. The collected samples were carefully labelled with animal identification, breed, sex, dental age and month of collection.

Gastrointestinal tract and faecal examination of animals

The gastrointestinal tracts of freshly slaughtered animals in various abattoirs of the valley were collected, tied off at both ends, brought to the laboratory and immediately processed to recover the cestode parasites. The abomasum and, the small and large intestines were thoroughly opened and examined separately for the presence of tapeworms using standard procedures and worms were identified following Soulsby (1982). The faecal samples were obtained directly from the rectum of the animals in suitable air tight containers, properly labelled and brought to the laboratory in 4% formalin and kept at 4°C until processing. The laboratory procedure was as per the methods of Soulsby (1982).

Statistical analysis

Percentages to measure prevalence and chi-square test to measure association between the prevalence of infection and the age, sex and breed of the host were the statistical methods applied using Statistical packages MINITAB software version 13.2 for Windows. Means and standard error of means were also calculated for each parameter tested in this study. The level of significance was statistically accepted at the 5% level ($p \leq 0.05$).

Results and discussion

The epidemiology of intestinal cestodes of goats in the present investigation was studied taking into consideration the seasons, sex, age and breed of the host. Out of the total of 1267 goats examined through GIT and faecal examination, 31.5% of the animals were found positive for infection (year 1: 31.4%; year 2: 31.6%). Overall, quite low prevalence of all the intestinal cestodes was observed in this study (Table 1). The present findings are in line with the observations of various workers in other parts of the country and world. Thangathurai *et al.* (2003) recorded the prevalence of 2.8% of *Stilesia* in goats in Bidder. Muraleedharan (2005) also reported quite low prevalence (0.94%) of *Moniezia* spp. in goats of Karnataka. Prevalence of *M. expansa* was recorded to be 2.6% in White improved goat and 2.2% in Alpen goat in Poland (Gorski *et al.*, 2004). The prevalence of *Stilesia globipunctata* was 0.3% and 0.2% of *Avitellina centripunctata* in goats in southern Mauritania (Jacquet *et al.*, 1995).

Table 1. Overall prevalence of different intestinal cestodes of goats in Kashmir valley, India

Parasite	Prevalence (%)
<i>Moniezia expansa</i>	4.1
<i>Avitellina centripunctata</i>	2.4
<i>Thysaneizia giardia</i>	2.1
<i>Stilesia globipunctata</i>	2.0

Table 2. Seasonal prevalence of intestinal cestodes of goats in Kashmir valley, India

Seasons	Year 1		Year 2	
	No. of hosts examined	No. infected (%)	No. of hosts examined	No. infected (%)
Spring	45	14 (31.1)	40	12 (30.0)
Summer	45	13 (28.8)	44	12 (27.2)
Autumn	45	17 (37.7)	39	16 (41.0)
Winter	40	6 (15.0)	31	4 (12.9)
Mean $\pm$ SE	43.75 $\pm$ 1.25	12.50 $\pm$ 2.32	38.50 $\pm$ 2.72	11.00 $\pm$ 2.51

Seasonal dynamics of infection

The data pooled for seasonal estimation of cestode infection revealed definite seasonality of infection in goats and the observations were statistically significant. The seasonal prevalence as per GIT and faecal examinations has been given in Table 2. The maximum infection was reported during autumn and lowest in winter ($p<0.05$). Our observations in this perspective seem to be in agreement with the agro-climatic conditions of the valley and the modes of animal rearing practiced. The valley has a temperate climate marked by well-defined seasonality consisting of four different seasons with wide variation in temperature and other weather conditions that variedly influence the occurrence of parasitic infections in domestic animals. During spring (March–May) and summer (June–August), the valley experiences maximum rainfall which seems to be conducive for parasite dissemination. During autumn (September–November) it is comparatively dry and in winter (December–February) there is maximum precipitation in the form of snow. These climatic conditions probably act as inhibitory conditions for the survival of intermediate stages of parasites as well as the intermediate hosts on the snow covered grass lands and pastures. A great variation in temperature between different seasons and a grazing break during winter season disrupts the continuous cycle of infection between host and pasture, due to which transmission of parasites does not occur throughout the year. This creates the ideal situation for the animals to remain escaped from infection in winters; however, as the spring advances and the animals start to return to the grazing areas, the overwintered infected mites in the soil and herbage get the chance to enter the intestinal tract of the animals wherein the cysticercoids from intermediate hosts are released and infection sets in. It is assumed that under the prevailing climatic conditions of the region, the tapeworm eggs, released through faeces in post autumn months and early winter, are ingested by mites and psocids in which they develop into larval (cysticercoïd) stage. Thus, the

infected mites overwinter and are available for infection to animals in ensuing grazing months. Shortage of trees and erect plants in pastures compels the goats to feed on the grass at soil level and thereby they are exposed to herbage contaminated with infected mites. This we consider to be an important risk factor for the otherwise browsing goats to get exposed to the soil and grass-transmitted cestode infections.

Influence of sex, age and breed on the prevalence of infection

The study revealed that male goats were comparatively more infected than females; however, the observations were not statistically significant (Table 3). The differences in the prevalence of infection between sexes could be attributed to differential susceptibility owing to hormonal control. Gorski *et al.* (2004) has also reported that males are more infected with *Moniezia* sp. than female goats in Poland.

After pooling all the data, age-wise epidemiological observations revealed highest prevalence in lower age groups of goats (Table 3). Generally the < 1 year age group was more infected ($p \leq 0.05$). With the increase in age the infection level decreased. That the lower age groups of animals were found to be infected more is because of the low resistance in young animals. The age resistance could be the reason for the recorded low prevalence of infection reported in adults. Our findings are in agreement with Rauf *et al.* (2005) and Thangathurai *et al.* (2003) who also reported young animals to be more prone to infection with tapeworms than adult.

Table 3. Influence of sex, age (years) and breed on the risk of goats to intestinal cestode infections in Kashmir valley, India

Factor	Level	Year 1			Year 2		
		No. of hosts examined	No. infected (%)	P-value	No. of hosts examined	No. infected (%)	P-value
Sex	Male	95	29 (30.5)	0.64	70	21 (30.0)	0.46
	Female	80	21 (26.2)		84	23 (27.3)	
Mean $\pm$ SE		87.50 $\pm$ 7.50	25.00 $\pm$ 4.00		77.00 $\pm$ 7.00	22.00 $\pm$ 1.00	
Age	<1	37	14 (37.8)	0.76	28	11 (39.2)	0.71
	1-2	40	13 (32.5)		38	13 (34.2)	
	2-3	39	10 (25.6)		31	8 (25.8)	
	3-4	34	8 (23.5)		28	6 (21.4)	
	>4	25	5 (20.0)		29	6 (20.0)	
Mean $\pm$ SE		35.00 $\pm$ 2.70	10.00 $\pm$ 1.64		30.80 $\pm$ 1.88	8.80 $\pm$ 1.39	
Breed	Kashmiri	80	22 (27.5)	0.83	73	20 (27.3)	0.81
	Bhakarwal	95	28 (29.4)		81	24 (29.6)	
Mean $\pm$ SE		87.50 $\pm$ 7.50	25.00 $\pm$ 3.00		77.00 $\pm$ 4.00	22.00 $\pm$ 2.00	

The breed-wise prevalence of infection is presented in Table 3; however, the observations were not significant statistically. The investigation revealed that the local Kashmiri breed was found to be less infected than the migratory Bhakarwal breed. This could be attributed to the adaptations of the local breed to the existing climatic and management conditions. Breed differences in parasitic resistance have also been reported for goats in Asia (Chauhan *et al.*, 2003) and Africa (Baker *et al.*, 1998). Gorski *et al.* (2004) from Poland also reported that White improved goat breed was most susceptible to helminth infections than Alpen goat breed.

In conclusion, the present study suggests that seasons affect the prevalence of intestinal cestodes significantly while the sex, age and breed of the host may not be significant factors in the onset of infection under temperate agro-climatic conditions of Kashmir valley (India). These findings may contribute to the existing epidemiological knowledge of the intestinal cestodes of goats and also improve the control strategies of helminthiasis in the region.

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Molecular characterization of intestinal fluke, *Artyfechinostomum sufrartylfex* (Trematoda: Echinostomatidae) using PCR amplification of rDNA ITS regions

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Abstract

Artyfechinostomum sufrartylfex Lane, 1915 is an echinostome intestinal fluke of pigs causing echinostomiasis, a zoonosis. *A. sufrartylfex* infection in humans has been sporadically reported to occur in few Southeast Asian countries including India. However, another echinostome species viz., *A. oraoni* has been found to be of endemic occurrence among the Oraon tribe of West Bengal. Discrete identification of the species implicated in infection becomes difficult if only based on morphological criteria of adult or operculate egg stages of the fluke. With the use of molecular tools assisting the conventional diagnostic techniques, we aimed at finding out species-specific molecular marker(s) for *A. sufrartylfex* that is prevalent in pigs of the North Eastern region of India. DNA of the parasite, collected from swine hosts from local abattoirs, was amplified for conserved rDNA Internal Transcribed Spacer (ITS) regions using universal trematode primers. The sequences obtained (Genbank Accessions- EF027100, EF27101) were compared with available sequences of echinostome and other digenean parasites. The BLAST search revealed a close similarity with other members of the family Echinostomatidae, the closest being *Echinostoma trivolvis* (ITS2) and *E. liei* (ITS1). Based on various tree construction methods phylogeny of *A. sufrartylfex* is discussed.

Keywords

Artyfechinostomum sufrartylfex; Digenea; Echinostome; Internal transcribed spacer; Ribosomal DNA; Polymerase chain reaction

Introduction

Artyfechinostomum sufrartylfex, (syn= *A. malayanum* Leiper, 1901 Railliet, 1925; *Euparyphium malayanum* Leiper, 1911; *Testifrontosa cristata* Bhalerao, 1924; *Paryphostomum mehrai* Jain, 1957; *Neoartyfechinostomum subhrai* Agarwal, 1963; *Echinostoma rehmani* Rao et Niphadker, 1963) is commonly found in intestine of pigs. *A. sufrartylfex* Lane, 1915 is an echinostome intestinal fluke, family echinostomatidae of pigs causing echinostomiasis. Although echinostomiasis occurs worldwide, most human infections were reported from East and Southeast Asia. Despite being rare, at least 19 species of echinostomes from 8 genera have been reported in humans from China, Indonesia, Japan, Korea, Malaysia, Russia, Taiwan and Thailand; sporadic cases have also been reported from Brazil, Egypt, Italy, Mexico, Romania (Haseeb and Eveland, 2000), France (Desclaux *et al.*, 2006) and Philippines (Eduardo, 1991; Blair *et al.*, 1999). In India, the occurrence of echinostomes has been reported from the states of Andhra Pradesh, Assam, Bihar, Tamil Nadu, Uttar Pradesh and West Bengal (Anonymous, 2005). *A. oraoni* has been found to be of endemic occurrence among the Oraon tribes of West Bengal (Bandopadhyay *et al.*, 1989; Maji *et al.*, 1995). Apart from pigs, dogs are also found to be infected (Deodhar *et al.*, 1967). The infection is zoonotic and usually asymptomatic. In humans, it has been sporadically reported to occur in few Southeast Asian countries including India.

The human infection is acquired by ingestion of poorly cooked freshwater fishes, prawns, crabs, molluscs and tadpoles (Adams and Motarjemi, 1999). The first human infection of *A. sufrartylfex* was reported in an 8-year old girl in Assam (Lane, 1915). Two other cases of *A. mehrai* infection were reported from India in 1962 and 1964 (Beaver *et al.*, 1984). Two *A. sufrartylfex* worms were recovered from a 35-year old Thai woman from stool after praziquantel treatment in 2001 (Wanachiwanawin and Ungkanont, 2001). In Korea, the intramolluscan life cycle stages of *E. recurvatum* were recovered from naturally infected snails, *Radix auricularia coreana* and the adult worms, from experimentally infected chick and ducks after 5-17 days post infection (Sohn, 1998). Identification of echinostome eggs as well as adult is difficult because of morphological similarity between biologically different taxa and historical nomenclatural problems (Kostadinova and Gibson, 2000). Collar spination, the first intermediate host (the snail species) and geographical distribution are the main diagnostic characters utilised for identification of the species; sometimes these characters are also of limiting value (Morgan and Blair, 1995). The application of DNA methods to studies on the systematics and population genetics of platyhelminth parasites has become widely accepted recently. The ribosomal non-coding DNA (ITS1 and ITS2) can be utilized for species level diagnosis as these are the most conserved genes in eukaryotic cells showing an extreme sequence similarity even between distant phylogenetic taxa as the rDNA cluster encodes structural components of ribosome (Hillis and Dixon, 1991). By PCR and sequencing approaches, taxonomic issues can be resolved quite accurately by comparing DNA, utilizing genetic markers in nuclear ribosomal DNA in particular (Blair *et al.*, 1996; Tandon *et al.*, 2007; Goswami *et al.*, 2009). In the present study, we amplified ITS1 and ITS2 regions of *A. sufrartylfex* with an aim to access their utility for species discrimination and echinostomid genera as well within the group.

Materials and methods

Preparation of whole mount

Collected parasites were washed in phosphate buffered saline and flattened in 70% alcohol. Whole mounts were prepared using ascending grades of alcohol and Borax Carmine; specimens were cleared in methyl benzoate and mounted in Canada balsam to confirm the morphological features.

Parasite material and DNA isolation

Live adult *A. sufrarytjex* flukes were obtained from the intestine of freshly slaughtered pig, *Sus scrofa domestica* at local slaughter houses of Meghalaya, India. To isolate DNA, adult worms were processed singly; they were first immersed in digestion extraction buffer (containing 1% SDS, 25 mg Proteinase K) at 37°C for overnight. DNA was then extracted from lysed individual worms by standard phenol chloroform technique (Sambrook *et al.*, 1989) and also extracted on FTA cards using Whatman's FTA Purification Reagent as described elsewhere (Prasad *et al.*, 2007). DNA from the eggs was extracted only with the FTA card technique. The isolated DNA samples were amplified for ITS1 and ITS2 segments in 25µl PCR reaction.

DNA amplification, Sequencing and its Analysis

The ITS regions of the rDNA were amplified from genomic DNA obtained from the adult stage, by PCR technique using the universal primers based on conserved ITS sequences of *Schistosoma* species (Bowles *et al.*, 1995) as detailed below:

- 1) ITS1 region- **BD1** (forward): 5'GTCGTAACAAGGTTTCCGTA-3' and **4S** (reverse): 5'TCTAGATGCGTTCGAA (G/A) TGTCGATG-3'
- 2) ITS2 region-**3S** (forward): 5'GGTACCGGTGGATCACTCGGCTCGTG-3' and **A28** (reverse): 5'-GGGATCCTGGTTAGTTTCTTTTCTCCGC-3'

The PCR amplification was performed following the standard protocols as described elsewhere with minor modifications (White, 1993; Prasad *et al.*, 2007). The DNA sequences were put to further analysis by using various bioinformatics tools such as, BLAST (<http://www.ncbi.nlm.nih.gov/blast>) and ClustalW (<http://www.ebi.ac.uk/clustalw>) for each query DNA sequence.

Molecular phylogenetic analysis

Phylogenetic tree building methods presume particular evolutionary models. Therefore, while interpreting the result obtained, we considered different tree building models to entertain possible explanations. ITS sequences were aligned in Bioedit (Hall, 1999) and entered in MEGA 4.0 for construction of phylogenetic trees using Maximum Parsimony and distance method namely Neighbor-Joining (Tamura *et al.*, 2007); the latter method was used so as to augment maximum parsimony as they are less likely to give errors when trees contain long branches (Blair and Barker, 1993).

Results

Morphology

Main characteristic features observed were: Body elongated, spined throughout the surface, collar spines 39, 5 spines in each corner group. Oral Sucker rounded ventral sucker postbifurcal, caeca wavy, testes branched, post-equatorial, cirrus sac small, ovary rounded, uterus intercaecal, genital opening preacetabular and postbifurcal. Anterior and posterior end is narrow, tapering.

PCR amplification and Sequence analysis

The ITS regions of *A. sufrartyfex* DNA were successfully amplified, by using the above mentioned primers. The obtained sequences were compared with other trematodes obtained from Genbank database. The length size of amplified DNA was 552 bp (ITS1) and 706 bp (ITS2), respectively, along with flanking regions.

BLAST search results (Figs. 1 and 2) show that most of the ITS1 sequence are more similar to *E. liei*, *E. revolutum*, *E. trivolvis*, *Hypoderaeum conoideum*, *E. albuferensis* and other echinostomid parasites of family Echinostomatidae and a few show similarity with *Fasciola gigantica* and *Fasciolopsis buski*. Similarly, in case of ITS2 sequences, maximum similarities were from among the family Echinostomatidae and few from the family Fasciolidae. In both ITS1 and ITS2 *Paragonimus westermani* and *Gatrodicroides hominis* were considered as outgroups. Alignments were made using default parameters.

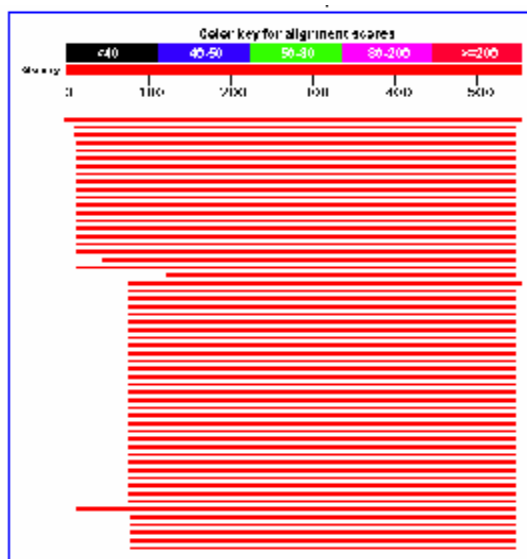


Fig. 1. BLAST search result for ITS1 sequences of *A. sufrartyfex*.

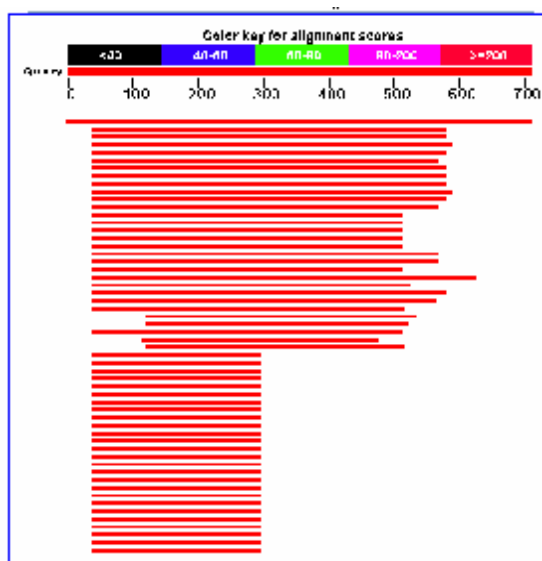


Fig. 2. BLAST search result for ITS2 sequences of *A. sufrartyfex*.

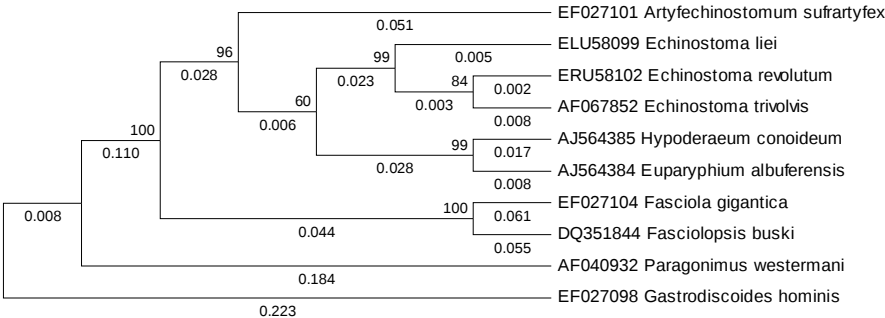


Fig. 3. Neighbor-Joining tree for ITS1.

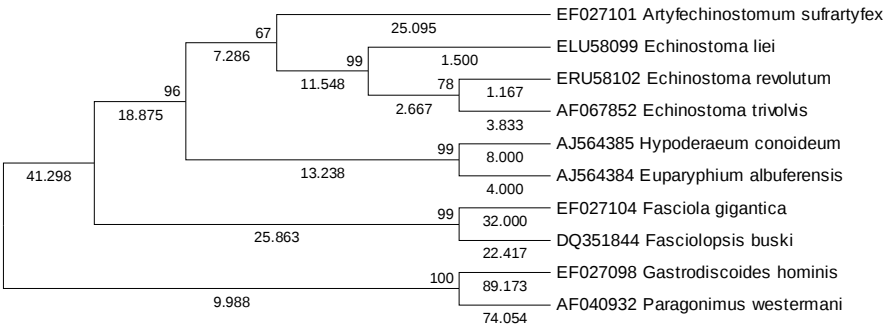


Fig. 4. Maximum Parsimony tree for ITS1.

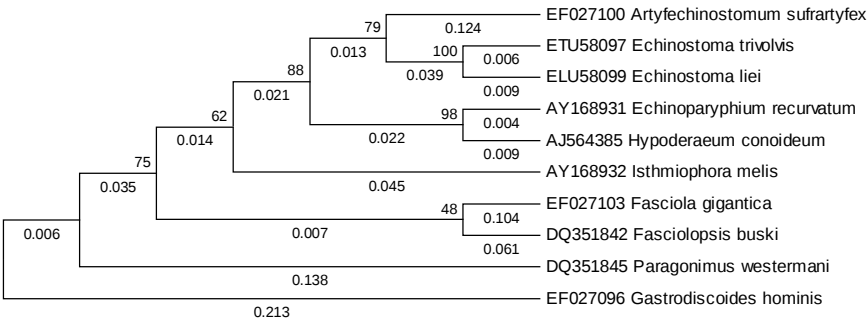


Fig. 5. Neighbor-Joining tree for ITS2.

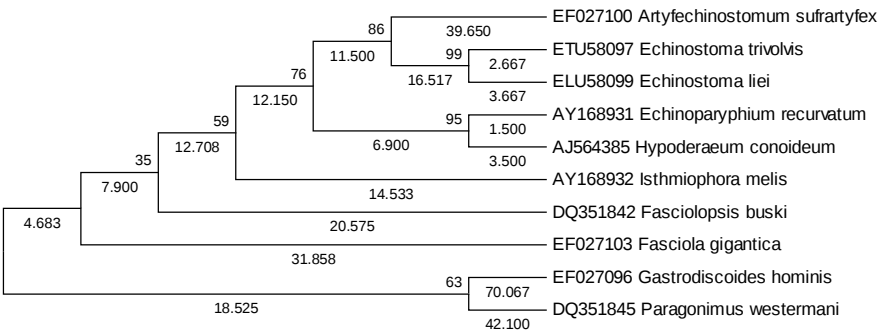


Fig. 6. Maximum Parsimony tree for ITS2.

Phylogenetic tree

Phylogenetic trees were constructed using Maximum Parsimony and Neighbor-Joining methods available in MEGA 4.0 version, by comparing the sequences of *A. sufrartylfex* and available ITS sequences for other digenean trematodes including echinostomatid and fasciolid species (Figs. 3-6). Phylogenetic analysis using various distance methods and character state method like maximum parsimony show that the topology is similar among trees obtained. Bootstrapping of the sequences with neighbour joining revealed significant support (the values 70% and above in the bootstrap test of phylogenetic accuracy indicates reliable grouping among different members of Echinostomidae).

Discussion

Platyhelminth species discrimination is based on morphological differences found on stained and mounted adult specimens (Miyazaki, 1974). The earlier taxonomic studies of *A. sufrartylfex* have been based on morphological, ecological and clinical manifestations. Based upon the morphological features parasite material was identified as that of *A. sufrartylfex* (Roy and Tandon, 1996). Lack of molecular data on genotypic diversity of other artyfechinostomatid flukes in India creates difficulty to perform fine scale phylogeographic analysis of populations and does not allow the origin of regional populations to be unambiguously determined. In recent times, molecular methods are utilised as a new tool for larval as well as adult identification which is based on DNA sequencing approaches (Jousson *et al.*, 1998). rDNA can also be utilised for clarification of digenean life cycles (Bartoli *et al.*, 2000; Jousson and Bartoli, 2000). The ITS1 region, which is more variable, can be utilised for intra-specific patterns of variation (Van Herwerden *et al.*, 1998, 1999). Due to the conserved nature of ITS2 sequence, it can be used as a diagnostic marker at the genus and species level (Adlard *et al.*, 1993; Kane and Rollinson, 1994, Blair *et al.*, 1997a, 1997b). Compared to ITS2, the ITS1 sequences in the present study showed a higher bootstrap value of 100%, confirming that it is a highly conserved monophyletic group. This is in accordance with other studies on trematodes that indicate sequences in the ITS2 might be less conserved than those in the ITS1 region (Luton *et al.*, 1992).

Based upon the tree constructed two distinct clusters are revealed in our study: one of Echinostomatidae and other for Fasciolidae. Our query sequence were found out to be more similar to *Echinostoma caproni*, *E. liei*, , *E. revolutum*, *E. trivolvis*, *E. paraensei*, *E. friedi*, *Hypoderaeum conoideum*, *E. albuferensis*, *E. recurvatum* and showed similarity with *F. gigantica*, *F. buski*, *P. westermani* and *G. hominis* (India).

In search of molecular markers for *A. sufrartylfex*, we characterized the ITS regions of rDNA. The present study herewith provides the first molecular characterization of this species using ITS sequences. However, to ascertain intra-specific strain variations, if any, and to determine the population structure and genetic variability, different geographical isolates of *A. sufrartylfex* from the region need to be studied with the use of additional molecular markers.

Acknowledgements

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Observations on the occurrence of *Opisthorchis pedicellata* in *Rita rita* from Varanasi, India

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Abstract

Opisthorchis pedicellata Verma, 1927 is a common trematode parasite occurring in the gall bladder of the freshwater fish, *Rita rita* (Ham.) from Varanasi, India. The seasonal prevalence of the fluke was studied for one annual cycle. The fluke occurred throughout the year but in varying intensities and showed seasonal cycle of occurrence. The percentage of infection varied from 28.0 to 86.0% and the number of parasites per infected fish varied from 1 to 7. The highest infection was observed in the month of September when 86.0% of the fishes were found infected with the fluke. The infection was lowest during December when only 28.0% of the fishes examined were found infected. The highest intensity was recorded in September and the lowest during the month of January and May. The incidence and intensity were found to be independent of the size of the fish but female fish were noted to be more heavily infected.

Keywords

Fish; Incidence; *Opisthorchis pedicellata*; Seasonal prevalence; Trematode; *Rita rita*

Introduction

Opisthorchis pedicellata is a common trematode reported in freshwater fishes of India. It was first described by Verma (1927) from *Rita rita* and *Bagarius yarrelli* from Allahabad. Subsequently, Mehra (1941) reported *O. pedicellata minuta* from *Mystus seenghala* and *Wallago attu*. In the present study, we report its occurrence in the gall bladder of *Rita rita* obtained from fish markets of Varanasi. This paper presents observations on the seasonal occurrence of *O. pedicellata* and relation between levels of infection with sex and length of the fish host.

Materials and methods

The live samples of fishes for the present study were collected during March, 2005 to February, 2006 from local fish markets of Varanasi. Data were maintained on total length and sex of each examined fish. A total of 389 fishes were examined for the presence of *O. pedicellata*. The recovered flukes were fixed and duly processed using standard protocol for whole mount study. The data on the number of flukes

collected from each fish were also recorded and analyzed on monthly basis during the said period.

Results

Seasonal changes in incidence and intensity of *O. pedicellata* infection

The data regarding the number of fishes examined from March, 2005 to February, 2006 and the incidence and intensity of *O. pedicellata* infection are shown in Table 1 & Fig. 1. Of the 389 fishes examined, 258 (66.3%) were found infected with the fluke. In total, 436 adult flukes were collected from the infected hosts, the intensity of infection being 1.6 worms per infected fish. The fluke occurred throughout the year but in varying intensities. Over the period of 12 months, the incidence of infection varied from 28.0 to 86.0%. The peak incidence (86.0%) was recorded in September and the minimum (28.0%) during the month of December. In the remaining months, the infection fluctuated between 30.4 and 80.0%. During the same period the intensity of infection also showed almost the same trend. The maximum intensity (an average of 2.7 flukes per infected host) was recorded in September and the lowest (1.1) during January and May. In the remaining months the intensity varied between 1.3 and 2.3.

Table 1. Seasonal variation in the incidence and intensity of infection of *Opisthorchis pedicellata* in *Rita rita* from Varanasi, India

Month	No. of fishes examined	No. of fishes infected	% of infection	Total no. of flukes collected	Mean intensity of infection
March, 2005	35	27	77.1	37	1.4
April	26	17	65.4	20	1.3
May	36	11	30.4	12	1.1
June	30	22	66.7	37	1.9
July	29	23	79.3	34	1.5
August	25	20	80.0	45	2.3
September	50	43	86.0	118	2.7
October	23	17	73.9	29	1.7
November	30	20	66.7	28	1.4
December	25	7	28.0	9	1.3
January, 2006	30	16	53.3	17	1.1
February	50	35	70.0	50	1.4
Total	389	258	66.3	436	1.6

Infection in relation to sex and size of the host

The data related to the host-sex and distribution of *O. pedicellata* is given in Table 2 and also depicted in Fig. 2. Out of the total 389 fish examined (193 males and 196 females) 124 males (64.3%) and 134 females (68.3%) were found to be infected with the fluke. The level of infection in males and females varied in different months. Females were found to be infected more in the month of March, April, May, June, July, October and November and the difference was significant. In August, males and females were infected to the same level. In the remaining months more males were

infected but this difference was not significant. The intensity of infection also appeared higher in females. The data related to the occurrence of fluke in relation to the size of the fish is shown in Fig. 2. The study revealed no occurrence of fluke infection in the fish under 21 cm. There was no relationship between the mean parasite burden and the size of the fish.

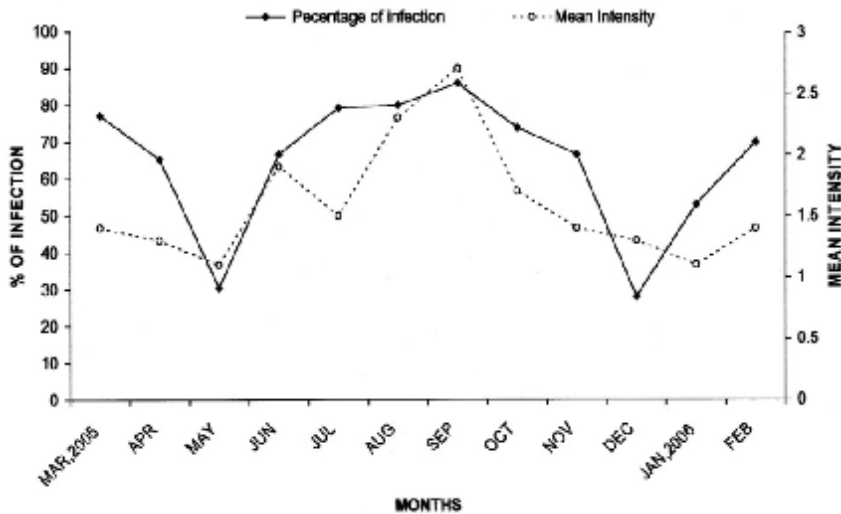


Fig. 1. Graph showing seasonal variations in the incidence and intensity of infection of *Opisthorchis pedicellata* in *Rita rita*.

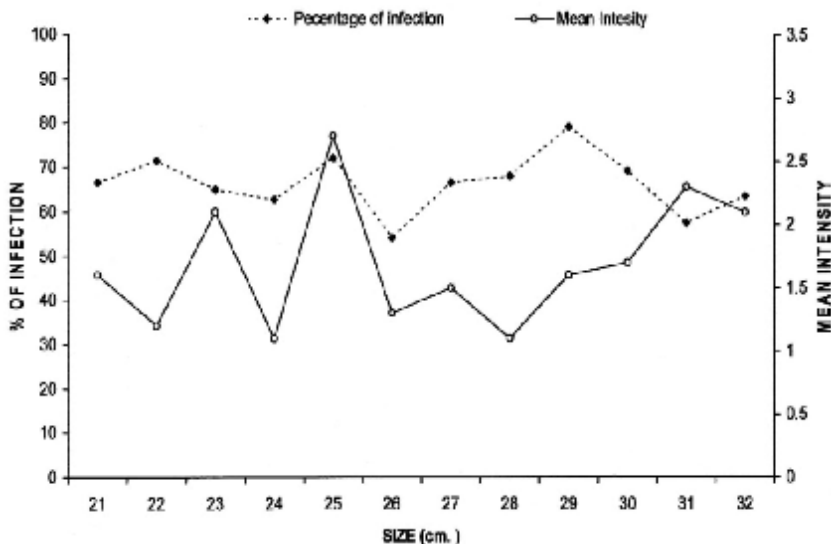


Fig. 2. Graph showing seasonal variations in the incidence and intensity of infection of *Opisthorchis pedicellata* in relation to size of *Rita rita*.

Table 2. Incidence and intensity of infection of *Opisthorchis pedicellata* in relation to sex of *Rita rita* from Varanasi, India

Month	No. of fishes examined		No. of fishes infected (%)		No. of flukes collected		Mean intensity of infection	
	Male	Female	Male	Female	Male	Female	Male	Female
2005	16	19	11(68.9)	16(84.2)	15	22	1.4	1.4
March								
April	14	12	8 (57.1)	9 (75.0)	9	11	1.1	1.3
May	15	21	4 (26.7)	7(33.3)	4	8	1.0	1.1
June	17	13	11(64.7)	11(84.6)	20	17	1.8	1.5
July	14	15	10 (71.4)	13(86.6)	14	20	1.4	1.5
August	15	10	12 (80.0)	8 (80.0)	24	21	2.0	2.6
September	23	27	20 (86.9)	23(85.2)	54	64	2.7	2.8
October	13	10	9 (69.2)	8 (80.0)	14	15	1.5	1.9
November	15	15	9 (60.0)	11(73.3)	12	16	1.3	1.5
December	10	15	3 (30.0)	4 (26.6)	4	5	1.3	1.3
2006	13	17	7 (53.8)	9 (52.9)	7	10	1.0	1.1
January								
February	28	22	20 (71.4)	15 (68.2)	27	23	1.5	1.4
Total	193	196	124 (64.3)	134 (68.3)	204	232	1.6	1.7

Discussion

Observations based on studies on the distribution of the parasites of fishes in temperate countries have been known to show seasonal variations but the factors involved are not fully understood. Among trematodes of freshwater fishes, the annual cycle of *Bunodera lucipercae* has been studied by number of workers (Dogiel, 1961; Cannon, 1972; Wootten, 1973). All these workers concluded that the seasonal cycle is temperature dependent. Seasonal cycle in definitive host has also been observed for *Crepidostomum spp.* (Thomas, 1957; Slusarski, 1958; Cannon, 1972). Awachie (1968) found well defined seasonal cycle in occurrence of *C. metoecus* and *C. ferionis* in trout. Seasonal cycles of *Allocreadium isoporum* and *Azygia lucii* have also been shown to be temperature dependent (Malakova, 1963; Halvorsen, 1972).

A review of the literature reveals that rather little information is available on seasonal cycles of digenetic trematodes from freshwater fishes of tropical countries. Pal (1963) observed seasonal variations in the parasitisation of *Hilsa ilisha* by *Sterrhurus monolecithus* and *Faustula brevicrus*. Rumpus (1975) studied the seasonal occurrence of *Nicolla gallica* in fishes and reported that seasonal cycles and intensity of infection of these flukes are related to the availability of infective larvae, changes in the feeding habits and general physiological conditions of fish host. Evans (1977) observed seasonal cycles of *Sphaerostomum bramae* and *Asymphyldora kubanicum* in the roach and stated that seasonal cycles and population intensity may be explained in terms of feeding habits of the host and seasonal fluctuations in the environmental temperature as well as the reproductive state of the host. Madhavi (1979) stated that seasonal cycle of *Allocreadium fasciatus*

depends on the availability of infective stages in the copepods which in turn, depends on a number of physiological features in the environments.

The only relevant study is that of Pal (1963) who observed that in tropical country like India temperature may not play an important role in determining seasonal cycles as there are no wide fluctuations. Observations on *O. pedicellata* indicate that its seasonal cycle depends on the availability of infective stages in the intermediate hosts which in turn depends on a number of physicochemical features of the environment. The peak invasion of *O. pedicellata* in the month of September could be related to peak occurrence of snails in this month (Madhavi, 1979). The peak occurrence of snail created the highest transmission of the fluke to the fishes. The present investigation indicates that the incidence of infection of *O. pedicellata* is independent of host size and there is neither premunition nor age resistance.

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Two new species of xiphidiocercariae from the freshwater snail, *Digoniostoma pulchella* (Bithyniidae) in Palakkad, Kerala, India

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Abstract

Two new species of xiphidiocercariae, *Cercaria* sp. XII Malabar n.sp. and *Cercaria* sp. XIII Malabar n.sp., found in the freshwater snail *Digoniostoma pulchella* (Bithyniidae) are described and compared with related species to establish the systematic position.

Keywords

Xiphidiocercaria; *Digoniostoma pulchella*; Malabar; Digenea

Introduction

A comprehensive survey of freshwater gastropods in reservoirs and ponds of Malampuzha in Palakkad district of Kerala for the larval trematodes revealed two species of xiphidiocercariae from the freshwater snail *Digoniostoma pulchella*. Detailed studies prove that they are hitherto unknown, undescribed and are therefore, reported here as new species. The new species are denoted by Roman numerals followed by Malabar, the region of collection.

Materials and methods

The bithynid snails, *Digoniostoma pulchella*, collected from reservoirs and ponds of Malampuzha in Palakkad district of Kerala, were brought alive to the laboratory and examined for cercariae. The newly emerged cercariae were subjected to detailed morphological studies by staining supravitaly with neutral red. Intramolluscan stages were studied by crushing the snails. Measurements were taken from 10% formalin-fixed larvae and are given in micrometers (μm) with mean values in parentheses. Sketches were drawn with the aid of a camera lucida.

Cercaria sp. XII Malabar n.sp.

Host: *Digoniostoma pulchella* (Benson)

Site of infection: Hepatopancreas

Locality: Malampuzha in Palakkad district, Kerala

Period of collection: May-August, 2005

Deposited in the Department of Zoology, University of Calicut, Kerala, No. Z/Par.Cer.2005 2a-d

Natural infection by this cercaria was found in 17 of 490 snails collected. The cercariae emerged from snails during the day time, with peak emergence from 9:00 am to 1:00 pm, and were found swimming actively with brief periods of rest.

Description (Fig. 1)

Non-virgulate xiphidiocercaria. Body elongate-oval, aspinose, $116-150 \times 83-120$ (138×103) in size. Tail aspinose, $98-120 \times 13-23$ (111×19) in size. Oral sucker round, $26-36$ (31) in diameter. Stylet prominent; stylet walls reinforced except at basal bulbous portion; $15-18$ (17) long, 5 wide. Basal bulbous portion 3 long; shaft 10 long; shoulders moderately developed, 5 wide. Ventral sucker round, $15-20$ (18) in diameter. Mouth sub-terminal, ventral. Prepharynx $3-7$ (6) long. Pharynx globular, muscular, $9-13$ (12) in diameter. Oesophagus $33-43$ (37) long. Caeca not discernible. Penetration glands 3 pairs, lateral to ventral sucker; anterior pair with hyaline contents; posterior two pairs with coarse granular contents; ducts of penetration glands open near base of stylet. Cystogenous glands numerous, round, with granular contents. Excretory bladder V-shaped; excretory ducts and flame cells not discernible.

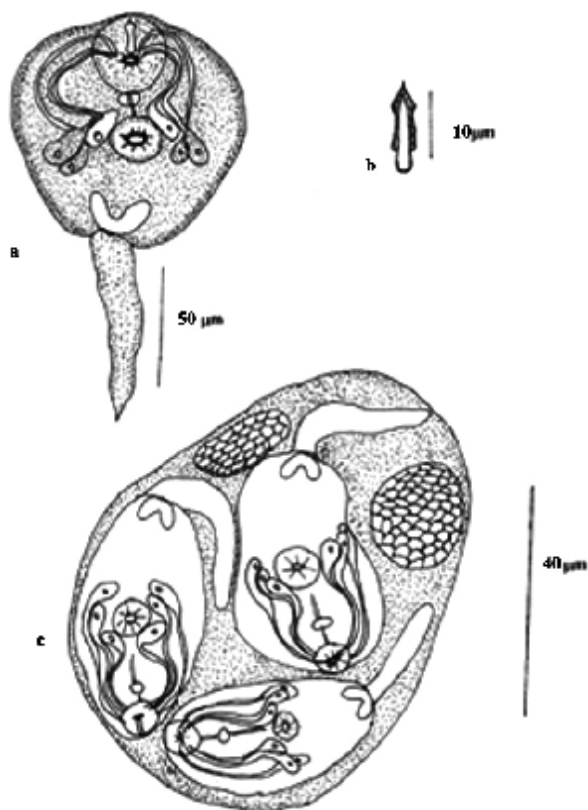


Fig.1. *Cercaria* sp. XII Malabar n. sp.

a . Cercaria b. Stylet c. Sporocyst.

Sporocyst

Sporocysts were recovered from the hepatopancreas of host snails. They were oval, sac-like, $105-155$ (150) long, $99-139$ (111) wide; contain $3-4$ developing cercariae and a few germ balls at different stages of development.

Remarks

Among the valid non-irgulate xiphidiocercariae with pharynx and 3 pairs of penetration glands, the present cercaria comes close to the descriptions of *Cercariae indicae* XVIII, *C. indicae* XIX, *C. indicae* XLVI and *C. indicae* XVI by Sewell (1922). But it is distinct from these cercariae in body size, shape and size of stylet and in the nature and arrangement of penetration glands.

Table 1. Comparison of *Cercaria* sp. XIII Malabar n.sp. with related cercariae

Character	<i>Cercariae indicae</i> XVIII Sewell, 1922	<i>Cercariae indicae</i> XIX Sewell, 1922	<i>Cercariae indicae</i> XLVI Sewell, 1922	<i>Cercariae indicae</i> XVI Sewell, 1922	<i>Cercaria</i> sp. XII Malabar n.sp.
Body	118 x 39; spinose	72-114 x 39; spinose	72-75 x 42-68; aspinose	56-121 x 50-75; aspinose	116-150 x 83-120; aspinose
Tail	118; aspinose	82 x 9; aspinose	93-107; aspinose	53 x 10.5; spinose	98-120 x 13-23; aspinose
Oral sucker	18	21	21	25-38	26-36 (31)
Stylet	Not described	Not described	12.5	21	15-18 x 4-5
Ventral sucker	9	11	11	21	15-20
Pharynx	Present	Present	4	Present	9-13
Penetration glands	3 pairs, anterior, lateral and posterior to ventral sucker; finely granular	3 pairs, anterior and lateral to ventral sucker; finely granular	3 pairs, lateral to ventral sucker; finely granular	3 pairs, round Ventral sucker; Anterior two pairs finely granular, posterior pair coarsely granular	3 pairs, anterior and lateral to ventral sucker; anterior pair hyaline, posterior two pairs coarse granular
Excretory bladder	Reniform	Bicornuate	Bicornuate	Bilobed	V-shaped
Host	<i>Melanoides tuberculatus</i> (Mueller)	<i>Melanoides tuberculatus</i> (Mueller)	<i>Melanoides tuberculatus</i> (Mueller)	<i>Melanoides tuberculatus</i> (Mueller)	<i>Digononiosom a pulchella</i> (Benson)

From Table 1 it would be evident that *C. indicae* XVIII differs further from the present cercaria in having spinose body, reniform excretory bladder and in the absence of intestine; *C. indicae* XIX and *C. indicae* XLVI differ in having bicornuate excretory bladder. Further, *C. indicae* XIX due to its spinose body and absence of intestine and *C. indicae* XVI with bilobed excretory bladder can be distinguished from the present cercaria. Besides, the snail host for the present cercaria is also different from that used by the other cercariae.

It is evident that the cercaria under report is different from the other related forms, and therefore, it is reported here as a new species and the name *Cercaria* sp. XII Malabar n.sp. is proposed after the name of the locality from where they were collected.

***Cercaria* sp. XIII Malabar n.sp.**Host: *Digoniostoma pulchella* (Ben)

Site of infection: Hepatopancreas

Locality: Malampuzha in Palakkad district, Kerala

Period of collection: May-August, 2005

Deposited in the Department of Zoology, University of Calicut, Kerala, India, No. Z/Par.Cer.2005 3a-d

Natural infection by this cercaria was found in 25 of 490 snails collected from Malampuzha in Palakkad district. Cercariae emerged from snails during day time with peak emergence from 9:00 am to 11:00 pm. They were sluggish and moving occasionally with lashing of tail. Most of the time they remained freely suspended in the water column.

Description (Fig. 2)

Non-irrigate xiphidiocercaria. Body club-shaped, aspinose, maximally wide at the anterior region; 60-105 × 36-50 (81 × 45) in size. Tail aspinose, 42-60 × 13-23 (56 × 17) in size. Oral sucker round, 17-36 (22) in diameter. Stylet dagger-shaped, thin-walled, 12-14 (3) long, 2-3 wide. Ventral sucker smaller than oral sucker, round, 8-14 (12) in diameter. Mouth sub-terminal, ventral. Prepharynx 2-3 long. Pharynx globular, muscular, 5-9 (7) in diameter. Oesophagus 17-28 (27) long. Caeca 13-15 (13) long, reaching near ventral sucker. Penetration glands 2 pairs, lateral to caecal

bifurcation; each gland with round nucleus and coarse granular contents; ducts of penetration glands unite and open near mid region of stylet. Cystogenous glands numerous with granular contents. Excretory bladder reniform; excretory tubules and flame cells could not be traced.

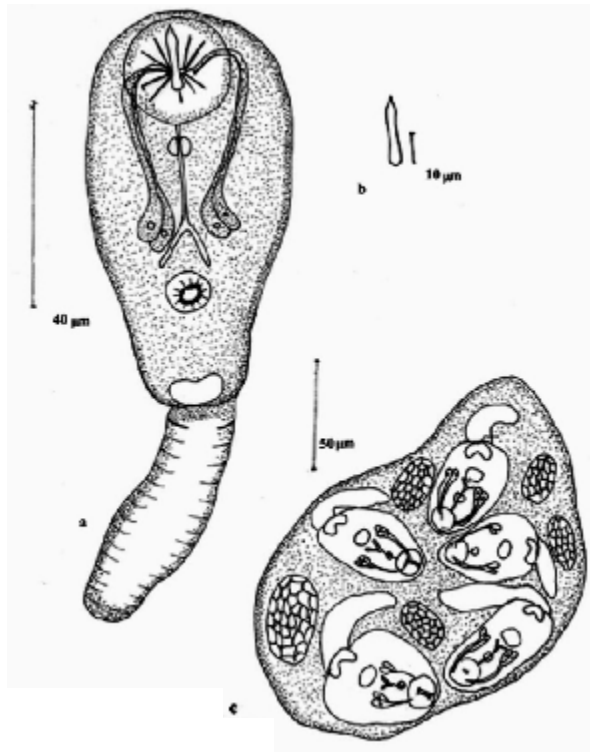


Fig. 2. . *Cercaria* sp. XIII Malabar n. sp.
a . Cercaria b. Stylet c. Sporocyst.

Sporocyst

Sporocysts were recovered from hepatopancreas of host snails. Sporocysts oval, sac-like, 165-231 (192) long, 99-115 (106) wide; contained 4-5 immature cercariae and a few germ balls at different stages of development.

Remarks

The present cercaria with spinose body and 2 pairs of penetration glands needs comparison with *Cercaria pusilla* described by Loos (1900), *Cercariae indicae* LVII by Sewell (1922) and *Cercaria cumanensis* by Nasir (1965). A comparison of characters of the present cercaria with its related cercariae is given in Table 2. It shows significant differences from the above three species in body size, shape and size of stylet, nature and distribution of penetration glands and in having aspinose tail. *C. pusilla* is further distinct from the present form in having 4 pairs of setae on body, inconspicuous ventral sucker, V- shaped excretory bladder and in the absence of intestinal caeca. *C. indicae* LVII and *C. cumanensis* differ again in the presence of spines on tail, absence of intestinal caeca, and in the shape of excretory bladder.

As the present cercaria could not be identified with any other known cercariae it is reported here as a new species and the name *Cercaria* sp. XIII Malabar n.sp. is proposed after the name of locality from where they were collected.

Table 2. Comparison of *Cercaria* sp. XIV Malabar n.sp. with related cercariae

Character	<i>Cercaria pusilla</i> Looss, 1900 XVIII Sewell, 1922	<i>Cercariae indicae</i> LVII Sewell, 1922	<i>Cercaria cumanensis</i> Nasir, 1965	<i>Cercaria</i> sp. XIII Malabar n.sp.
Body	70-100 x 30-50; spinose; with 4 pairs of setae	82-125 x 21-57; spinose	63-75 x 30-39; spinose, with 13 rows of hair like processes	60-105 x 36-50; spinose
Tail	45-75 x 18-25; spinose	46-107 x 17; spinose	60-87 x 12-15 x 18-24; spinose	42-60 x 13-23; aspinose
Oral sucker	18- 25	25	18-24	17-36
Stylet	12-15	12.5	13-16	12-14 x 2-3
Ventral sucker	Not conspicuous	14	10-12	8-14
Pharynx	6-10	7	7-9	5-8
Penetration glands	2 pairs, anterior to ventral sucker' coarsely granular.	2 pairs, pyriform, pre and para- acetabular coarsely granular	2 apirs, pre and para-acetabular coarsely granular	2 pairs, anterior to ventral sucker; coarsely granular
Excretory bladder	V-shaped	I-shaped	Y-shaped	Reniform
Host	<i>Digoniostoma cerameopoma</i> (Benson)	<i>Digoniostoma cerameopoma</i> (Benson)	<i>Marisa cornuarictis</i> (L.)	<i>Diagoniostoma pulchella</i> (Benson)

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Population studies of *Camallanus fotedari* Raina and Dhar, 1972 in its host, *Nemachilus kashmiriensis* Hoar

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Abstract

Freshwater fishes belonging to four families were surveyed but only *Nemachilus kashmiriensis* Hoar was found to be infected with *Camallanus fotedari* Raina and Dhar, 1972. Out of 389 fishes examined, 154 (39.5%) were infected with the parasite at study site *Lidder II*, whereas 144 fishes at study site *Lidder I* had no infection. A total of 338 parasites with mean intensity (2.16 ± 1.8) were recovered throughout the year. Maximum (61.3%) prevalence of infection was found in October and minimum (11.7%) in April. In contrast, the mean intensity of infection was maximum (4.1 ± 4.9) in April, and minimum (1.69 ± 0.8) in October. The age structure of the parasite population varied with seasons; both juveniles and gravid females were recovered throughout the year. A positive correlation ($r = +0.96$) was observed between prevalence and mean length of the fish. Similarly, the mean intensity of infection increased with the increase in length of host which was highly significant ($r = 0.96$). Host sex was not a significant ($|z| = 1.89$) factor, although female fishes were more infected than male fishes. The population of the parasite was over dispersed and fitted best in negative binomial. The variance- to- mean ratios were higher throughout the year. 'k' value was maximum (3.28) in October. The parasite was host- and site- specific.

Keywords

Population dynamics; Kashmir; Nematode; Fish; Prevalence; Age; Over-dispersion

Introduction

Parasite ecology is concerned with the distribution and abundance of parasites in time and space both on and inside their hosts and it involves the study of factors which regulate hosts parasite relationships at different levels. The quantitative studies of the parasites populations have been carried out throughout the globe and many factors like density-dependent and density-independent have been found responsible for regulation of parasite population (Morevac, 1985; Amin, 1986; Lasee 1989). *Camallanus* is a pathogen resulting in heavy loss of blood of their hosts. The early signs of the parasite's presence are typically unnoticed. In the end, the anus and reproductive organs of the fish become so irritated and inflamed that secondary

infection sets in and the swelling causes enough hemorrhaging in the victim to bleed to death or it dies from internal bacterial infection. A detailed study on the population structure of this parasite in its host, *Nemachilus kashmiriensis* Hoar has been done in many parts of the world (Stromberg and Crites, 1975; Chopra *et al.*, 1984; Moravec, 1985; Kumar *et al.*, 1985; Jackson and Tinsley, 1995; Levsen, 2001; Wu *et al.*, 2007).

Ecological studies on helminth parasites prevailing in the Kashmir region have been carried out in the recent past (Dhar and Peerzada, 1992; Chishti and Peerzada, 1998, 2004; Khan *et al.*, 2004) but there has been no report on the ecology of this parasite. Therefore, the present investigation was undertaken to fill this lacuna in ecoparasitology.

Materials and methods

Bionomy of the fish host

Nemachilus kashmiriensis Hoar is a carnivorous and bottom feeder fish. It resides mostly under stones and shows little movement in its habitat. It likes shallow waters with a cover of vegetation. The main food items of this fish are insect larvae, gammarids, crustaceans, molluscs and occasionally annelids.

The fishes for the present investigation were collected with the help of local fisherman and brought live to the laboratory for helminth parasite examination. All segments of the intestine, body cavity and viscera were searched for the parasites and the techniques used for preservation and permanent mounts were the same as reported earlier (Dhar and Peerzada, 1992).

Ecological Analysis

To access the relationship between host sex and infection, a modified chi square test called "Odds ratios" ($\hat{O}$) (Agresti, 1984) was used. The ratio $\hat{O} = \frac{n_{11} \times n_{22}}{n_{12} \times n_{21}}$ where, n_{11} , n_{22} , n_{12} and n_{21} are the cell frequencies of the contingency table. Further, it can be interpreted as a measure of association that is if $\hat{O} = 1$, there is no association between the two variables. If $\hat{O} > 1$ or $\hat{O} < 1$, then it implies that there is association between the two variables. Independence was tested by using Z test.

$$|z| = \log \hat{O} \div (\log \hat{O}) \text{ where } \hat{O} (\log \hat{O}) = \sqrt{1 \div n_{11} + 1 \div n_{12} + 1 \div n_{21} + 1 \div n_{22}}$$

If $|z| > 1.96$ then it implies that association is significant at 5% risk level, otherwise not.

To study the association between infection parameters and host factors like age, weight and length, simple correlation was applied:

$$r = \frac{\sum x y}{\sqrt{\sum x^2 \sum y^2}}$$

To test whether the "r" values obtained were significant or not another test was applied:

$t = r\sqrt{n-2} / \sqrt{1-r^2}$, where “r” is the coefficient of correlation and “n” is the number of observations.

Dispersion and distribution of the helminth parasites were studied using the variance to mean ratios. These ratios were calculated from the frequency tables made for the annual occurrence, seasonal occurrence and for the size classes of the host. The reason for this was that $s^2 > \bar{x}$ is applicable to over dispersion, $s^2 < \bar{x}$ is relevant to under dispersion and $s^2 = \bar{x}$ is related to random distributions (Valtonen, 1983).

The frequency distributions were calculated for the better understanding of the nature of over dispersion. The negative binomial distribution was given by the expansion of the formula $(q-p)^{-k}$ (Crofton, 1971) where

“p” is the probability of infection

“q” is the chance of failure

“k” is the negative binomial constant or carrying capacity of the microhabitat and is also the measure of aggregation.

“k” was calculated using the formula

$\log(N/N_0) = k \log(1 + \bar{x}/k)$, where N is the number of samples, N_0 is the number of samples containing zero number of parasites and $\bar{x}$ is the average number of parasites per sample. The maximum likelihood method was used to estimate parameter “k” (Bliss and Fisher, 1953).

$p = \bar{x}/k$, $q = 1 + p$ and pkq is the negative binomial variance

The ecological terms used in this study are in accordance with the nomenclature given by Margolis *et al.* (1982). In order to study the maturation cycle of the parasite the nomenclature used by Williams (1979), Amin (1987) and Lasee (1989) was followed.

Results and discussion

In the present study, eight fish species were investigated for helminth infections in three snow-fed streams of south Kashmir. Different parasite species belonging to four helminth groups viz. Acanthocephala, Cestoda, Nematoda and Trematoda were recorded (Table 1). *Camallanus fotedari* infected the stone loach, *Nemachilus kashmiriensis* at study site *Lidder II*, while the other hosts were not infected with this parasite. Thus, the parasite was host and site-specific. The reason for its limited host range could either be the host feeding preferences or/and availability of the infection in the habitat. The fish is carnivorous (Subla and Das, 1970). The habitat at *Lidder II* had rich invertebrate community and low water current that provided excellent environment for fish feeding on invertebrates, which also act as intermediate hosts for helminths. Both these factors contributed in the dynamic host-parasite relationship and parasite community richness in this host at *Lidder II*. Similar results on other parasites in different hosts have been attributed to host feeding preferences and availability of intermediate host in the habitat (Amin, 1986; Kennedy, 1974). Table 1 shows that the fish had eight different endoparasites in this habitat, while in rest of the fishes there were three to five parasite species only. The reason for low parasites richness in these hosts was their food habits. They were omnivores with 80% plant matter in their diet (Jan and Das, 1970), therefore, less animal matter in diet probably contributed to low endoparasitic infection in the host. In *Lidder I* the

habitat conditions were quite reverse to *Lidder II*. The invertebrate community consisted of 90% insect larvae, of which the ephemeropteran larvae alone contributing 38% (Kumar and Baghat, 1978). These ephemeropteran larvae are intermediate host for another nematode, *Rhabdochona* (Moravec, 1976). The abundance of this larva in the habitat left hosts with limited dietary preferences and all hosts in this habitat including *Nemacheilus kashmiriensis* had high (63.6%) prevalence of *Rhabdochona* infection. Similar studies have been carried out on endoparasites of Wisconsin fishes (Amin, 1987) in two different habitats where the same host was found infected with different parasite species owing to the host role changes.

Table 1. Prevalence of helminth parasite infection in snow fed stream fishes of Kashmir

	Neo	Pompho	Adeno	Allo	Filo	Phy	Camma	Eust	Diplo	Clino	Habitat	
											Sample	
N.K.	4.5	4.5	-	-	63.6	-	-	-	-	-	LI	144
	43.1	8.2	0.25	0.77	51.9	46.5	39.5	62.2	-	-	LII	389
O.P.	14.5	13.5	19.0	-	39.6	-	-	-	40.2	-	LI	199
	28.5	28.5	14.0	14.2	14.2	-	-	-	57.8	-	LII	14
	84.9	11.3	5.5	-	18.8	-	-	-	37.7	-	Brengi	53
	50.0	19.8	13.0	-	45.0	-	-	-	64.0	-	Arpath	100
S.E.	14.2	71.4	-	-	25.0	-	-	-	14.2	-	LI	17
	28.5	100	-	10.5	57.1	-	-	-	50.0	-	LII	08
	-	16.6	-	-	-	-	-	-	50.0	-	Brengi	12
	14.2	64.2	28.5	-	-	-	-	-	28.5	-	Arpath	14
S.N.	33.3	33.3	18.3	-	-	-	-	-	33.3	-	LI	10
	-	-	-	-	-	-	-	-	48.2	-	LII	04
	-	-	-	-	-	-	-	-	14.2	-	Brengi	-
	-	40.0	-	-	-	-	-	-	41.1	-	Arpath	05
C.L.	-	-	-	-	-	-	-	-	-	-	LI	11
	-	-	-	-	-	-	-	-	-	18.1	LII	10
	-	-	-	-	-	-	-	-	-	50.0	Brengi	---
	-	10	-	-	-	-	-	-	-	-	Arpath	12
S.P.	7.1	78.5	42.5	7.1	-	-	-	-	50	7.1	Arpath	13
S.L.	33.3	80.5	33.3	-	-	-	-	-	33.3	33.3	Arpath	10
S.H.	33.3	33.3	48.5	-	-	-	-	-	33.3	33.3	Arpath	24

N.K. = *Nemachilus kashmiriensis*; O.P. = *Oreinus plagiostomus*; S.N. = *Schizothorax niger*; C.L. = *Crossochilus latius*; S.P. = *Schizothorax planifrons*; S.L. = *S. longipinnis*; S.H. = *S. hugelli*.

Population study of *Camallanus fotedari* in *Nemachilus kashmiriensis*

In the present study, out of 389 fishes examined, 154 (39.5%) were found to be infected with *C. fotedari* (Table 2). Maximum prevalence (61.3%) was observed in October and minimum (11.1%) in April. The maximum prevalence in October was a

Table 2. Seasonal occurrence, distribution and maturation of *Cammallanus fotedari* in its host *Nemachilus kashmiriensis* Hoar

Months	No. of fishes examined (No. infected)	Prevalence (%)	No. of Worms	M.I. (±SD)	Male worms %	Female worms (%) JF Adults	— x	s ²	k	s ² / x̄	
May	40 (7)	17.5	17	2.4 (2.5)	12.5	12.5	66.6	0.60	1.36	0.29	2.20
June	17 (9)	52.9	18	2.0 (4.8)	28.5	-	71.4				
July	54 (21)	38.8	44	2.0 (0.8)	28.5	-	71.4	0.85	1.43	0.67	1.60
August	43 (20)	46.5	35	1.7(1.2)	21.4	35.7	42.8				
Sept	51 (28)	54.9	50	1.7 (0.8)	20.0	36.3	40.2	0.98	1.06	3.28	1.08
Oct	44 (27)	61.3	43	1.6 (0.8)	77.7	50.0	22.2				
Nov	31 (12)	38.7	32	2.6 (0.7)	72.7	18.0	9.0	1.31	4.44	0.40	3.39
Dec	21 (11)	52.3	35	3.1(0.9)	53.8	30.7	15.3				
Jan	22 (6)	27.2	23	3.8 (1.0)	57.6	30.3	6.0	0.94	4.68	0.18	4.98
Feb	10 (3)	30.0	7	2.3 (3.2)	-	100	-				
March	22 (6)	27.7	12	2.0 (1.0)	37.5	25.0	37.5	0.51	2.40	0.41	4.70
April	34 (4)	11.7	17	4.2 (4.9)	38.8	33.3	61.1				
Total	389 (154)	39.5	333	2.1 (1.8)				0.83	1.78	0.53	2.14

result of recruitment of the parasites because the infra population of parasites showed a maximum (77.7%) in males and minimum (22.2%) in adults at this stage. The greater percentage of males in a population at a particular point of time is considered a measure of recruitment of the parasite (Awachie, 1964; Amin, 1975; 1985, Amin and Burrows, 1977; Valtonen, 1980; Zdzitowiecki, 1986). From Table 1 it is evident that during October and in following months the infra population comprised more juvenile females than adults. The mean intensity of infection was minimum (1.6 ± 0.8) in October and maximum in April (4.2 ± 4.9). These results show that maximum hosts were infected during October but with minimum parasites per host and minimum hosts were infected with maximum parasites during April. It was perhaps due to recruitment of parasites during October that large numbers of hosts were infected and establishment of infection had started in April. The maturation of the parasite had also begun. Similar maturation cycle has been reported in *Bothriocephalus acheilognathi*, infecting fishes in a thermally altered reservoir (Granth and Esch, 1983). Moravec (1979) reported spring to summer as suitable time when maximum development of the parasites occurred due to warm climate. The decrease in temperature during winter months also showed a drop in the prevalence of infection. But with the increase in temperature there was increase in prevalence of infection. Chopra *et al.* (1984) correlated increase in temperature with increase in infection in spirurid nematodes infecting coldwater fishes of Gharwal (India). Malik and Singh (1992) attributed increase in nematode infection in hill stream fishes to fluctuation in water temperature. However, the maturation of the parasite followed a definite pattern, with male and juvenile females dominating the population during autumn and adults during spring to summer. Such a seasonal maturation cycle has also been reported in *C. oxycephalus* in white bass (Stromberg and Crites, 1975), in which maturation cycle of the parasite had synchronized with the breeding cycle of the host. This might also be true in present study. The presence of adults throughout the year might be the leftovers in the parasite population, quite similar to observations made by Moravec (1985).

The variance- to- mean ratios were higher in different bi monthly samples. This shows that the population of the parasite was highly over dispersed. The observed frequency was in agreement with the expected observations of theoretical negative binomial. Over dispersion is common among helminth parasites to the extent that Crofton (1971 a, b) included over-dispersion as a parameter in the definition of parasitism. It implies that considerable proportion of the worms is to be found in a relatively small number of hosts (Kennedy, 1968; Pennycuick, 1971; Anderson, 1974; Amin, 1981; Valtonen, 1983; Agarwal 1986; Madhavi and Rukmini, 1991).

Host factors

The prevalence and mean intensity of infection increased with increase in host size. The size class 10.1-11.5 cm had maximum prevalence (42.8%) of infection (Table 3). Similarly the mean intensity of infection was maximum (2.4) in size class 10.1-13.0 cm. A positive correlation was observed between host size, prevalence and mean intensity of infection. These results were also confirmed by *t* test. Host factors like size and age have been studied in relation to infection parameters. The effects of length and weight on the incidence of infection have been carried out by Fox (1962),

Colley and Olson (1963), Hanek and Fernando (1978), Kumar *et al.* (1985), Amin (1986), and Lasee (1989). These authors have attributed increase in infection with increase in host length and weight to the increase in food intake with age. The present results are also similar to these observations made by the authors on the helminths from different hosts all over the globe.

Table 3. Host size and infection parameters of *Camallanus fotedari* in its host *Nemachilus kashmiriensis*

Length class	No. of fishes		% Infected	No. of parasites	Mean intensity
	Examined	Infected			
>8.5	62	23	37.1	44	1.9
8.6-10	138	54	39.1	104	1.9
10.1-11.5	126	54	42.8	130	2.4
11.6-13	54	20	37	49	2.4
13.1-15	9	3	33.3	6	2.0
	r=0.96	t=5.92(0.001)			r=0.96

Table 4. Host sex and infection parameters ($|z|=1.89$) of *Camallanus fotedari* in its host *Nemachilus kashmiriensis*

Sex of the host	No. of fishes		% Infected	No. of worms	Mean intensity
	Examined	Infected			
Male	250	108	40.2	205	1.9
Female	139	62	44.6	128	2.0
		$\hat{O}=1.9$			$\hat{O}=2.0$

Host sex-wise data showed female fishes were more infected than male fishes (Table 4). The odds ratio was also more in favour of the female than male fishes. But the $|z|$ test did not confirm the same. The higher infection in females than males can be attributed to their aggressive feeding during post spawn period. As host size has been found significantly influencing parasite infection parameters, the size of female fish in samples could have also tilted the ratio in favour of female fish. In most of the cases host sex has not been found significantly affecting the parasite occurrence. These results are similar to those reported by Lawrence (1970), Cannon (1972), Stromberg and Crites (1975), Muzall (1980), Sutherland (1989) and Lasee (1989).

Site specificity (Table 5)

The population of the parasite was found occupying the last segment of the intestine and occasionally during heavy infections the parasite also migrated to the anterior segment. The influence of season did not seem to work on the change in site of infection. It also showed that parasite did not share this segment with any other parasite avoiding the interspecific competition for space and resources conversely in acanthocephalan niche segregation and competitive exclusion of parasites has been reported (Kennedy, 1974).

Table 5. Seasonal distribution and site specificity of *Camallanus fotedari* in its host *Nemachilus kashmiriensis*

Months	Intestinal Segments			
	I	II	III	IV
April			2	15
May			1	16
June			3	15
July			7	37
August			0	35
September			10	40
October			5	38
November			4	28
December			2	33
January			0	23
February			0	7
March			0	12
Total			45	288

Conclusion

From the results obtained it can be safely concluded that *C. fotedari* is host- and habitat- specific. Although a large number of hosts were examined, only *Nemachilus kashmiriensis* was infected with the parasite throughout the year. It completed its life cycle in its host with recruitment occurring in autumn season and the seasonal maturation occurred with increase in water temperature. Large-sized fish had more infection, while the sex of the host did not influence infection significantly. The parasite remained confined to the last part of the intestine throughout the year. The parasite population was highly over dispersed.

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Pathogenicity and population dynamics of *Procamallanus spiculogubernaculus* Agarwal- a nematode parasite of fresh water fish, *Heteropneustes fossilis* (Bl.)

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Abstract

Pathogenicity and population dynamics of the nematode parasite *Procamallanus spiculogubernaculus* Agarwal in the fresh water fish *Heteropneustes fossilis* (Bl.) have been studied. The nematode was found attached to the stomach wall of the fish by its buccal capsule. The gastric mucosal tissue of the plug underwent a number of cellular changes. Widespread histolysis was evident in the mucosal plug. Portions of the body of the nematode were seen throughout the stomach wall of the fish. The incidence, intensity and infection of the nematode were higher during summer season in comparison to winter season. Single infection was more common than concurrent infection in the fish. Concurrent infection caused exclusion, niche segregation and reduction in the number of helminth parasites in the fish.

Keywords

Pathogenicity; Population dynamics; Nematode; Parasite; Fish

Introduction

The pathogenicity and population dynamics of helminth parasites have been studied in a number of fishes. Gupta *et al.* (1984) have studied population dynamics of helminth parasites in *Channa punctatus*. Agarwal (1986) studied the dynamics and regulation of endohelminths in three species of fishes at Raipur, India. Niyogi *et al.* (1982) studied the population dynamics of caryophyllaeids in *Clarias batrachus*. Sinha (1993) studied the pathogenicity of *Pallisentis ophiocephali* in the intestine of *Channa punctatus*. Sinha and Mehrotra (1991) studied the pathogenicity of *Bothriocephalus aceilognathi* in the intestine of *Xiphophorus helleri*. In the present study we report the pathogenicity and population dynamics of *Procamallanus spiculogubernaculus* Agarwal in the stomach wall of *Heteropneustes fossilis* (Bl.).

Materials and methods

The fishes were collected from local ponds around Jamshedpur. For histopathological studies both infected and uninfected stomach samples were fixed in aqueous Bouin's and Zenker's fixatives, dehydrated in the usual manner, cleared in xylene and embedded in paraffin wax. Histological sections were cut at 6-7 μ m and stained with Haematoxylin- Eosin and Mallory's triple stain.

Nematodes collected from the infected fish were fixed in 70% warm alcohol and preserved in 70% alcohol with a few drops of glycerine. Cestodes were fixed and preserved in 2% formaldehyde after flattening them. Population dynamics was calculated using the standard formulae. Incidence (percentage of infection), intensity (total worm burden/number of fishes infected) and index of infection [number of worms $\times$ number of helminth infected fish/ (number of fish examined)²] were for one annual cycle constituted the parameters.

Results

The study revealed that 82% of *H. fossilis* (80% male and 84% female) were infected by the helminth parasites. The incidence of heminthic infections was higher during the summer as compared to winter season. The incidence of *P. spiculogubernaculus* was higher (78%) in comparison to *L. fossils* (3%) and *C. biloculus* (3%). Infection by a single species of endohelminth was observed in 72% and by two species in 8% male fishes, whereas it was 74% by one species and 10% by two species in the female fishes (Figs. 3 & 4). However, concurrent infection by more than two species of endohelminths was not observed throughout the study period. The intensity of nematode infection was greater in summer months (13%) as compared to winter months (7%). The highest intensity of infection of the nematode was recorded in June in the



Fig. 1. Transverse section of stomach of *H. fossilis* showing gastric plug (P) of the fish lodged inside the buccal capsule (BC) of the nematode (PS). Note lysis (D) of mucosal tissue $\times 100$, H & E.



Fig. 2. Transverse section of stomach of *H. fossilis* showing the portion of body of the nematode (PS) in the stomach wall $\times 100$, H & E.

male as well as female fishes, whereas it was lowest in January. The index of infection of the nematode was highest in June, whereas it was lowest in January (Table 1).

Table 1. Incidence, intensity and index of infection of *Procamallanus spiculogubernaculus* Agarwal in *Heteropneustes fossilis* (Bl.)

Months	Total worm burden		Incidence (%)		Intensity		Index of infection	
Sex of Fish	M	F	M	F	M	F	M	F
Mar	325	287	83.8	91.8	10.5	13	7.4	11.0
April	394	419	85.4	94.4	11.3	12.3	8.2	11.0
May	407	614	91.7	97.7	12.3	14.3	10.4	13.6
June	535	507	97.7	97.4	12.7	13.3	12.2	12.7
July	351	443	82.4	87.5	12.5	12.7	8.5	9.7
August	218	297	69.0	78.1	10.9	11.9	5.2	7.3
September	107	199	66.7	79.2	8.9	10.5	4.0	6.6
October	83	145	52.6	68.1	8.3	9.7	2.3	4.5
November	51	89	53.8	64.7	7.3	8.0	2.1	3.4
December	77	124	55.6	57.7	7.7	8.3	2.4	2.8
January	74	107	45.8	48.3	6.7	7.6	1.4	1.8
February	167	235	67.7	75.7	8.0	8.4	3.6	4.8

M- Male

F- Female

H. fossilis was found infected by the nematode *P. spiculogubernaculus* and cestodes *Lytocestoides fossilis* and *Crescentrovitus biloculus*. *P. spiculogubernaculus* was present in the stomach, whereas *L. fossilis* and *C. biloculus* were encountered in the intestine of the fish. *P. spiculogubernaculus* was seen attached with the stomach wall of *H. fossilis* by its buccal capsule. The gastric mucosa was protruded in the form of a plug which was lodged inside the buccal capsule of the nematode (Fig. 1). Histolysis of mucosal tissue of the plug was noticed at many places. The epithelial cell of the mucosal tissue of plug like projection displayed ulceration and desquamation caused by strangulation. Further, fragments of mucosal tissue and blood cells were also seen inside the nematode's buccal capsule.

Portions of the body of the nematode were also seen in the submucosa of the stomach wall of the fish (Fig. 2). They were seen surrounded by fibroblasts, histiocytes, eosinophils and lymphocytes although capsule formation was not observed around the nematode inside the stomach wall. The blood supply to the parts of the submucosa containing nematode was also found to be increased. Dilated and congested blood capillaries were seen at a number of places in the infected stomach. Further widespread haemorrhage was noticeable at a number of places in the severely infected fish.

Discussion

The higher incidence and intensity of *P. spiculogubernaculus* infection as seen here have also been reported by Kennedy (1969), Gupta *et al.* (1984) and Niyogi *et al.*

(1982). Amin (1987) has also reported the incidence and intensity of helminths to be higher in the summer months. The higher incidence of nematode infection observed in the female fish has also been reported by Niyogi *et al.* (1982), Gupta *et al.* (1984) and Amin (1987). This may be due to the fact that during the breeding season female fish becomes sluggish, weak and becomes more susceptible to infection as it stops feeding during this period. These factors may cause a higher incidence of nematode infection in *H. fossilis* during the breeding season.

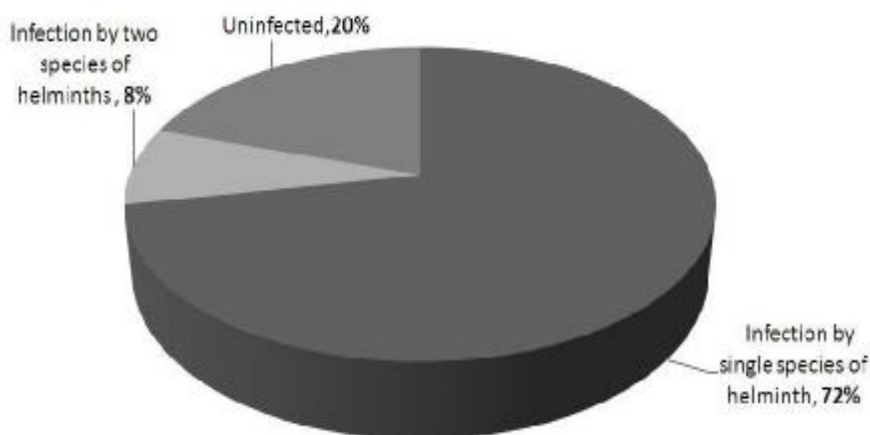


Fig. 3. Incidence of infection of helminth parasites in male *H. fossilis*.

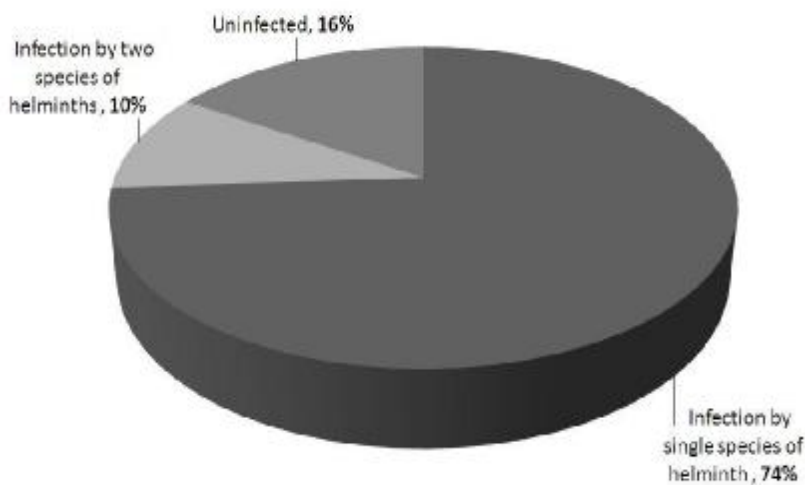


Fig. 4. Incidence of infection of helminth parasites in female *H. fossilis*.

In the present study mode of attachment of *P. spiculogubernaculus* was found to be similar to that of *Cucullanus minutus* (Janiszewska, 1939) and *Bothriocephalus gowkongensis* (Scott and Grizzle, 1979). The lysis of cells at the site

of attachment observed in the present infected fish has also been reported by Jain and Pandey (1976) in *H. fossilis* infected by *Capingentridentes moghei*. Similarly the cellular infiltration around the body of the nematode in the stomach wall of the host as seen during the present study has also been reported by Bullock (1963) in salmanoid fishes infected with *Acanthocephala jacksoni* and by Williams and Richard (1968) in *Raja radiata* infected with *Pseudoanisakis rotundata*. The dilation of blood vessels in infected fish as observed here has also been reported by Janiszewska (1939) in infected fish. Buturo (1980) has also reported increased blood supply of infected organ in cyprinid fry. Smyth (1976) considered vasodilation as a pathological response caused by invading helminth parasite.

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Distribution and public health importance of *Anopheles minimus* Theobald, 1901 (Diptera: Culicidae) in India

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Abstract

The distribution and public health importance of *Anopheles minimus*, the major vector of malaria in north-eastern region of India, has been reviewed. This species which was believed to have disappeared from north-eastern region of India, has staged a come back with all its biological characteristics intact. The role of *A. minimus* in malaria transmission in the region is also highlighted. An in-depth study on the presence of sibling species, their behaviour and vectorial capacity is emphasized, which is of considerable importance in relation to malaria epidemiology and control in India.

Keywords

Anopheles minimus; Assam; Malaria; sibling species; public health importance; Vector

Introduction

During the 1940s *Anopheles minimus* was considered to be one of the most notorious and important malaria vectors in the north-eastern region of India, comprising of seven states, namely Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland and Tripura, as well as in the Terai region of Uttar Pradesh (Viswanathan, 1941). During that period, the distribution and bionomics of this species were extensively studied by researchers in Assam (Thomson, 1941), North Bengal (Krishnaswami, 1952) and in terai region of Uttar Pradesh (Chakraborty and Singh, 1957). However, in recent years, rapid urbanization, industrialization, deforestation, population migration under new developmental projects apart from the use of DDT and other residual insecticides under the patronage of National Malaria Eradication Programme (NMEP), *A. minimus* is claimed to have disappeared from Uttar Pradesh (Nagpal *et al.*, 1983) and was also believed to have disappeared from the north-eastern region (Rao *et al.*, 1973; Rajagopal, 1976). Presently, there is no comprehensive information available on the taxonomy, distribution and public health importance of this vector in India.

In recent years the north-eastern region, particularly the state of Assam has witnessed a large number of malaria outbreaks (Dev and Sharma, 1995; Dev *et al.*, 2001) and many researchers have highlighted the role of this species in malaria transmission (Prakash *et al.*, 1996; Dev, 1996). Therefore, the information generated during the last few decades towards understanding of distribution and role in malaria transmission, with particular reference to north-eastern region of India, is compiled and presented in the present communication.

Systematics and nomenclature

There was a great confusion regarding the nomenclature of *A. minimus*. For some time this species was regarded as *A. christophersi* (Theobald), but later as *Anopheles minimus* (Ewards, 1915). The type specimen of this species was first noted by Theobald (Christophers, 1933). The identification of other related species, *A. fluvitiles*, *A. aconitus* and *A. minimus* was very uncertain group was re-examined giving more emphasis on intra-specific variations (Harrison, 1980). In another study, presence of two sibling species of *A. minimus* was reported from Thailand (Sucharit *et al.*, 1988). The first one is typical *A. minimus*, while the second one is *A. minimus* species 'C'. There were marked differences noted in the feeding and resting behaviour of these sibling species.

Prevalence and distribution

A. minimus is the mosquito of hills and foothill regions. It is found in the low lying areas of Brahmaputra valley of Assam as well as in Shillong (Meghalaya) and deltaic West Bengal (Dash *et al.*, 1984; Krishnaswami, 1952). In India, this species is widely prevalent in the eastern, northern and north-eastern region (Nagpal *et al.*, 1983; Dev and Sharma, 1995). It has a wide distribution in the eastern states of Assam, Bengal, Orissa, Bihar, Meghalaya, Nagaland, Tripura and also in the state of Goa. It is scarcely present in Andhra Pradesh, Tamil Nadu, Kerala, Karnataka, and Uttar Pradesh. However, the species was not found in Maharashtra, Gujarat, Rajasthan, Punjab, Haryana, Jammu & Kashmir and Delhi (Rao, 1984).

Subsequent collections in which the species continued to be recorded did not however reflect its distribution. Gilroy (1939) reported high density of *A. minimus* in the tea gardens of Darjeeling District of North Bengal. In Tamil Nadu, Russell and Rao (1940) and Russell and Jacob (1942) could not collect even a single specimen of *A. minimus* from their extensive survey in Tamil Nadu for several years. It may, however, be noted that the collection of one specimen of *A. minimus* from Thanjavur district of Tamil Nadu needs re-examination and reconfirmation. Since the presence of one specimen of this species can not be claimed its establishment in this region. On the other hand, in North Bengal, this mosquito was present in good numbers (Krishnaswami, 1952). During 1949 to 1951, this species was regarded as major malaria vector in Terai region of Nainital District of Uttar Pradesh (Srivastava and Chakravorty, 1952). Senior-White and co-workers (Senior-White *et al.*, 1949) reported the presence of this species from hill tracts of Koraput District of Orissa. However, subsequent surveys conducted in nine districts of Orissa revealed that this species was completely absent from those regions (Dash *et al.*, 1984). In a recent

study, however, this species was once again reported in the hill tracts of Koraput District of State (Gunasekaran *et al.*, 1994).

Distribution in North-eastern region of India

Available data on its prevalence suggest that *A. minimus* has made the entire north-eastern region its territory (Kamal and Das, 2001). Misra (1956) reported this species an established malaria vector in the foothill regions of the erstwhile North Eastern Frontier Agency (NEFA), the present state of Arunachal Pradesh. Subsequent surveys conducted in Tirap, Lohit, Lower Subansiri and Chang Lang Districts of the state revealed that this species was completely absent from these Districts (Malhotra *et al.*, 1978). However, several researchers have reported and also incriminated this species as malaria vector from Tirap District of Arunachal Pradesh (Dutta and Baruah, 1987; Dutta *et al.*, 1992).

In Assam, this species was regarded as the most important mosquito species responsible for malaria transmission (Thomson, 1940). The presence of this species has been reported from various places of Assam viz Goalpara, Lakhimpur, Digboi, Limbuguri, Sibsagar and Toklai (Puri, 1948). It was reported to be an important vector species particularly in the teagardens (Kar, 1950; Gilroy, 1958) and encountered in almost entire state of Assam with varying degree of density (Wajihullah *et al.*, 1992; Nandi *et al.*, 1993; Jana-Kara *et al.*, 1995; Prakash *et al.*, 1997a). In Meghalaya, this species could not be collected in a survey conducted by Rajagopal (1976). Later, Kareem *et al.* (1983) reported this species from forest areas of Assam-Meghalaya border. In Manipur, this species was detected in adequate density (Rajput and Singh, 1986, 1987, 1988). However, in subsequent surveys, it was absent from this region (Malhotra *et al.*, 1983). Das and Baruah (1985) recorded high density of this vector in Mizoram, where it was reported to be absent in earlier studies (Malhotra *et al.*, 1982, 1984). In the foot hill areas of Nagaland, this mosquito was collected in high densities and it was incriminated as a malaria vector (Bhatnagar *et al.*, 1982; Misra *et al.*, 1993). In Tripura, Misra and Dhar (1955) recorded this species and labeled it as malaria vector. This information clearly suggests that *A. minimus* has made the entire north-eastern region, particularly Assam as its territory. In recent years, there have been several ecological changes in Assam, particularly urbanization, industrialization and deforestation. It is also possible that *A. minimus* is establishing as a major vector species due to increased paddy cultivation sequel to removal of jungles, thus disrupting the ecological niche of *A. dirus*, another malaria vector of this region (Dutta *et al.*, 1989).

Public health importance

There is doubtless involvement of *A. minimus* in malaria transmission in many parts of north-eastern region as it has been incriminated and re-incriminated by several researchers. Before recognition of *A. dirus* in malaria transmission was realized in Assam, *A. minimus* was considered the principal vector of malaria in this region. Sen (1948) made a review of the dissections of *A. minimus* in West Bengal and showed that this species acted as a major malaria vector in the state. Further studies conducted in Assam, Arunachal Pradesh, West Bengal, Tripura, Bihar, Mizoram, Nagaland and Orissa also confirmed its role in malaria transmission. Incrimination of this species in very high densities in Assam (Dutta *et al.*, 1994) and in extremely low densities in Arunachal Pradesh (Dutta *et al.*, 1993) and in Nagaland (Nandi *et al.*,

1993) clearly indicates its transmission potential irrespective of adult vector density of *A. minimus*. In Bangladesh, Burma and Nepal, which share their borders with many states of the north eastern region, hyper-endemic malaria transmission, particularly in forest belts has been attributed to *A. minimus* (Brydon *et al.*, 1961; Khin and Winn, 1971; Khan and Talibi, 1972; Khin and Winn, 1976; Myo-Paing *et al.*, 1988; Shresta, 1996). This species has also been found to be an important malaria vector in countries such as Thailand (Harinasuta *et al.*, 1976; Ratanatham *et al.*, 1988), Vietnam, (Spring *et al.*, 1993) and China (Wu *et al.*, 1993). There is a possibility that with other known malaria vectors in the region (Dutta *et al.*, 1989; Dutta *et al.*, 1991; Bhuyan *et al.*, 1997; Prakash *et al.*, 1997b), this species may have increased the risk of malaria transmission.

Being such an important species in relation to malaria transmission, particularly in north-eastern region of India, studies should be initiated on determining the sibling species of *A. minimus* in India, as has been observed in other neighboring countries (Ismail *et al.*, 1974; Sucharit *et al.*, 1988; Wu *et al.*, 1993; Dev, 1996). Unfortunately there has been no focus in this direction. Like many other anopheline complexes the existence of *A. minimus* sibling species cannot be overlooked (Baimai *et al.*, 1988; Subba Rao *et al.*, 1988). The fact that *A. minimus* disappeared from terai region of Uttar Pradesh (Nagpal *et al.*, 1983) but reappeared in the north eastern part of India suggests the existence of sibling species complex within its range of distribution. Indeed, based on the population and cytogenetic evidence (Green, 1990) and behavioral studies (Nutsathapana *et al.*, 1986), *A. minimus* sibling species have been documented in Thailand. Population of *A. minimus* from Assam, Arunachal Pradesh, Meghalaya, Manipur, Nagaland, Tripura and Mizoram, where substantial increase in overall malaria incidence and in the incidence of *Plasmodium falciparum* malaria has been reported (Yadav and Sharma, 1995), should be screened for cytogenetic studies.

Studies conducted by various researchers have revealed the fact that *A. minimus*, which was earlier believed to have disappeared from north-eastern region of India, is still present in adequate numbers in the region. Probably this vector might have reduced itself in number or re-established in those areas with poor or no spray coverage and it was still acting as principal malaria vector in north-eastern region, particularly in Assam. This species has once again staged a comeback with all its biological characteristics intact. An in-depth study on its seasonal prevalence, bionomics, behaviour and vectorial capacity, therefore, would be of considerable importance in relation to malaria control in these areas.

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Efficacy of *Eucalyptus* (a homeopathic medicine) in combination with artesunate to clear *Plasmodium berghei* infection in Balb/C mice

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Abstract

Malaria remains a major cause of mortality specially in developing nations. On account of parasite having become resistant to the conventional antimalarials, Artemisinin based Combination Therapy (ACT) is the most reliable treatment option. Artemisinin (Qinghaosu) is a sesquiterpene lactone endoperoxide isolated from *Artemisia annua* L. But, poor availability and high risk of recrudescence due to its short half life limits its effectiveness as monotherapy. ACT offers hope to improve efficacy and delay development and thereby prolonging the useful therapeutic life of existing drugs. In the present study efficacy of the combination of artesunate and homeopathic drug eucalyptus mother tincture (ϕ) has been checked against rodent malarial parasite, *Plasmodium berghei* (NK65). Homeopathic drug eucalyptus reduces fevers that accompany infectious illness. Out of different concentrations of eucalyptus, (ϕ) 1:2 was found to inhibit high percentage of parasitaemia ($p < 0.0005$) as compared to control on day 7. So, it was combined with 100 mg/kg of artesunate. A 7-day oral administration of artesunate plus eucalyptus (ϕ) prevented parasitaemia considerably with an extremely significant difference ($p < 0.0001$) on day 7. However, no recrudescence was observed and the mice survived up to 30 days. The preliminary observations demonstrated that the short treatment course of artesunate plus eucalyptus (ϕ) inhibits blood stage rodent malaria infection; so, the combination can be established as a safe ACT; however the study needs to be further explored.

Keywords

Plasmodium berghei; Artesunate; Recrudescence; Combination therapy; Homeopathy; *Eucalyptus*

Introduction

Malaria, a disease of antiquity, has proved to be a formidable deterrent to the cultural and socio-economic progress of man in the tropical, sub-tropical and monsoon prone zones of the world. Despite more than a century of eradication efforts, the disease is endemic in many parts of the world. Malaria causes about 300 million cases and at least one million deaths each year, 90 percent of which in Africa and most of them children under five (Snow *et al.*, 2001; WHO, 2004). The current approaches to

curtail malaria include the vector control, vaccination and chemotherapy. However, the drug resistant malaria has become a major problem in malaria control. Resistance *in vivo* has been reported against almost all antimalarial drugs (Zucker and Campbell, 1992; Sharma, 1997). Artemisinin and its derivatives represent a new class of antimalarial that is effective against drug-resistant *P. falciparum* strains and therefore, they are of utmost importance in the current antimalarial campaign (WHO, 1998; Balian, 2001). However, their activity is not long lasting, with significant decrease in effectiveness after one to two hours; artemisinin is given alongside other antimalarials. Such treatment is known as Artemisinin based Combination Therapy (ACT). In combination with more slowly eliminated antimalarials, the addition of artesunate, artemether or dihydroartemisinin consistently improves cure rates and has the advantage of producing a more rapid clinical response to treatment and reduction of malaria incidence (Price *et al.*, 2000; Carrara *et al.*, 2006). Artemisinin based combinations provide mutual protection against resistance, high efficacy, excellent tolerability and reduced transmissibility (Bangchand and Congpuong, 2007). Four ACTs are currently recommended by WHO, artesunate + mefloquine (AS+MQ), artesunate + sulphadoxine-pyrimethamine (AS+SP), artesunate + amodiaquine (AS+AQ) and artemether + lumefantrine. But the combinations containing SP and AQ are both threatened by resistance (WHO, 2006). Drug combination therapies have been devised to delay the development and spread of resistant malaria parasites. However, poor design often leads to ineffective combinations (Martinelli *et al.*, 2008).

Homeopathy has been cited by the World Health Organization as one of the systems of traditional medicine that should be integrated with conventional medicine to ensure adequate global healthcare (Bannerman *et al.*, 1983). Eucalyptus mother tincture is a homeopathic drug which is obtained from the leaves of “fever tree” *Eucalyptus* sp. It was listed in the 1885 edition of the British Pharmacopoeia. The major constituent in eucalyptus leaves is a volatile oil known as eucalyptol (1, 8-cineol). The oil possesses many qualities, including being a powerful antiseptic, antibiotic, antiviral and antifungal agent (Santhil, 2007).

For the present study, *P. berghei* (NK65), a lethal rodent parasite has been used to study the *in vivo* antimalarial efficacy of artesunate in combination with homeopathy drug eucalyptus and to monitor effect of drugs on body weights of mice in various experimental groups.

Materials and methods

White Swiss mice *Mus musculus* of Balb/C strain (weighing 22-30 g and 4-6 weeks old) of either sex were used as experimental model for present study. These mice were obtained from the Central animal house, Panjab University, Chandigarh and fed with a standard pellet diet and water.

Maintenance of strain

Strain of *P. berghei* (NK- 65) was maintained *in vivo* in Balb/C mice by weekly passages of blood from infected to normal mice. Blood stage infection was initiated by intraperitoneal inoculation of 1×10^6 infected RBC's from infected mice to naïve

mice in citrate saline (0.85% w/v sodium chloride, 3.8% w/v sodium citrate). The total number of RBCs was determined by using a Neubaur Chamber (Waynforth and Flecknell, 1992).

Parasitaemia was checked daily by preparing thin blood smears on slides through tail vein incision of infected mice. Smears were air dried, fixed in methanol for 1 min and then stained with 3% Giemsa stain {3% Giemsa stock in 0.95% (w/v) sodium phosphate dibasic, 0.9% (w/v) potassium phosphate monobasic; pH 7.0} for 25-30 min. After staining, the smears were washed in running water, air-dried and observed under microscope at 100 x using immersion oil (Wilcox, 1960). The percentage parasitaemia was calculated as:

$$\frac{\text{Number of infected erythrocytes/reticulocytes}}{\text{Total number of erythrocytes/reticulocytes}} \times 100$$

Experimental design

Different concentrations of eucalyptus {mother tincture (ϕ) of any homeopathic drug consists of 1 part of drug and 6 parts of nascent alcohol} were checked *in vivo* for their antimalarial efficacy. Four groups (E1-E4) with 6-8 mice were first injected with 1×10^6 *P. berghei*-parasitized red blood cells and then given 0.2 ml of eucalyptus/day/mouse OD orally in different concentrations to different groups (E1-1:2, E2-1:4, E3-1:25) except E4 which was kept as a control.

Parasitaemia was checked on day 0, day 3, day 5 and day 7 by study of Giemsa stained blood smears. The concentration showing maximum parasite inhibition was used in combination therapy with artesunate. Artesunate is water-soluble component of artemisinin. Falcigo tablet containing 50 mg artesunate base was used in present study. The tablet was diluted in 1 ml 5% Na_2HCO_3 and 5 ml 0.9% NaCl.

For the final experiment, five groups having 8-10 mice each were used. Groups were designated as G-I (normal), G-II (5% Na_2HCO_3 +0.9% NaCl), G-III (artesunate 100 mg/kg), G-IV (artesunate + eucalyptus) and G-V (artesunate + sulphadoxine/pyrimethamine). All groups except G-I were injected with 1×10^6 *P. berghei* parasitized red blood cells. Artesunate was given 0.2 ml/mice *bi daily* on first day and once in a day for 5 days where as homeopathic drug was given 0.2 ml/mice/day. All the mice were kept for follow up studies up to 28 days. Thin blood smears from mice of various groups were prepared on day 0, day 3, day 5, day 7, day 14, day 21 and day 28 to study clearance and recrudescence of parasite in respective groups. The weights of all the mice of various groups were also noted on day 0, day 7, day 14, day 21 and day 28 to check the adverse effect of drugs on the body weight.

Statistical analysis

Data has been presented as mean and standard deviation (SD). Statistical evaluation of differences between the experimental groups was determined by the Student's *t*-test with the level of significance of $p < 0.05$ using Graph Pad Software.

Observations

Plasmodium berghei (NK 65) has been found to be lethal to Balb/C strain of mice. Gradual increase in infection was observed after inoculation of 1×10^6 *P. berghei* infected red cells into six naïve mice leading to death of mice by 7th day due to heavy infection ($49.5 \pm 3.535\%$)

Course of Parasitaemia in experimental groups

All mice of E-1 to E-4 groups were inoculated with 1×10^6 *P. berghei* parasitized red cells on day 0. Various concentrations of eucalyptus were employed to evaluate its antimalarial efficacy. Blood smears from all groups were prepared on day 5 and day 7 post inoculation (Fig. 1). Control mice (E4) died within 7 days of heavy infection. Parasitaemia in group E1 was the lowest when compared with E2 and E3 on day 7. No mortality was observed in E1.

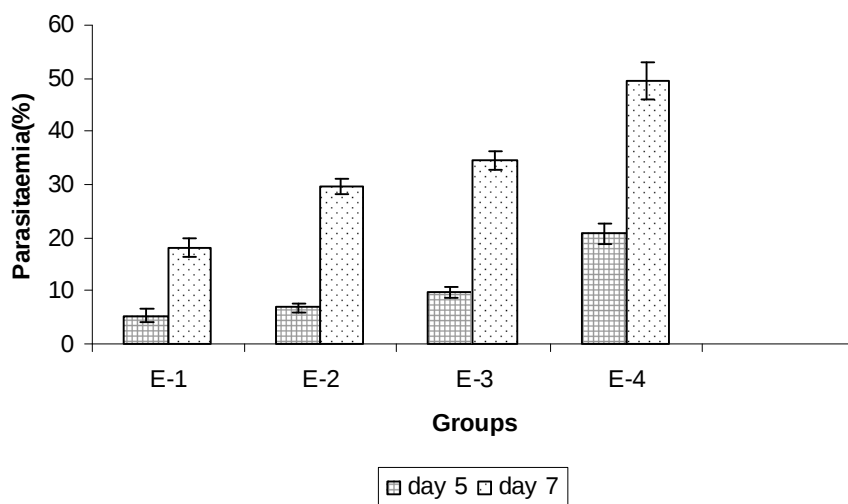


Fig. 1. Histogram showing course of parasitaemia (Mean $\pm$ S.D.) in E-1 to E-4 on day 5 and day 7, *extremely statistically significant ($p < 0.0005$).

For the final experiment 1:2 concentration of eucalyptus was combined with artesunate in combination therapy. Drugs were administered as given in materials and methods. Mice receiving only vehicle (G-II) nascent alcohol showed $3.72 \pm 2.37\%$ parasitaemia on day 3 post inoculation which increased to $25.1 \pm 12.9\%$ on day 5 ($p < 0.1431$) which was not statistically significant when compared with control group. All the mice died on day 7 post inoculation. In G-III the parasitaemia on day 7 was $1.3 \pm 0.1\%$. It was also extremely statistically significant ($p < 0.0005$) when compared to control. The infection was cleared after 7th day but recrudescence occurred by day 21. A steady decrease in infection was observed in G-IV and a negligible infection i.e. $0.33 \pm 0.3\%$ was observed on day 28. All the mice survived during the follow up period. G-V was given 3 day course of artesunate + sulphadoxine/pyrimethamine. The parasitaemia on day 3 was found to be $0.9 \pm 0.9\%$ which continuously decreased up to $0.16 \pm 0.2\%$ on day 7 post inoculation. After day

7, the infection was cleared from the blood as observed by blood smears till day 28 (Fig. 2).

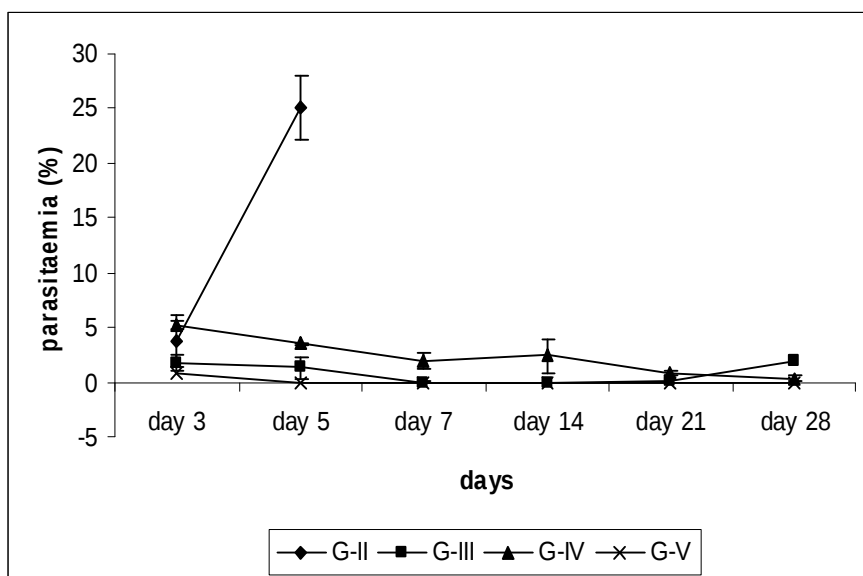


Fig. 2. Graph showing course of parasitaemia (Mean $\pm$ S.D.) in G-II to G-V on various days of study, *Extremely statistically significant ($p < 0.0005$), # All the mice died.

Effect of drugs on body weight

A gradual increase in the body weight was observed during the course of study up to 28 days in G-I. On day 0, 25.2 ± 1.13 g weight was observed which increased to 28.5 ± 1.05 g on day 28. In infected control group (G-II), the weight showed a decrease after a week i.e. on day 0 weight was recorded as 24.5 ± 0.18 g which decreased to 20.5 ± 0.23 g on day 7 post inoculation. After which all the mice died due to heavy infection. The body weight of the mice in G-III showed a continuous decrease till day 14, after which the body weight increased till day 28. In G-IV, the body weight decreased slightly till day 7 but a gradual increase in the body weight were recorded on day 14, day 21 and day 28 i.e. 25.2 ± 0.18 g, 26.1 ± 0.33 g and 28.4 ± 0.30 g, respectively. In G-V also the body weight of mice decreased till day 7 but on day 14 the body weight again increased which was similar to that on day 0. Not much difference in body weight was recorded after this (Fig. 3).

Discussion

Plasmodium berghei has been reported to show preference for reticulocytes in late phase of infection (Carter and Walliker, 1975). Parasite establishes itself by manipulating host's defense (Heussler *et al.*, 2001). *P. berghei* (NK65) strain of rodent malaria parasite is lethal to Balb/C strain of laboratory mice. When injected with 1×10^6 *P. berghei* infected red blood cells, mice died within 7 days.

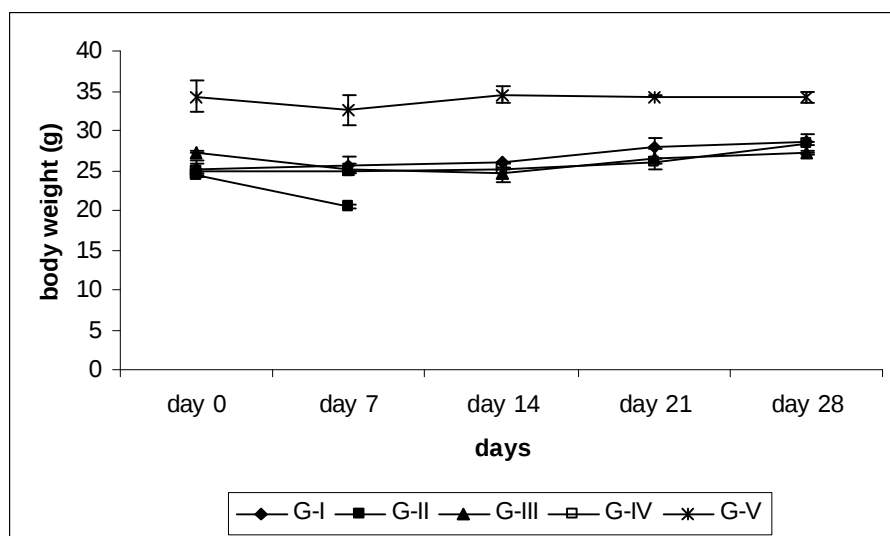


Fig. 3. Graph showing body weights (Mean $\pm$ S.D.) in G-I to G-V on various days of study, *Extremely statistically significant ($p < 0.0005$), # All the mice died.

The murine malaria model is well established for investigations of malaria infection in mammalian hosts and for evaluation of antimalarial efficacy (Peters and Robenson, 1999). In the present study the efficacy of artesunate and homeopathic drug eucalyptus in combination therapy against *P. berghei* infected Balb/C mice has been evaluated. The objective of the treatment in uncomplicated malaria is to provide a rapid resolution of symptoms and cure of the infection (i.e. prevention of recrudescence) with minimum adverse effects.

Homeopathic medicine can be very effective for treating some of the symptoms. It can also be effective for reducing some of the side effects. Homeopathic remedies are also safe when taken as directed because they are virtually nontoxic. They are also inexpensive and often do not require any prescription (Sylvia, 2000). For the present study eucalyptus mother tincture (a homeopathic medicine) was used along with artesunate (100 mg/kg) and their efficacy in inhibiting the blood stage *P. berghei* was checked. The quantitative estimation of alkaline phosphatase activity, bilirubin, urea and creatinine in the serum of mice treated with artesunate (100 mg/kg) + china (homeopathic medicine) showed that they remain within the normal range after the 7 day treatment regimen however, artesunate alone (100 mg/kg) showed elevated levels of alkaline phosphatase activity, bilirubin, urea and creatinine which proved that the ACT with the homeopathic drug does not cause any toxic effects and do not disturb the normal functioning of liver and kidney whereas, artesunate monotherapy caused jaundice like symptoms and also impaired the renal function (Rajan, 2009).

In a previous study the maximum cumulative dose of oral artesunate used in studies was 600 mg given over 5 days, which was followed by a recrudescence rate of 10-28% with uncomplicated *P. falciparum* malaria infection (Karbwan et al., 1994). Previous studies have also reported that once daily administration of

artemisinin derivatives provides equivalent cure rates to more frequent administration (Chotivanich *et al.*, 2000). In a 7 days regimen 10 mg/kg concentration of artesunate reduced the parasitaemia till day 7 post inoculation but after cessation of drug, infection again increased continuously leading to death of mice whereas, in 100 mg/kg the infection was cleared after 7 days but recrudescence was observed after 10 days. However, 14 days treatment with artesunate has been reported to completely prevent parasitaemia and enhance survival up to 60 days (Bonginkosi *et al.*, 2003). No toxicity was observed at a total dose of artesunate of 1400 mg/kg given over 14 days in the same study.

Artesunate is readily hydrolyzed to dihydroartemisinin (DHA), probably by blood esterases and the hepatic cytochrome P450 3A4, as in the case with closely related compounds artelinic acid and arteether (Grace *et al.*, 1999). However, duration for antimalarial activity is short. In the present study 100 mg/kg of artesunate given over 7 days has been observed to clear the infection by 7th day of follow up studies. The antimalarial activity of artemisinin and its derivatives is extremely rapid and most patients show clinical improvement within 1-3 days after treatment. The drug is postulated to cause free radical damage to the parasite membrane system. They stop the parasite development in early stages and prevent subsequent resetting and cytoadherence, both of which are important pathophysiological mechanisms in severe malaria (Goldsmith, 1995). Cumming *et al.* (1997) reported that the iron liberated from hemoglobin by the parasite, reduces the peroxide bond in artemisinin generating high-valent iron-oxo species, resulting in a cascade of reactions, which damage the parasite leading to its death. Inhibition of digestive vacuole cysteine protease activity of malarial parasite is also done by artemisinin. They found artemisinin to be potent inhibitor of hemozoin formation activity of malarial parasite. AS+SP was used as positive control. In this group the infection was completely cleared after 7 days. Artesunate + eucalyptus treated mice showed gradual decrease in parasitaemia and clearance was observed by day 28. The effect of drugs on body weight of mice was also checked. In G-III, G-IV and G-V the body weight of mice decreased up to day 14 but after that it increased. In case of artesunate monotherapy the body weights became normal during follow up period. In combination therapies, G-IV and G-V it became normal by day 28. From the present study it can be concluded that a mutual protection is provided by artesunate and eucalyptus in clearing blood stage *P. berghei* infection and preventing recrudescence in Balb/C mice.

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***Setaria cervi* (Nematoda: Filarioidea): Absence of γ -glutamyl cyclotransferase activity**

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Abstract

One of the six constituent enzymes of γ -glutamyl cycle i.e. γ -glutamyl cyclotransferase (γ -GCT; EC 2.3.2.4) was not detected in the bovine filarial species *Setaria cervi* which favours the operation of circumstantial glutathione degradative pathway in them.

Keywords

Glutathione; γ -Glutamyl cycle; γ -Glutamyl cyclotransferase; Filariasis; Nematode; *Setaria cervi*

Introduction

Glutathione (GSH) is the most abundant low molecular weight thiol in cells from bacteria to mammals (Meister and Anderson, 1983). Filarial species have also been known to possess sufficient amount of GSH (Singh *et al.*, 1997; Tiwari *et al.*, 2003). GSH has been proposed to constitute the antioxidant system responsible for the long term existence of filarial worms in mammalian host by protecting them from the reactive oxygen species produced by the normal metabolism and the immune cells of the host (Rzepczyk and Bishop, 1984; Schirmer *et al.*, 1987; Callahan *et al.*, 1988; Callahan *et al.*, 1990; Brophy and Pritchard, 1992; Selkirk *et al.*, 1998). The enzymes involved in the synthesis and degradation of GSH therefore deserve interest and need to be studied in filarial parasites.

There is a continuous turnover of GSH in all cells, at rates that can vary widely, even in different tissues in the same organism (Kosower and Kosower, 1978). Enzyme systems synthesize GSH, utilize GSH, and regenerate GSH as per the γ -glutamyl cycle that comprise of six enzymes: glutamate cysteine ligase (GCL; EC 6.3.2.2), glutathione synthetase (GS; EC 6.3.2.3), γ -glutamyl transpeptidase (γ -GT; EC 2.3.2.2), γ -glutamyl cyclotransferase (γ -GCT; EC 2.3.2.4), 5-oxoprolinase (5-OP; EC 3.5.2.9) and cysteinyl glycine dipeptidase (DP; EC 3.4.13.19). GSH is synthesized by the sequential actions of GCL and GS. Breakdown of GSH is initiated by γ -GT, which catalyses the transfer of γ -glutamyl group of GSH (and of other γ -glutamyl compounds) to acceptors (amino acids, dipeptides, GSH, H₂O). γ -Glutamyl amino acid and cysteinyl glycine are formed in the above reaction. γ -Glutamyl amino acid acts as substrate for γ -GCT, which

converts it into 5-oxoproline and the corresponding amino acid whereas cysteinyl glycine is split by DP to cysteine and glycine. The conversion of 5-oxoproline to glutamate is catalysed by 5-OP, which along with glycine and cysteine joins the cycle to reconstitute GSH (Meister, 1974).

Due to the importance of GSH in filarial nematodes, there is the need for the elucidation of whole γ -glutamyl cycle in them. Although all the enzymes of γ -glutamyl cycle have been identified in mammals (Orlowski and Meister, 1973; Werf *et al.*, 1975; Oppenheimer *et al.*, 1979; Kozak and Tate, 1982; Seelig and Meister, 1982; Tate and Meister, 1985; Orlowski and Meister, 1970; Meister, 1973; Meister, 1974), no attempts have been done for its full characterization in filarial parasites. While, both the GSH biosynthesizing enzymes have been identified in some filarial species *viz* *Onchocerca volvulus* and *S. cervi* (Tiwari *et al.*, 2003), its full degradative pathway is still not clear. Except the γ -GT in *S. cervi* and *Acanthocheilonema viteae* (Singh *et al.*, 1996; Gupta *et al.*, 2005a) no other enzyme of GSH degrading pathway has been characterized in any of the filarial species. The present work is an attempt to further study its degradative pathway using *S. cervi* as a model filariid. Attempts have been made for elucidation of the activity of γ -GCT, the enzyme succeeding γ -GT in γ -glutamyl cycle by following the sensitive RP-HPLC based assay system.

Materials and methods

Classical methods for γ -GCT assay involve spectrophotometric determination of L- γ -glutamyl-p-nitroanilide released from L- γ -glutamyl-L-y-glutamyl-p-nitroanilide produced during the reaction or by following the release of 5-oxoproline from L- γ -glutamyl-L-a-aminobutyrate (Orlowski *et al.*, 1969; Orlowski and Meister, 1973). Coupled enzyme procedure is the other commonly employed method in which the γ -GCT activity has been determined by linking alanine liberation with NADH oxidation (Board *et al.*, 1978a). These methods can be used on fully/partially purified enzyme, but are unsuitable for crude preparations. Another drawback is their low sensitivity. Radioactive method (Meister, 1985) is although sensitive but hazardous. In the present study a RP-HPLC method has been employed for the detection of γ -GCT activity in crude preparations employing L- γ -glutamyl-L-alanine as substrate. Hydrolysis and autotranspeptidation of substrate by interfering γ -GT has been prevented by adding the inhibitor (acivicin) of this enzyme in the assay system.

A 10% (w/v) *S. cervi* homogenate was prepared in 150 mM KCl containing 5 mM 2-mercaptoethanol and 1 mM MgCl_2 (Meister, 1985). The homogenate was centrifuged at 100,000 *g* and the supernatant was used for γ -GCT activity determination.

Results and discussion

γ -GCT activity was not detected in either crude extract or cytosolic fraction of *S. cervi* homogenate. However, under the specified experimental conditions, γ -GCT activity was calculated to be around 0.234 ± 0.047 $\mu\text{mol/min/mg}$ protein in the cytosolic fraction of the rat kidney (Wistar strain male) homogenates which confirms the validity of the method of estimation. The detailed specifications of the RP-HPLC based assay system are explained in Fig 1.

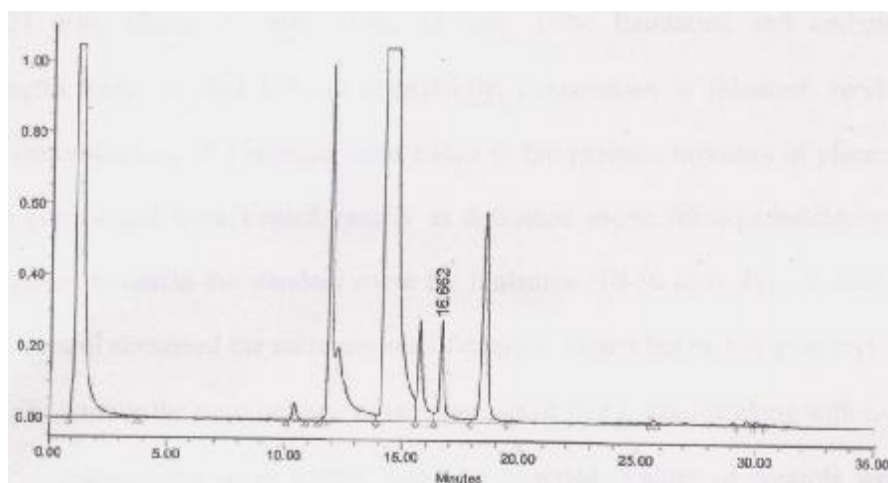


Fig. 1. Chromatographic profile for the RP-HPLC method employed for the determination of γ -glutamyl cyclotransferase activity.

Peak with retention time of 16.6 is for L-alanine (the reaction product), identified in the case of rat kidney enzyme. Assay system (1 ml) contained 0.2 M Tris HCl buffer (pH 7.5), 3 mM L- γ -glutamyl-L-alanine, 5 mM acivicin and a suitable amount of enzyme preparation. Followed by incubation for desired period at 37°C the reaction was stopped by the addition of 5% sulfosalicylic acid. After centrifugation at 10,000 g for 10 min the supernatant was used for product estimation after derivatization with o-phthalaldehyde (Lindroth and Mopper, 1979). One unit of enzyme activity is defined as the amount that catalyses the formation of 1 fmol of L-alanine per minute at 37°C. Waters Nova-Pak C₁₈ column (3.9 mm x 150 mm; 4 μ m particle size) was used along with Nova-Pak C₁₈ guard cartridge (3.9 mm x 20 mm) for separation at a flow rate of 1 ml/min with solvent A (0.15 M sodium acetate, pH adjusted to 7.0 with acetic acid/methanol (1/24, v/v)) and solvent B (100% methanol). Gradient (expressed as percentages of solvents) used was: 1 min, 10%; 2 min, 15%; 6 min, 28%; 7 min, 32%; 9 min, 36%; 10 min, 44%; 12 min, 48%; 19 min, 50%; 21 min, 60%; 25 min, 68%; 27 min, 100%; 29 min, 10%; 35 min, 10%. Excitation and emission wavelengths were 330 and 418 nm respectively. **Preparation of standard:** varying known concentrations of L-alanine were added to the reaction mixtures in place of enzyme aliquot and were treated exactly as described above for o-phthalaldehyde derivatization to obtain the standard curve for L-alanine (10-50 μ M). **Preparation of control:** control contained the same amount of enzyme aliquot but no L- γ -glutamyl-L-alanine. To remove the error because of the presence of free L-alanine along with L- γ -glutamyl-L-alanine, one more control was also operated. Values of controls were negated from the experimental to obtain the exact amount of L-alanine formed.

This observation may be an indication of the presence of incomplete γ -glutamyl cycle in filariids. There might be following other reasons for the non-appearance of γ -GCT activity in *S. cervi*: firstly, the parasite could have limited enzyme expression; secondly, the substrate specificity of filarial enzyme may possibly be different with that of mammalian enzyme.

There are few examples in the biological systems where γ -glutamyl cycle is not complete or more appropriate to say that operates in a way different from that of mammals with only few operative enzymes. There are evidences for the absence of γ -GCT and 5-OP in *Saccharomyces cerevisiae* and *Escherichia coli* (Jaspers *et al.*, 1985; Suzuki *et al.*, 1999). Only a circumstantial degradative pathway involving γ -GT and DP has been identified in these organisms along with both the GSH biosynthesizing enzymes. According to the proposed model for glutathione

metabolism in *E. coli*, GSH was cleaved/ hydrolysed by γ -GT to generate glutamate and cysteinylglycine, and the resultant cysteinylglycine was cleaved by enzyme(s) with cysteinylglycinase activity into cysteine and glycine to be used as a cysteine source and glycine source, respectively (Suzuki *et al.*, 1999). Similarly, *S. cerevisiae* has been proved to have GSH degrading pathway involving γ -GT and leading to the formation of either glutamate or transpeptidation products. This picture is consistent with a dual role of γ -GT functioning either as a hydrolase or a transferase of GSH (Jaspers *et al.*, 1985). γ -Glutamyl cycle is also not absolute in erythrocytes (Board and Smith, 1977; Srivastava *et al.*, 1976) with the absence of γ -GT. γ -GCT is widely distributed in mammalian tissues and has been purified from human and sheep brain, hog and rat liver, human erythrocytes and rat kidney (Orlowski *et al.*, 1969; Adamson *et al.*, 1971; Orlowski and Meister, 1973; Board *et al.*, 1978b; Taniguchi and Meister, 1978). By cumulating the results of the present and previous studies, it is established that both the GSH synthesizing enzymes GCL and GS are present in filarial worms *S. cervi*. As far as other γ -glutamyl cycle enzymes are concerned, the chief GSH degrading γ -GT has been recognised in the filarial worms, however, the presence of γ -GCT has not been detected. This favours the operation of circumstantial glutathione degradative pathway in filarial group of nematodes.

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Immunological markers for exposure, infection and infectivity of lymphatic filariasis for identifying transmission foci and evaluating the interventions

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Abstract

Repeated annual rounds of Mass drug administration (MDA) with Diethylcarbamazine (DEC) and albendazole for 5 to 6 years has been the strategy to eliminate the lymphatic filariasis in endemic areas. India is one of the 48 countries implementing this elimination programme out of 82 endemic countries with indigenous transmission. Worldwide, over 570 million people are covered under annual treatments. In an effort to assess the impact of DEC salt distribution to supplement MDA, we carried out sample survey to screen individuals for microfilaraemia (infectivity), antigenemia (infection) and antibody (exposure) in a *Wuchereria bancrofti* endemic area in South India. Microscopic examination of blood smears for microfilaria and Enzyme-linked Immunosorbent Assays (ELISAs) using Og4C3 and IgG4 test kits were carried out from blood samples drawn from 204 individuals. Prevalence of microfilaraemia, antigen and antibody were 6.4, 22.1 and 16.2 per cent respectively. Antigen assay could detect about 16% more infections, with 98% sensitivity and 75% specificity. Antibody prevalence as an indicator of exposure was lower than infection with low sensitivity. The results suggest that antigen assay using Og4C3 can be considered an appropriate immunological marker to delimit the areas with filarial infection (identifying the transmission foci) and evaluate the impact of MDA. The limitations of antibody assay using IgG4 to assess the risk of exposure are discussed.

Keywords

MDA; DEC; Monitoring; Microfilaria; Antigen; Antibody; Prevalence

Introduction

Lymphatic filariasis (LF) is a major vector-borne parasitic disease, which is reported to be endemic in 81 countries of the world (WHO, 2008). Nearly one billion people are estimated to be at risk, and over 120 million have already been affected by it

(WHO, 1998). India contributes about 42% of the global burden of this disease. The International Task Force for Disease Eradication (1993) considered lymphatic filariasis to be one of only 6 eradicable or potentially eradicable diseases (WHO, 2001). In 1995, the World Health Report identified LF as the second leading cause of permanent and long-term disability worldwide. The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was launched in 1997 (WHA.50.29) and currently over 570 million people are covered under this elimination programme in as many as 48 countries (Ottesen *et al.*, 2008), having indigenous transmission of the disease. Mass annual single dose drug administrations (MDA) with DEC co-administrated with albendazole for 5-6 years and distribution of mass DEC-fortified salt are the two recommended strategies towards filariasis elimination. Monitoring and evaluation of the programme implementation and impact is an inbuilt component of the programme. Appropriate epidemiological indicators need to be identified for situation analysis and impact assessment towards making evidence based decisions. Search for sensitive, simple, rapid, cheap and reliable tests for diagnosing the infection and exposure among the target community is need of the hour. In view of reports that more than five rounds of MDA are required to reach the level of elimination, supplementary measures assume importance in enhancing the effect of MDA towards elimination of LF. In an effort to identify such a measure, a field study was carried out with mass distribution of DEC-fortified salt (Nandha and Krishnamoorthy, 2007) covering 104 villages in South India with evidence of indigenous transmission of *Wuchereria bancrofti*. The persistent foci of infection with *W. bancrofti* were assessed through antigenemia survey among children. Following the intervention, the impact was assessed using epidemiological indicators such as microfilaria prevalence (marker of infectivity), prevalence of filarial antigen (marker of infection) and antibodies (marker of exposure). We present here the relationship between these indicators as well as the scope of their use in delimitation (identification of endemic foci) and evaluation of the impact of interventions against LF.

Materials and methods

Study area

The study was conducted in Villupuram district of Tamil Nadu, India, which is endemic for lymphatic filariasis caused by *W. bancrofti*. Two blocks viz., Ginjee and Kanai with a population of 163310 and 122524 were selected to demonstrate mass DEC-fortified salt as a supplementary strategy to eliminate lymphatic filariasis in this district. The number of villages in the respective blocks was 104 and 88 and all these villages were covered by MDA with DEC. At the end of five annual rounds of MDA, a situation analysis was carried out to identify the foci of infection persisting after the intervention, which require supplementary measures in achieving elimination of LF. Blood samples were collected from these villages to screen for microfilaraemia, antigenemia and antibody. The sample size was calculated based on the expected microfilaria prevalence of 1%. Blood samples were drawn by finger prick method between 2000 and 2200 hr and all the available individuals were sampled. Written consent was obtained from the individuals and parents of children below 15 years of age.

Sample size

Out of 9271 blood samples collected in the study area, 204 samples from individuals residing in three villages of the block were processed for all the parameters. Microfilariaemia (mf) was detected from the blood smears on slides and the sera samples were subjected to detect antigen and antibody levels.

The blood smears were dehaemoglobinized, stained with JSB stain (Das *et al.*, 1995) and examined under a compound microscope at a magnification of 100 x for the presence of mf covering all the fields of the smear. The number of mf in each positive slide was recorded.

Measurement of circulating filarial antigen

In our study we have used commercially available monoclonal antibody based assay (Og4C3) for the detection of circulating filarial antigen. By this assay sera samples collected from the individuals were detected and quantified for the presence of *W. bancrofti* antigen. The test was performed with 50 µl sera samples (equivalent to 100 µl of blood), using TropBio ELISA kit (JCU Tropical Biotechnology Pvt Ltd, Townsville, Australia). Catalogue no. 03-010-01). For this each of 50 µl sera samples were added with 150 µl of sample diluent and boiled for five min in a boiling water bath. Then the sera samples were centrifuged at 10,000 rpm for 10 min and 50 µl of the clear supernatant was added to the test well. The seven standards with known antigen content supplied in the kit were also added parallelly to wells. The plates were then incubated overnight at 4°C. The plates were then washed three times with wash buffer and added with 50 µl of sec antibody and incubated at 37°C for an h. After the wash, the plates were added with the 50 µl of the peroxidase conjugate and incubated at 37°C for one h. With further washing of the plates the wells were added with 100 µl of the ABTS chromogen as a substrate and incubated for one hour. After incubation the plates were read at 414 nm in an ELISA reader. Standard curve relating optical density (OD) and the antigen content was prepared and used for deciding positivity and negativity. Any sample which had an OD value equal or above than the value of the standard four were considered positive.

Measurement of filarial specific antibodies

The antibody level of the sera samples from the individuals were determined by ELISA using the standard procedure (Voller *et al.*, 1976) by reacting of the sera with *Brugia pahangi* adult antigen. Briefly, the antigens were coated (0.5 µg/well) on to 96- well polystyrene plates (Nunc-U.S.A) and incubated overnight at 4°C. The sera samples from the experimental and control animals (diluted 1:100) were reacted for 2 h after blocking the wells with 1% Bovine Serum Albumin (BSA). Anti-mouse peroxidase conjugate (Sigma Chem. Co., USA) was allowed to react with the antigen-antibody complex, followed by the addition of o-Phenylenediamine dihydrochloride (Sigma Chem. Co., USA) as a chromogen in a substrate solution after another wash. After 20 min of incubation the reaction was arrested with 5N H₂SO₄ and O.D. was read at 492 nm in an ELISA reader (Titertek Multiscan., Lab systems). The cut-off OD value was determined based on the average O.D. value of normal sera added with 2 standard deviations.

Results

Samples drawn from three villages, viz., Pakkam, Alampoondi and Puthagaram, with population ranging from 1259 to 4643, were tested for the prevalence of microfilariaemia, antigen and antibody. The results of screening of 204 samples are shown in Table 1. Microfilaria (mf) was detected in 13 blood smears and the overall mf prevalence was 6.3, with a range between 2.4 and 7.8% in different villages. Mf prevalence increased with age, reaching its peak in 21-30 years of age and remained in a plateau form thereafter (Fig. 1). As many as 45 sera samples showed positive reaction to antigenemia test with an overall prevalence of 22.1%. The peak prevalence of antigenemia was in 31-40 years of age. Antibody prevalence also showed a similar age related prevalence. The overall positivity of sera samples for antibody was 16.7%, which was lower by 6% compared to antigenemia prevalence.

Table 1. Samples (n=204) and prevalence of microfilaria, antigenemia and antibody

Test	No. positive	% positive
Microfilaria detection	13	6.37
Antigen assay	45	22.06
Antibody assay	34	16.19

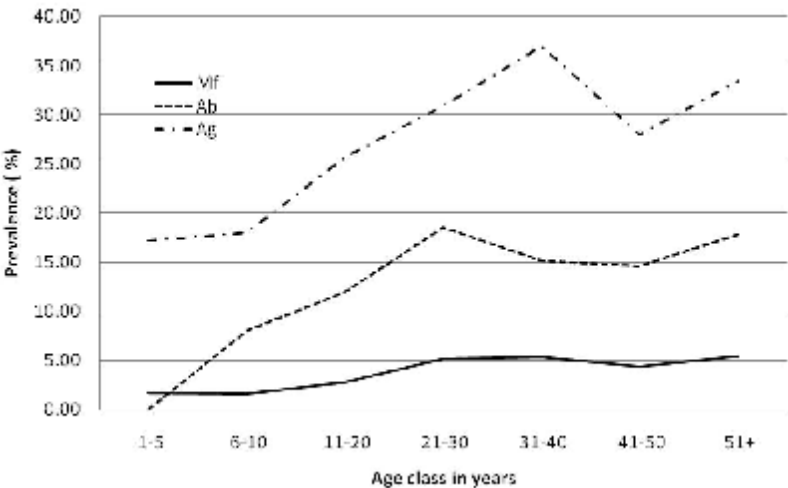


Fig. 1. Age specific prevalence of infectivity (mf), infection (Ag) and exposure (Ab).

Considering presence of mf as a gold standard in detecting the infection, the sensitivity of antigen was as high as 98% and the specificity was 75%. Ag prevalence was 15.8% higher than mf prevalence. True positives along with positives by antigen were 14% and true negatives with false positives by antigen test were as high as 99%. Similar comparison with antibody showed 9% sensitivity of antibody but specificity was as high as 87%. True positives along with positives by antibody test were about 2% and true negatives with false positives were 97%. The results

suggest that antigen test with high sensitivity of 98% can be a method of immunological test to detect infection with LF.

Considering antigen test result as a gold standard to compare with antibody detection, the sensitivity was only 13% and the specificity was 87%. Positive predictive value was 18% and the negative predictive value was 78%. This indicates that the response of the individuals exposed to infection and subsequent establishment of infection are not related and may be due to heterogeneity in immune mediated response.

Discussion

The infection with a given parasite or pathogen can be diagnosed based on signs and symptoms as clinical presentation, demonstration of parasite or pathogen in the sample specimens, presence of parasite product (antigen) or on the basis of host response to infection (antibody). Therefore, the clinical manifestation, a delayed outcome of infection would be useful for individual treatment only. But detection of parasite provides information on the potential risk of transmission and the host response reflects the risk of exposure to infection. Parasite detection can be done either by microscopy or by using molecular tools (probes). However, the host response is based on immunological markers.

The early irreparable lymphatic damage by the filarial parasite is the main cause for clinical manifestations of LF (Shenoy *et al.*, 2007). Such damages cannot be detected until outward signs are observed. Normally, the acute manifestations, which include ADL (filarial fever), are ignored and the external manifestation of lymphodema/hydrocele occurs after a long incubation period (Pani *et al.*, 1995). Therefore, delayed diagnosis may not be of therapeutic advantage.

A review of the literature reveals that only few of the promising tools are available for filarial antigen and antibody detection. For antigen detection the two commercially available tools are the ICT card (Weil *et al.*, 1997), and Og4C3 ELISA system (More and Copeman, 1990), whereas for antibody detection two rapid kits available are, WB RAPID and panLFRAPID cassette tests (Rahmah *et al.*, 2007) and also the Bm14 ELISA tests (Weil and Ramzy, 2007). A multicentric evaluation study showed that sensitivity and specificity of panLFRAPID were 96.5% and 99.6%, respectively; the specificity of 98.9-100% and the sensitivity of 96-100% have been reported for Bm14 ELISA (Noordin *et al.*, 2007). Og4C3, an antibody-based diagnostic assay developed from *Onchocerca volvulus*, is known to have greater diagnostic value in terms of high sensitivity and specificity (Gyapong *et al.*, 1998). Our observation (unpublished) also confirms this as it could detect 98% of known positives and about 17% more than that detected by parasite demonstration (mf examination).

Another advantage of using immunological markers is that samples can be drawn at any time unlike the night blood samples required for demonstrating mf. Antigen detection, therefore, is considered potential for application in detecting the foci of infection as well as evaluating the interruptions. MDA with DEC co-administered with Albendazole has been advocated as the strategy for elimination of LF (WHO), aiming at interruption of transmission. Reduction in mf prevalence is the

outcome of repeated rounds of MDA, which limits the application of mf survey in view of huge sample size. Therefore, it is considered ideal to use as more sensitive indicator, also revealing relatively higher levels of prevalence, which could reduce the sample size. Presence of mf is considered an indication of patent infection, indirectly confirming the presence of fecund adult worm (both male and female worms). However, in the case of infection with non-fecund female in the absence of male or single sex (male) infection post patent infections cannot be detected by screening for mf. Therefore, tools such as antigen detection are considered useful for delimitation and impact evaluation surveys. Recent reports on the impact evaluation of MDA programme showed the utility of immunological markers (Kumari *et al.*, 1994; Lammie *et al.*, 2004; Helmy *et al.*, 2006; Ramzy *et al.*, 2006; Weil *et al.*, 2008).

The risks of exposure can be considered most useful indicator, as only certain proportion of infective larvae could successfully enter into the lymphatic system and establish infection. It is the matter of fact that LF elimination programme primarily aims at interruption of transmission, thereby reducing the risk of exposure. But, the available antibody assay tests are limited with low sensitivity and hence warrant more studies. However, an earlier study evaluated ELISAs for anti-*Brugia malayi* immunoglobulins, IgG and IgG4 from 1561 subjects in French Polynesia for the serodiagnosis of *W. bancrofti* infection, compared with the test for *Onchocerca gibsoni* circulating antigen (Og4C3) as a 'gold standard'. The sensitivity of the ELISA-IgG and ELISA-IgG4 assays was reported to be high but specificity was low. Helmy *et al.* (2006) reported that 52.5% of IgG4 positive individuals became negative after two years of single dose treatment with DEC and Albendazole. This indicates that the response to the infection is influenced by the MDA. However, realizing the importance of a tool to assess the risk of exposure in the impact evaluation of MDA, there is a need to develop more sensitive antibody assays. Until such a tool is developed, immunological markers to detect antigen can be considered the method of choice in evaluating intervention programmes. Also, it has its potential in using it as a tool to identify the foci of infection and certification of elimination.

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Nanotechnology-based detection system for the control of infectious diseases – an overview

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Abstract

Infectious diseases like malaria, filariasis, dengue, chikungunya are widely prevalent in India and many tropical countries and are transmitted by mosquitoes. Despite the availability of vector control measures and potent drugs, these diseases continue to be a global threat. Recently, filariasis has been identified as one of the neglected diseases and efforts are underway to eliminate lymphatic filariasis on a global scale. Diagnostic tools are essential at every step of the elimination programme, initially for mapping endemic areas then for monitoring the progress of the programme and finally for verifying the absence of infection. To address the shortcomings of the traditionally used and available tools, research is needed to develop novel diagnostic assays and/or adaptations of currently available assays that can rapidly identify infection in human and vector in field setting. Molecular technologies have moved parasite-detection systems from traditional methods to improved PCR-based assays that have considerable potential for field use. At VCRC, a simple, rapid and inexpensive method for extraction of filarial DNA from mosquito vector, useful in Ssp1 PCR assay for xenomonitoring of infection with *Wuchereria bancrofti* has been developed. Like many other PCR systems, it relies on resolving the PCR product by agarose gel electrophoresis, followed by EtBr staining and detection of parasite specific DNA band based on their molecular size, which is a time-consuming and cumbersome process. To overcome these problems, more convenient field portable PCR detection schemes are sought. One such new approach is nanotechnology based sequence specific electrochemical detection of the PCR products, which would solve the problem of false positives associated with nonspecific amplification. Nanotechnology based detection of pathogens/parasites, a promising diagnostic approach to infectious diseases, will be discussed.

Keywords

Nanotechnology; Nanoparticles; Biodiagnostics; Methods; DNA; RNA; Diagnosis; Xenomonitoring

Introduction

Over the past decade, many important technological advances have been made in the use of nanotechnology in a wide spectrum of research areas including biomolecular detection. Nanotechnology is defined as the design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometer (10⁻⁹ m) scale. The concept of nanotechnology was first introduced by Richard Feynman in 1959 and the term nanotechnology used by Norio Taniguchi in 1974 at the University of Tokyo. The most significant aspect of this technology is its broad appeal and is the confluence of many sciences viz. biology, chemistry, engineering, materials, mathematics, and physics and many other areas in education. The new analytical tools and perspectives offered by this technology have significant and varied applications in the field of medical sciences. One of the major developments in nanotechnology and nanoscience is the production and application of nanoparticles (NPs) in biological and medical sciences. By controlling the particle sizes and shapes in the 1-100 nm range (Figs. 1 & 2) virtually all material properties can be controlled. The impact of advances in nanotechnology is particularly relevant in biondiagnostics, where nanoparticle based assays have been developed for specific detection of bioanalytes/pathogens of medical importance. This article reviews nanotechnology based detection systems that are medically relevant and have the potential to be incorporated in the diagnosis and control of infectious diseases.

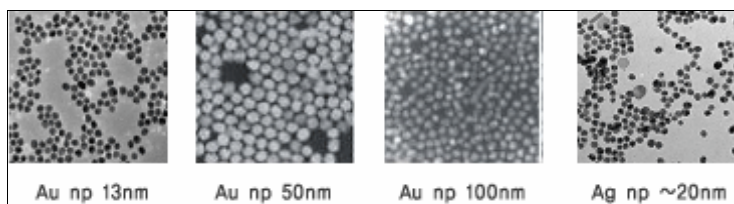


Fig. 1. Various sizes of the metal nanoparticles with specific emissive, absorptive and light scattering properties.

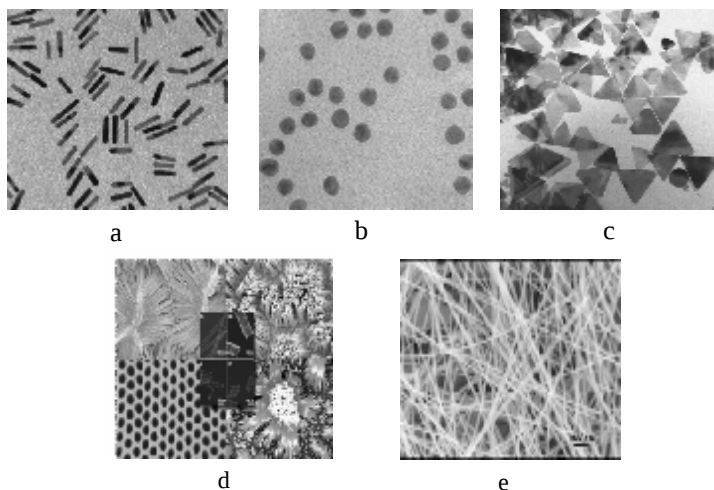


Fig. 2. Nanoparticles produced from different materials in different shapes such as (a) rods, (b) spheres, (c) triangles, (d) tubes and (e) wires.

Nanoparticles/Nanomaterials

Biological tests measuring the presence or activity of selected analytes become quicker, more sensitive and more flexible when nanoscale particles are put to work as tags or labels, with numerous advantages over more traditional procedures, for example fluorescence and chemiluminescence technology. Nanoparticles (NPs) have high surface areas and unique physical (such as particle aggregation and photoemission, and electrical and heat conductivities) and chemical (such as catalytic activity) properties that can be easily tuned, making them ideal candidates for developing biomarker platforms. NPs can be produced from different materials in different shapes such as rods, spheres, triangles, tubes and wires (Fig. 2 a-e). NPs can be classified based on the type of material into metallic (Gold, magnetic NPs), semiconductor (Quantum dots - ZnS and CdS) and polymeric NPs.

Gold nanoparticles (Au NPs)

Gold NPs are associated colloids with dimensions ranging from 0.8-250 nm (Hayat, 1970). Gold NPs show easily tuned physical properties, including unique optical properties arising from their surface plasmon resonance (SPR), robustness, and high surface areas, making them ideal candidates for developing biomarker platforms. Modulation of these physico-chemical properties can be easily achieved by adequate synthetic strategies and give gold nanoparticles advantages over conventional detection methods currently used in clinical diagnostics. The surface of gold nanoparticles can be tailored by ligand functionalization to selectively bind biomarkers. Thiol- linking of DNA and chemical functionalization of gold nanoparticles for specific protein/antibody binding are the most common approaches (Xu *et al.*, 2006). Simple and inexpensive methods based on these bio-nanoprobes were initially applied for detection of specific DNA sequences and are presently being expanded to diagnosis of infectious diseases.

Nanoparticle-based molecular diagnostics

Nucleic acid sequences unique to every living organism and every bacterium, virus, or pathogen provide practical targets for the identification and diagnosis of various diseases. With the advent of rapid sequencing capabilities, sequence information is now available for many diseases, including those associated with bioterrorism and warfare. To more effectively combat these diseases in the medical arena and accelerate response to bioterrorism threats, early and accurate detection of DNA markers is crucial.

Nanoparticle-based DNA detection

In this area, multidisciplinary teams of researchers including chemists, biochemists, biologists and physicists have been evaluating the prospect of using assays based upon nanomaterials to compete effectively with the polymerase chain reaction (PCR) coupled with molecular fluorophore assays (Tyagi and Kramer, 1996; Lander, 1999; Dugggan *et al.*, 1999; Fang *et al.*, 1999). PCR, a technology that allows duplication of portions of prospective targets, represents the ultimate in terms of sensitivity (Saiki *et al.*, 1985) but has significant drawbacks including complexity, sensitivity to contamination, cost, and lack of portability and major challenges with respect to multiplexing (detecting multiple targets in a single assay) (Kirk *et al.*, 2002). Many researchers view these limitations as some of the biggest impediments to moving

nucleic-acid-based detection to point-of-care settings. These settings require straightforward, inexpensive, and disposable detection formats that have rapid and accurate readouts and require limited processing and user expertise. For nanomaterials to compete in the area of nucleic-acid detection, they have to make a compelling case, with PCR and molecular fluorophore technology setting the benchmarks for comparison.

Optical methods of DNA detection

Nanoparticle-based optical DNA detection assays include different methods such as colorimetry (Elghnani *et al.*, 1997), SPR (He *et al.*, 2000), Fluorescence resonance energy transfer (FRET) (Ray *et al.*, 2006) and surface enhanced Raman spectroscopy (SERS) (Cao *et al.*, 2002). Optical detection methods relying on nanoparticle materials functionalized with oligonucleotides have shown enhanced sensitivity and selectivity compared to conventional assays based on molecular probes. *Mycobacterium tuberculosis* has been detected using gold nanoparticles resulting in the color change of the solution from red to purple color due to the hybridization event by colorimetric method.

Novel DNA assays utilizing distance-dependent optical properties of gold particle-modified oligonucleotides have been developed (Mirkin *et al.*, 1996). The hybridization-induced cross-linking of colloidal particles triggered a red-to-purple color change in solution (due to a red shift in the surface plasmon resonance of the gold nanoparticles). Such particle-based optical assays have been combined with simultaneous multiple-target detection capability (Taton *et al.*, 2001). A scanometric DNA array, based on silver amplification of a hybridization event, represents another important contribution from Mirkin's laboratory (Taton *et al.*, 2000). Labeling the DNA target with a nanoparticle offered greater mismatch discrimination and higher sensitivity (compared to the common use of fluorophore tags). The same group reported on a highly sensitive Raman spectroscopic method for detecting DNA and RNA based on gold nanoparticle probes labeled with oligonucleotides and Raman-active dyes (Cao *et al.*, 2002). The gold nanoparticles facilitated the formation of silver coating that acted as a surface-enhanced Raman scattering promoter, to yield femtomolar detection limits.

Spectral coding opens new opportunities in gene expression studies, high-throughput screening, and medical diagnostics (Han *et al.*, 2001; Nicewarner-Pena *et al.*, 2001). Nie and co-workers described a multicolor optical coding based on embedding different sized quantum dots into polymeric microbeads at precisely controlled ratios (2001). 'Bar-coded' metallic microrods (with segments of up to five different metals), synthesized by plating into the pores of a host membrane, were shown extremely useful for effective optical identification (Nicewarner-Pena *et al.*, 2001). Most early devices have relied on optical transduction of the hybridization event. Typically, the hybridization event (or patterns across the chip) is being detected by a confocal scanning laser system in connection to the use of fluorescent tagging agents (Piumno *et al.*, 1995). While fluorescent detection is successfully used, it is hampered by the need for bulky and costly control instrumentation. Mass-sensitive devices generating frequency signals offer label free sensitive detection but not suitable for routine diagnostic applications

Nanoparticle-based electrical DNA detection

Electrochemical devices have received considerable recent attention in the development of sequence specific DNA hybridization biosensors (Mikkelsen, 1996; Palecek and Fojta, 2001). Electrical DNA biosensors rely on the conversion of the Watson–Crick base-pair recognition event into a useful electrical signal. Electrochemical devices offer elegant routes for interfacing—at the molecular level—the DNA recognition and signal transduction elements, and are uniquely qualified for meeting the size, cost, low-volume, and power requirements of decentralized DNA diagnostics. The high sensitivity of electrochemical biosensors, coupled with their inherent miniaturization, compatibility with modern microfabrication technologies, low-cost and power requirements, and independence of sample turbidity, make such devices excellent candidates for centralized and decentralized genetic testing. While the use of electrochemical DNA biosensors or chips is at an early stage, easy-to-use hand-held electrical DNA analyzers are already approaching the marketplace (Chan *et al.*, 2000; Napier *et al.*, 2000), and are expected to have a considerable impact on future DNA diagnostics (Wilson, 1998). Electrochemical transduction of DNA hybridization events has commonly been achieved in connection to electroactive indicators/intercalators or enzyme tags. The use of nanoparticle tracers is relatively new in electrical detection, and offers unique opportunities for electrochemical transduction of DNA sensing events.

Coupling DNA detection with electrochemical readout has been widely studied (Drummond *et al.*, 2003). Redox-active nanoparticle probes are attractive because their electrochemical signal can be systematically tuned by changing their compositions and their binding properties to various biomolecules can be controlled. Recently, new assays were developed that involve electrochemical stripping of the nanoparticle portion of DNA-nanoparticle conjugates (Wang *et al.*, 2001). These systems employ a sandwich assay in which target capture strands are attached to magnetic beads (Wang *et al.*, 2003). Once the target oligonucleotide hybridizes to the capture strand, it is then labeled with an oligonucleotide-functionalized inorganic nanoparticle probe that code for the target strand of interest. The sandwich system can be magnetically separated and transferred to an electrochemical cell where the nanoparticles are dissolved and detected electrochemically. Different nanoparticles yield different voltammetric signals, depending upon their composition. The magnitude of the recorded signal corresponds to the concentration of target DNA, thus making this method amenable to multiplexing and quantification. However, the optimized detection limit of this assay is at 270 pM target concentration, still necessitating target amplification with PCR.

Applications of nanoparticle-based diagnosis

Gold nanoparticles have found promising applications in signal enhancement of the standard enzyme-linked immunosorbent assays (ELISAs) where they can be conjugated with the antibodies (Tanaka *et al.*, 2006) or coupled with silver-enhancement (Gupta *et al.*, 2007). Recent examples include the development by Tanaka *et al.* (2006) of a novel enhancement for immunochromatographic test strips where both the primary and the secondary antibodies are conjugated with AuNPs. Electrochemical approaches based on derivatization of electrodes with AuNPs have recently been applied to the label-free detection of the carcinoembryonic antigen (CEA) (Ou *et al.*, 2007; Lin *et al.*, 2007). Au-nanoprobe method has been developed

and successfully applied to the detection of single nucleotide polymorphisms (SNPs) and mutations associated with disease or metabolic variation (Doria *et al.*, 2007). This technique facilitates characterization of individual genetic variability, which has been associated with individual susceptibility to several multifactorial diseases such as cancer, diabetes, and also with individual response to therapeutics. As a model system, electrochemical DNA detection of *Salmonella typhimurium* using the PCR amplicons of *gyrB* gene as the specific site was accomplished (Rebecca *et al.*, 2006). A rather successful application of the Au-nanoprobe method to clinical diagnosis was in the rapid and sensitive detection of *Mycobacterium tuberculosis* (the etiologic agent of human tuberculosis) in clinical samples (Baptista *et al.*, 2006). In order to improve sensitivity and easily achieve quantification of target DNA/RNA samples, the detection system was directly integrated in an amorphous/nanocrystalline silicon device, without the need to functionalize the glass surface with the sequence of interest (Martins *et al.*, 2007). Other systems based on non-cross-linking DNA hybridization method (Taton *et al.*, 2001; Huber *et al.*, 2004; Hong *et al.*, 2005) and double-probe cross-linking method (Thaxton *et al.*, 2006) have been described and applied to detection and characterization of human SNP sequences.

Only very few Au-NP-based strategies have proven suitable for direct use on biological samples in clinical diagnostics. Most AuNP-based methods are rapid and easy to perform, especially when compared with the most commonly used molecular procedures, such as polymerase chain reaction (PCR) or real-time PCR. Even though these methods are widespread, the high costs involved and the need for highly skilled and trained operation make them difficult to implement at point-of-care and in remote regions or low-resource countries. The AuNP based methodologies that have already reached the clinical setting may help solve this issue. Even though a large number of methodologies and techniques have been presented for the detection of DNA/RNA through AuNPs, most have done so in controlled experimental settings and/or using synthetic or previously prepared molecules as targets. Baptista *et al.* (2005; 2006) developed a colorimetric method which utilized gold nanoparticles for rapid and sensitive direct detection of *M. tuberculosis* in clinical samples with high efficiency after an initial round of PCR. Gold-nanoparticle-based methods have been used extensively for detection of specific DNA and RNA sequences. This nanoprobe assay has a total work-up time of 2 h, and the reaction is performed in a single tube, which reduces carryover contamination. The method is inexpensive and can be performed with simple laboratory equipment, a PCR thermocycler and spectrophotometer, with visual detection made possible by a sharp color change. The overall cost was less than US \$0.35 per sample (including first-round PCR), which is considerably lesser than any other molecular methods (Baptista *et al.*, 2006). Few methods have been applied to the detection of DNA/RNA directly in clinical samples; these are summarized in Table 1.

Table 1.* Methodologies based on spherical gold nanoparticles for detection of nucleic acid sequences (DNA/RNA) directly in clinical samples. The underlying principle of the methods is listed, with limit of detection, molecular target (gene, locus, organism, etc) and type of sample

Detection	Detection limit	Clinical biological targets / sample	References
Naked-eye (dry-reagent dipstick) (dry-reagent dipstick)	2 fmol–25 fmo la,b	Hepatitis C virus (HCV) / RNA, human plasma SNPs (mannose-binding lectin gene, MBL2) / genomic DNA, human whole blood	Glunou <i>et al.</i> 2003 Litos <i>et al.</i> 2007
Naked-eye (electrostatic interactions of unmodified AuNPs)	100 fmol ^a	SNPs associated with long QT syndrome—KCNE1 gene/genomic DNA	Li and Rothberg 2004.
Light-scattering imaging (cross-linking aggregation)	33 zmol	Methicillin-resistant <i>Staphylococcus aureus</i> and <i>Staphylococcus epidermidis</i> , (mecA gene)/DNA, cultured bacteria	Storhoff <i>et al.</i> 1998.
Naked-eye or UV–visible Spectroscopy (non-cross-linking aggregation)	375 zmol–4.2 pmol; 100 nmol L ⁻¹ ^a	K-ras oncogene/genomic DNA, colorectal adenocarcinoma cell lines; β -thalassemia <i>Mycobacterium tuberculosis</i> /DNA, clinical specimens	Baptista <i>et al.</i> 2006 Doria <i>et al.</i> 2007
Light-scattering imaging (sandwich hybridization)	250 zmol–10 amol	Coagulation genes (Factor V, Factor II, and MTHFR—associated with thrombotic disease)/genomic DNA, human placenta; Gene expression in human brain/ Methicillin-resistant <i>Staphylococcus aureus</i> (mecA gene)/DNA, cultured bacteria	Huber <i>et al.</i> 2004

^a - May involve PCR

^b - May involve RT-PCR

* - From Baptista *et al.* (2008)

Lymphatic filariasis, one of the most infectious diseases affecting humans in over 80 endemic countries, mainly in tropical and subtropical belts, is caused by the lymphatic- dwelling nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. Globally, more than 90% of the infections are caused by *W. bancrofti* (Melrose, 2002). The global programme to eliminate lymphatic filariasis was launched with a time limited goal for disease elimination and diagnostic tools are essential at every step of the programme. At Vector Control Research Centre, a simple, rapid and inexpensive method for the extraction of filarial DNA from the vector, *Culex quiquefasciatus* useful in Ssp1 PCR assay has been developed for the rapid detection of the infection in vectors (Vasuki *et al.*, 2003). Like many other PCR systems, it relies on resolving the PCR product by agarose gel electrophoresis, followed by EtBr staining and detection of parasite specific DNA band based on their molecular size, which is a time consuming and cumbersome process. To overcome these problems, nanotechnology based sequence-specific electro-chemical detection of the PCR products is being developed to detect the filarial infection in the vectors. Such attempts will improve the detection process and may obviate the need for PCR, thereby leading to successful advancements through nanotechnology in reducing the time and cost.

Conclusion

Nanotechnology offers refinements and improvements in the conventional diagnostic techniques in medical fields especially in the clinical laboratories. Advances in nanotechnology are providing nanofabricated devices based on nanoscale particles that are small, sensitive and inexpensive enough to facilitate direct and speedy observation, manipulation and analysis of single biological molecule from single cell. This technology enables detection of a few microorganisms or molecules in relatively small sample volumes that would not be possible with conventional techniques. This opens new opportunities and provides powerful tools in the fields such as genomics, proteomic, molecular diagnostics and high-throughput screening. Nanotechnology, by reducing the time required for the detection has a positive impact on both decision-making and treatment costs.

Future trends

Although significant advances have been made involving nanotechnology and the use of NPs for diagnostics applications, technical and practical problems need to be solved. These include tight control over particle synthesis (size, size dispersion, capping agents) and functionalization, as small variations can drastically change their properties and behavior in diagnostic methods. One should note that most of the proposed systems must still be taken from proof-of-concept to use in generalized laboratorial settings, and from there to common clinical and diagnostic situations. Many questions on sample handling and preparation still need to be addressed, as many of these Au-NP systems are rather unstable in complex media, which may hinder application at point-of-care.

Further developments should include convenient high-throughput analysis, either by multiplexing and use of instrumentation, or by means of different chemistry that may vary the physicochemical properties of the AuNPs. Additional advances

will be directed towards creation of integrated platforms to test for a variety of analytes simultaneously without loss of sensitivity, providing fast, specific, and low cost analysis at point-of-care. This could bring from bench to bedside instruments that will enable faster screening of alterations (DNA associated with human pathology, gene expression profiles, biochemical analysis, and pathogen identification), enabling tailored therapeutic intervention, prevention, and reduction of the significant number of hospital admissions that result from therapeutic incompatibility and letdown. The potential effect of nanoparticles on organisms in natural environments is still unknown. Hence, there is a need to understand the potential hazards of nanoparticles on human health and on other organisms, while the use of nanoparticles in various scientific researches and medical applications continues.

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Structural analysis of purine nucleoside phosphorylase of *Schistosoma mansoni*

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Abstract

Schistosomiasis is a chronic, debilitating, major human parasitic disease caused by trematode parasites of the genus *Schistosoma* infecting over 200 million people in more than 74 countries throughout the world. Purine Nucleoside Phosphorylase (PNP) [EC 2.4.2.1] of nucleotide salvage pathway is reported to be a good drug target for Schistosomiasis. The complex of human PNP with guanosine where guanosine is a natural substrate of *Schistosoma mansoni* PNP as well, retrieved from Protein Data Bank (PDB), was analysed using HyperChem 7.5 for the identification of its substrate-binding amino acid residues. The corresponding amino acid residues in the *S. mansoni* PNP were identified by manual inspection of the sequence alignment of human and *S. mansoni* PNP. The amino acid residues of *S. mansoni* PNP with a potential for hydrogen bond formation with guanosine were identified by calculating the inter-atomic distances between the guanosine and amino acid residues using HyperChem 7.5. Computed Atlas of Surface Topography of Proteins (CASTp) was used to identify the substrate-binding pocket of *S. mansoni* PNP. Structure alignment of *S. mansoni* PNP and human PNP was carried out using Combinatorial Extension (CE) algorithm which indicated an average RMS deviation of 1.20 Å. However, some of the amino acid residues involved in substrate-binding have significant different spatial orientations. These residues are His 88, Tyr 90, Val 217, Thr 244, Asn 245, His 259. This observation highlights the significance of PNP as a drug target for *S. mansoni*.

Keywords

Structural analysis; Purine Nucleoside Phosphorylase; *Schistosoma mansoni*; Substrate-binding; Combinatorial Extension algorithm; Drug target

Introduction

Schistosomiasis, also known as Bilharzia or bilharziasis is a chronic and debilitating major human parasitic disease caused by trematode parasites of the genus

Schistosoma infecting over 200 million people. At least 600 million people are at risk of contracting schistosomiasis in 76 tropical and subtropical countries throughout the world (<http://www.who.int/tdr/diseases/schisto/direction.htm>). It is second only to malaria in terms of public health importance (Bergquist, 1995; Bergquist and Colley, 1998). The World Health Organization's latest report states that the number of deaths per year due to schistosomiasis could be as high as 200,000 and estimates are that 600 million people live in areas at risk (<http://www.who.int/tdr/diseases/schisto/direction.htm>). Chemotherapy is currently the main method of control for schistosomiasis. Several drugs have been developed for the treatment of schistosomiasis, which includes praziquantel, oxamniquine, hycanthone, etc. Nevertheless most of the existing drugs have their own disadvantages (Ribeiro-dos-Santos *et al.*, 2006). This sufficiently advocates the need for development of alternate drug with improved efficacy and safety.

***S. mansoni* purine nucleoside phosphorylase**

In *S. mansoni*, most of the cells have an active turnover of their nucleic acids, which through degradative processes result in the release of adenine, guanine and hypoxanthine. These free purines are reconverted to their corresponding nucleotides through salvage pathway. Due to the inability of schistosomes to synthesize purines de novo, they essentially rely on the salvage of exogenous purine bases into the corresponding purine nucleotides needed for the cellular metabolism (Kanaani *et al.*, 1995).

Purine Nucleoside Phosphorylase (PNP) [EC 2.4.2.1] catalyzes the reversible phosphorolysis of purine nucleosides to generate the corresponding purine base and ribose (deoxyribose) 1-phosphate and has previously been described as participating in the purine-salvage pathway in *S. mansoni* (Pereira *et al.*, 2003). PNP is a crucial enzyme for the survival of this pathogen. Therefore it has been proposed to be a potential target for antischistosomal chemotherapy.

Materials and methods

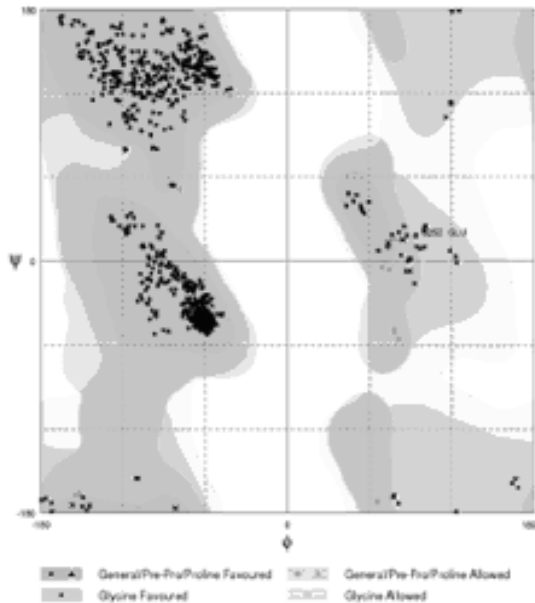
The crystal structure of *S. mansoni* was retrieved from Protein Data Bank (PDB) (<http://www.pdb.org/pdb/home/home.do>) (Berman *et al.*, 2000) and was assessed using RAMPAGE server (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) (Lovell *et al.*, 2003) for its Ramachandran plot occupancy. Owing to the absence of crystal structure of *S. mansoni* PNP complexed with its substrate (guanine) in the PDB, the complex of human PNP with guanosine where guanosine is a natural substrate of *S. mansoni* PNP as well, retrieved from PDB, was analysed using HyperChem 7.5 for identification of its substrate-binding residues. Further, the amino acid residues with a potential for hydrogen bond formation with guanosine were identified by calculating the inter-atomic distances between the guanosine and amino acid residues of human PNP using HyperChem7.5.

The corresponding amino acid residues in the *S. mansoni* PNP were inferred by manual inspection of the sequence alignment of human and *S. mansoni* PNP. Computed Atlas of Surface Topography of Proteins (CASTp) (<http://sts.bioengr.uic.edu/castp/calculation.php>) (Dundas *et al.*, 2006) was used to

identify the substrate-binding pocket of *S. mansoni* PNP. Structures of *S. mansoni* PNP and human PNP were aligned using Combinatorial Extension (CE) algorithm (http://cl.sdsc.edu/ce/ce_align.html) (Shindyalov and Bourne, 1998) using default parameters.

Results and discussion

Ramachandran plot of the crystal structure of *S. mansoni* PNP indicated the presence of 98.3% of the residues in favoured region while 1.6% of the residues lie in allowed region and 0.1% of the residues are outliers (Fig. 1). This suggests that the crystal structure of *S. mansoni* PNP is good enough to be used as a target for structure-based drug design.



Evaluation of residues			
Residue [A 4 :SER]	(66.14,-172.17)	in Allowed region	
Residue [A 57 :ASN]	(74.98, -5.47)	in Allowed region	
Residue [A 145 :LEU]	(80.55, 14.85)	in Allowed region	
Residue [A 222 :SER]	(-151.55,-166.26)	in Allowed region	
Residue [A 223 :THR]	(77.34, -49.20)	in Allowed region	
Residue [B 57 :ASN]	(69.69, -3.94)	in Allowed region	
Residue [B 145 :LEU]	(78.10, 15.58)	in Allowed region	
Residue [B 169 :ASP]	(-41.51, 118.49)	in Allowed region	
Residue [B 223 :THR]	(81.29, -55.83)	in Allowed region	
Residue [C 7 :ALA]	(-78.48, 51.34)	in Allowed region	
Residue [C 145 :LEU]	(82.86, 17.97)	in Allowed region	
Residue [C 222 :SER]	(-154.07,-166.74)	in Allowed region	
Residue [C 223 :THR]	(77.99, -50.29)	in Allowed region	
Residue [B 252 :GLU]	(93.75, 18.31)	in Outlier region	
Number of residues in favoured region	(~98.0% expected)	:	795 (98.3%)
Number of residues in allowed region	(~2.0% expected)	:	13 (1.6%)
Number of residues in outlier region		:	1 (0.1%)

Fig. 1. Ramachandran plot of *S. mansoni* PNP.

residues with inter-atomic distances below 3.2Å and the appropriate orientation of hydrogen bond donor and acceptor atoms have a potential to form hydrogen bond. CASTp showed a pocket that contained the guanosine-binding amino acid residues which was inferred to be the substrate-binding pocket (Fig. 3). It has surface area of 735.9 Å² and volume of 1667 Å³.

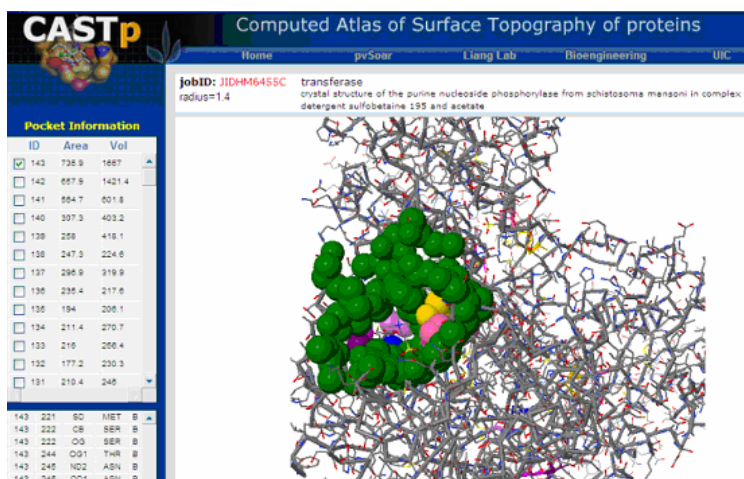


Fig. 3. Substrate (guanosine)-binding pocket of *S. mansoni* PNP.

Structural alignment of the human and *S. mansoni* PNPs carried out using CE algorithm (Fig. 4) to delineate their possible structural dissimilarities indicated that the two molecules have average RMS deviation of 1.20 Å which is quite a significant structural similarity. However, some of the amino acid residues involved in substrate-binding have significant different orientations. These residues are His 88, Tyr 90, Val 217, Thr 244, Asn 245, His 259. This observation highlights the significance of PNP as a drug target.

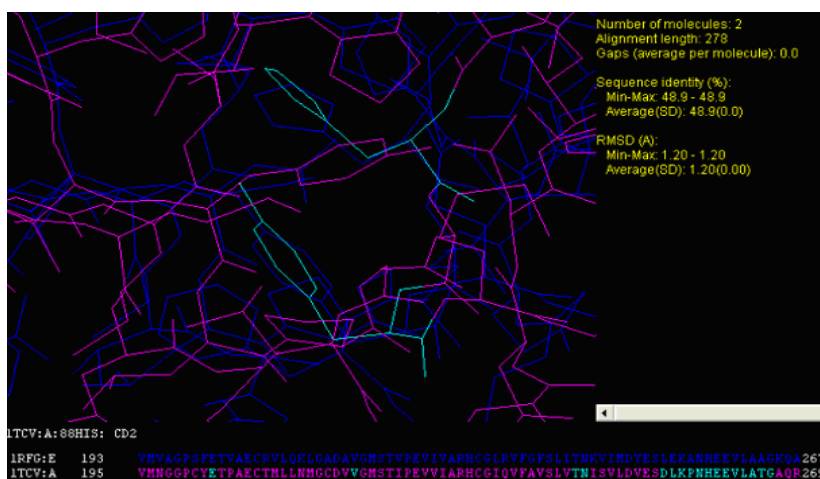


Fig. 4. Structural alignment of *S. mansoni* PNP and human PNP.

Conclusion

Structural analysis of *S. mansoni* PNP and its comparison with human PNP further corroborate the fact that it is a potential target for antischistosomal chemotherapy and can be explored for structure-based drug design.

Acknowledgements

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Inaugural Session

From Left: Dr. J. K. Saxena (Secretary, ISP); Prof. V. Tandon (President, ISP); His Excellency the Governor of Meghalaya, Shri R. S. Mooshahary; Hon'ble Vice-Chancellor, NEHU, Prof. P. Tandon; Prof. R. N. K. Hooroo (HOD, Zoology, NEHU) and Dr. A. K. Yadav (Organizing Secretary).



Participants of the Congress

Cost-effective medium for the production of mosquito pathogenic bacilli

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Abstract

Industrial wastes consist of organic and inorganic materials, which are discharged in large scale, in abundance from factories, fisheries, poultries and food processing industries. Development of suitable technologies are necessary to reutilize these bio-organic wastes, for the benefit of mankind. It can be achieved through bio-degradation of such unused disposals. This in turn would help to reduce environmental accumulation of waste. Sugarcane processing industries discard bagases, in bulk, as biological wastes, globally. They consist mainly of sugar (sucrose) and trace quantity of proteins and lipids. Soyabean, a food product from agricultural industries, contains mainly proteins. We have developed a cost-effective technology to utilize these bioorganic wastes as culture media to produce mosquitocidal biopesticides, *Bacillus sphaericus* (Bs) and *B. thuringiensis* serovar israelensis (Bti), capable of synthesizing mosquitocidal toxins. Biochemical studies indicated that the mosquitocidal spore/crystal toxins produced from the experimental medium (Bagases, BA + Soyabean, SB) were higher as compared to that of the conventional medium (Luria Bertani, LB). The bacterial toxins produced in these media (LB, BA, SB, BA + SB) were bioassayed against the mosquito vectors (*Culex quinquefasciatus*, *Anopheles stephensi*, *Aedes aegypti*) and the toxic effect was found to be significant. Cost-effective analysis indicates that the use of combination of bagases waste and Soyabean as bacterial culture media is effective and economical, for the production of these mosquito pathogenic bacilli. This study is, therefore, important as it explores the possibility of cost-effective utilization of environmental bio-organic wastes, for the production of mosquitocidal biopesticides.

Keywords

Bio-degradation; Bagases; Soyabean; *Bacillus sphaericus* (Bs); *B. thuringiensis* serovar israelensis (Bti); *Culex quinquefasciatus*; *Anopheles stephensi*; *Aedes aegypti*; Cost-effectiveness

Introduction

Biological control of mosquitoes, using *Bacillus sphaericus* (Bs) (Neide) and *B. thuringiensis* serovar israelensis (Bti) (deBarjac), is gaining widespread acceptance in the control of pests and vectors of diseases, especially mosquitoes, principally due to the widespread occurrence of chemical insecticide contamination and the development of resistance in insects. Since the discovery of the mosquitocidal property of Bs and Bti, various commercial formulations of these agents have been developed and evaluated. The attributes of their preparations such as large-scale production, high efficacy, specificity, long shelf-life, cost-effectiveness, transportability and relatively low risk of development of resistance, as well as, their safety to non-target organisms, ensure a promising future for these agents, in practical application, for mosquito control (Mulla *et al.*, 1988; Kumar *et al.*, 2000; Poopathi and Tyagi, 2006).

At the time of sporulation, the Bs synthesizes proteins that assemble into crystals, which are composed of two major polypeptides, with molecular masses of 51 and 42 kDa, whereas Bti synthesizes four polypeptides (mol.wt:135, 125, 68, and 28 kDa), referred to as Cry4A, Cry4B, Cry11A and Cyt 1A, respectively (Delecluse *et al.*, 1995). These crystal proteins interact synergistically, causing toxicity against mosquito larvae, upon ingestion along with food materials (Poncet *et al.*, 1995). The mode of action of these bacterial toxins in mosquitoes involves highly specific binding to cells of midgut brush border membrane (Ravoahangimalala and Charles 1995; Silve-Filha *et al.*, 1997; Poopathi *et al.*, 2002a).

Despite, many advantages of Bs and Bti based biopesticides, their application is limited due to high production costs (Obeta and Okafer 1984; Poopathi *et al.*, 2002b, 2003; Lachhab *et al.*, 2001). In the conventional process, these biopesticides are commercially produced utilizing synthetic medium, (Nutrient Yeast Extract Salt Medium, NYSM) comprising glucose, peptone, beef extract, yeast extract and mineral salts and, at times, extra ingredients need to be added to enhance the sporulation process. The cost of Bs/Bti biopesticide production depends on many factors, however, the raw material cost is one of the most important criteria which comprises > 70 % of the overall production cost (Ejiofer, 1991). Therefore, selection of growth medium or raw material is critical for commercial production of these biopesticides. In order to encourage the commercial production of biopesticides, utilization of less expensive raw material is advisable (Mummigotti and Raghunathan, 1990). Several raw materials (industrial and agricultural byproducts) have been tested successfully, as alternative culture media, for the production of Bs/Bti (Obeta and Okafer, 1984; Poopathi *et al.*, 2002b, 2003; Saalma *et al.*, 1983a, b; Ventosilla and Guerra, 1997; Poopathi and Anup Kumar, 2003; Poopathi, 2005).

Bagases (BA) from sugar industries and the cheaply available Soya bean (SB) are very good sources of carbon, nitrogen and other nutrient sources, for the microbial synthesis of mosquitocidal toxins. The present study indicated that a judicious combination of both these ingredients yielded a superior level of bacterial growth, than the conventional medium (LB). Besides, it was found to have higher yield in terms of biomass and toxin production. This study shows that agro residues

and Soya bean can be utilized as a suitable substrate for biopesticide production. Therefore, the objectives of the present study were: (1) to demonstrate that a combination of BA and SB could be used as a cheap source of nutrients to produce Bs/Bti biopesticides, (2) to compare the mosquito toxicity levels of Bs/Bti produced from the test medium, and (3) to assess the cost-effectiveness of the test medium.

Materials and methods

Microorganisms

Bacillus sphaericus (IAB-59) and *B. thuringiensis* serovar israelensis (IPS-82) used in this study, was obtained from the Bacteries Entomopathogenes, Institute Pasteur, Paris, France (courtesy: Dr. Jean Francois Charles).

Preparation of bacterial culture medium

The studies were conducted with LB (Luria Bertani Medium), as reference medium, which contains (gm/l) peptone, 20, yeast extract, 10, and NaCl, 10, (pH 7.8). Bagases (BA) was collected from sugarcane industries, air-dried, powdered and stored at room temperature (32°C). Soya bean powder that is cheaply available in the market was also used for the present study. A known quantity of these dried BA and SB materials (20 gm/l each) were boiled separately in ordinary tap water for 15 min. After cooling, the extracts were removed and the pH was adjusted (7.8). The extracts (BA/SB) were dispensed separately into three Erlenmeyer flasks (2 liter capacity each) for culturing Bs and Bti and plain medium (control, without Bs and Bti) respectively. Similarly, the extracts of BA and SB were combined (1:1) and dispensed separately into three Erlenmeyer flasks for culturing Bs/Bti and plain medium. The conventional medium (LB) was also maintained along with. All the bacterial culture media were autoclaved (at 120°C / 20 lb / in² / 20 min).

Inoculum

A small amount of Bs and Bti lyophilized primary powder was inoculated separately in 2 ml each LB medium and allowed to grow for 12 h at 32°C as pre-culture. A small volume of these pre-cultures (50µl each) was inoculated into culture media. The cultures were allowed to grow under constant agitation (120 rev/min) under room temperature (32°C) in an orbital shaker. Culture samples (2 ml) were drawn from each culture medium at 6 hr intervals from 0 to 72 h. The pH and culture turbidity were measured using a digital pH meter (Genei, India) and SP75 UV-VIS spectrophotometer (Sanyo, UK). These were also examined microscopically for detection of sporulation and production of crystalline inclusions.

Separation of spore/crystal toxin from culture media

After complete sporulation of Bs/Bti from the media, the spores/crystals were harvested by centrifugation (10,000 x g / 30 min / 4°C) using SORVALL Evolution RC super speed centrifuge (Kendro, USA), by discarding the culture supernatants (Payne and Davidson, 1984). The pellets containing the cell mass were washed three times each with 0.1 M NaCl and sterile double distilled water (10,000 x g / 15 min / 4°C) and finally these were treated with protease inhibitor (phenyl methyl sulphonyl fluoride, 1mM, Sigma), resuspended in sterile water and stored at -20°C, until further use.

Protein analysis

Protein concentration in samples is an indication of toxin production from Bs/Bti and was determined with Folin-phenol reagent, using bovine serum albumin (BSA) as standard (Lowry *et al.*, 1951). A small quantity of the Bs/Bti spore/crystal mixture complex was centrifuged (10,000 x *g* /30 min /4°C) and the pellets were solubilized in alkaline buffer (NaHCO₃, 50 mM, dithiothreitol, 10 mM, pH 10) and incubated for 3 h at 32°C. After centrifugation and extraction, the solubilized protein was quantified using UV-VIS spectrophotometer (optical density at 620 nm).

Electrophoresis

A total of 10µg protein equivalent samples (Bs/Bti spores/crystals) from test and conventional media were mixed with equal volumes of sample buffer (β-mercaptoethanol, 0.5M and SDS, 3%), boiled for 5 minutes and separated by electrophoresis in 10% sodium dodecyl sulphate- polyacrylamide gel (SDS-PAGE) unit (Genei, India) (Lammeli, 1970). The protein bands were stained with Coomassie Brilliant Blue R-250 and visualized.

Toxicity assays

Mosquito larvae (*Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*), received from the Division of Mosquito Rearing and Colonization (R & C), Vector Control Research Centre, Puducherry, India were used in the present study. These larvae were reared in the laboratory as per the standard larval rearing procedures (Poopathi *et al.*, 1999). The bioassays for Bs/Bti were carried out according to World Health Organization (WHO 1982, 1985). A stock solution was prepared from Bs/Bti (5 gm/100 ml water for Bs and 100 mg /100 ml water for Bti) and homogenized, using glass beads, in a mechanical shaker. From the stock solution, serial 2-fold dilutions were made to obtain dosages ranging from (mg/l) 33.33 to 0.02 for Bs and 13.33 to 0.33 for Bti. Bioassays were conducted in disposable wax coated paper cups (250 ml capacity). Test media were prepared by adding appropriate volumes of Bs/Bti in 150 ml of water and 15 early third instar larvae from each of the three mosquito species were introduced separately in each of the test concentrations. While food supplement (dog biscuit and yeast mixture, 2:1) was provided for larvae under Bs treatment, no food was provided for Bti treated larvae as recommended by WHO. The bioassays were conducted at room temperature (32°C) and larval mortality was assessed after 24 and 48 hr for Bti and Bs treatment, respectively. Abbott's formula was used to correct the control mortality (< 20%) (Abbott, 1925). Moribund larvae in the replicates were counted as dead.

Data analysis

The data from the growth and sporulation of Bs/Bti cultured from the test (BA, SB and BA + SB) and control (LB) medium were subjected to the students 't' test, to analyze the significance of difference ($P \leq 0.05$), using SPSS package (SPSS-2001, window version 13.0). The LC₅₀ and LC₉₀ values were estimated by Probit regression analysis, using a software package 'ASSAY' (courtesy: Dr. C. F. Curtis, London School of Tropical Medicine and Hygiene, UK).

Results

The duration of growth and spore production of bacteria (Bs) in the experimental (BA, SB and BA + SB) and in the conventional culture media (LB) is shown in Fig. 1. In general it was observed that in all the culture media, after a lag phase of about an hour, there was a rapid multiplication of bacterial cells and maturation of spores. With increasing culture time, culture density increased (optical density at 650 nm) and reached a plateau (range 1.5 to 3.3). This process of multiplication lasted until 48 h, followed by cell lysis, which released the delta-endotoxin (crystal toxins) into the media. The maximum growth and release of endotoxins was completed at 72 h of growth. Thus, the overall growth and production of Bs spore/crystal toxins in the experimental media were comparable with that of the conventional medium (LB).

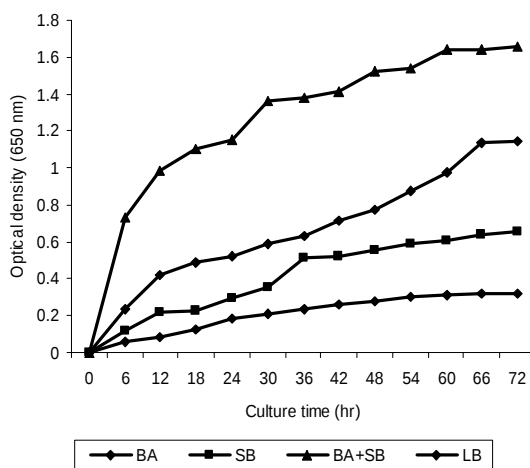


Fig. 1. Growth pattern of *Bacillus sphearicus* cultured in different media.

As shown further, from Fig.1, it is worth to mention that in all the three experimental media, between 60 to 72 h of fermentation (peak growth period), an appreciable amount of spores/delta endotoxin crystals were released, which indicated that the bacteria was able to grow and utilize the nutrients contained in all the media. The growth pattern studies (measured by OD values at 650 nm) showed that the range for LB was 1.00 to 1.14, whereas, for the combination medium (DRB + BFW), it was 1.40 to 1.70, which indicated that it had the highest bacterial growth rate ($P < 0.05$).

The growth rate of SB was equivalent to that of LB ($P > 0.05$), whereas BA showed the least growth, since, the degree of sporulation is lesser, but, a combination strategy with SB exhibited a higher growth rate amongst all the other media. Similar observation was found on the growth pattern of Bti as shown in Fig. 2.

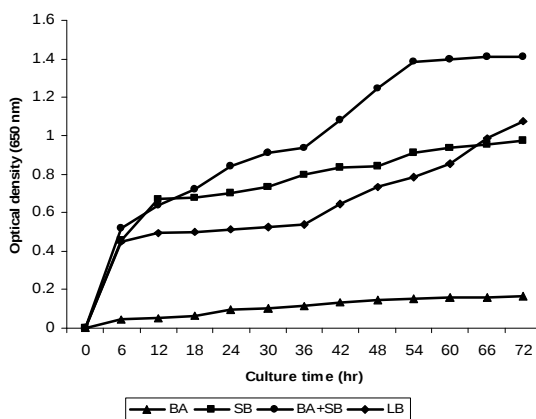


Fig. 2. Growth pattern of *Bacillus thuringiensis servar israelensis* (Bti) cultured in different media.

The protein concentration, which is an indication of the capacity for the toxin production, in the various media, exhibited the following trend, viz., BA + SB > LB/SB > BA (Figs. 3 and 4). This indicated that the efficacy of Bs and Bti toxin production was the maximum in the combination medium (2.91 g/l). It was further observed that the overall growth and production of toxins in the SB alone was comparable to that of LB, and the mean values were not significantly different ($P > 0.05$). The yield of Bs bacterial cells/crystals, which is an indication of biomass production, was also highest for the combination medium (80 gm/l) than the LB (1.8 gm/l) (Fig. 5). Similar trend was also observed for the growth and sporulation of Bti toxins (Fig. 6). Microscopic observation of Bs and Bti spores/crystals obtained from BA + SB and LB after 72 h growth period indicated that the sporulation in the former was better than the latter medium.

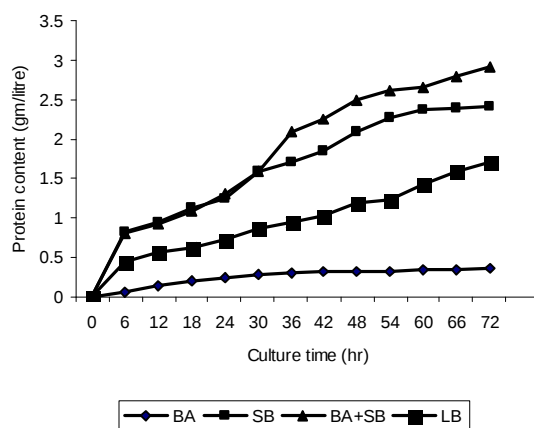


Fig. 3. Spore/crystal concentration of *B. sphaericus* cultured from different media.

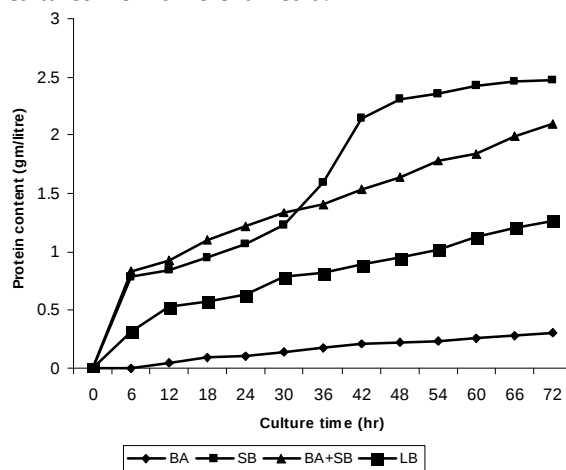


Fig. 4. Spore/crystal concentration of *Bti* cultured from different media.

Analysis of the protein profile of Bs and Bti spore/crystal toxins produced from the new (BA + SB) and the conventional culture medium (LB) by SDS- PAGE (10%) was also done and it was found that the major mosquitocidal polypeptides present in the parasporal crystal proteins of Bs and Bti (Bs : 51 and 42 kDa and Bti: 134, 125, 67 and 27 kDa proteins) produced from the new and conventional culture media (BA + SB and LB) were clear and conspicuous (Fig. 7). There was no variation in the protein pattern between the toxins produced from the conventional and new media. The protein profiles, as indicator of expression of binary (Bs) and multiple (Bti) toxins, were consistent with their larvicidal activity in the laboratory experiments.

Toxicity tests with mosquito larvae (bioassays) were performed with Bs and Bti toxins produced from the conventional and the new culture media. The lethal

concentrations were expressed in milligram of toxins per liter. The laboratory reared mosquito species of *C. quinquefasciatus*, *A. stephensi* and *Ae. aegypti* were used for bioassays. The comparative toxicities of Bs produced from the culture media are shown in Table 1. The LC50 and LC90 values for Bs produced from LB against *C. quinquefasciatus* were (mg/l) 0.13 and 0.73, respectively. These toxicity values were statistically similar to those of Bs produced in all three experimental media (BA + SB, BA, SB), since, fiducial limits were overlapping. The other two mosquito species had similar effects of toxicity when tested with the cultures grown in the conventional and test media. Table 2 also represents data on the efficacy of Bti produced from the new culture medium in comparison with that of the conventional medium. The Bti from BA + SB was highly effective against all the three mosquito species tested and was found to be comparable with that of conventional medium. It is worth mentioning here, that, Bti produced from both the culture media exhibited a higher lethal effect on the larvae than Bs, obviously, due to the presence of multiple toxins in Bti. The present study reveals that this new medium resulted in higher maximum specific growth rate, sporulation, toxin yield and entomotoxicity.

Cost analysis indicated that the quantity of bagases and soya bean powder needed to prepare one liter of culture medium was 20 gm respectively

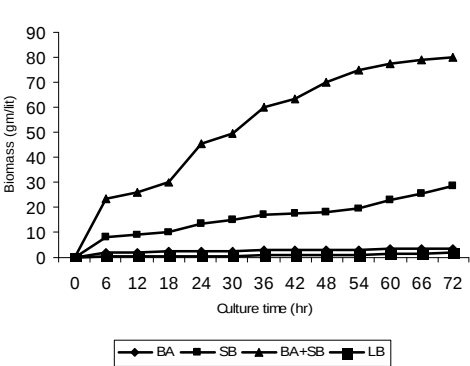


Fig. 5. *Bacillus sphaericus* biomass production in cultured from different media.

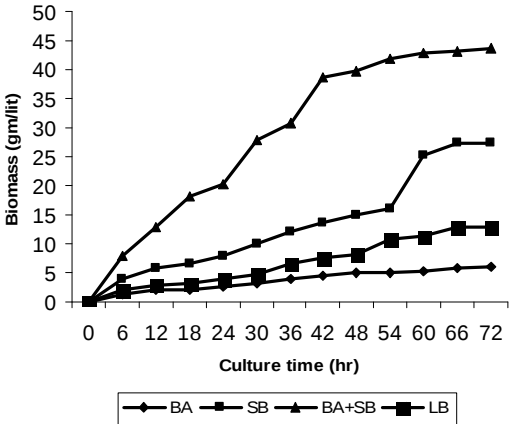
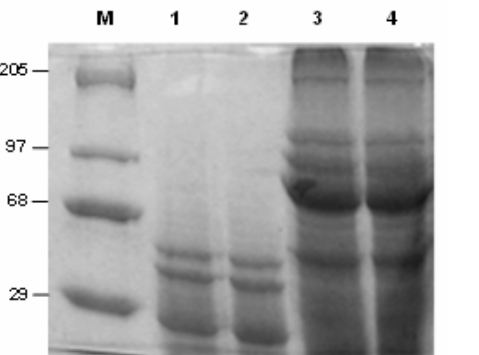


Fig. 6. *Bti* biomass production in cultured from different media.



Lane M: Protein Marker, lane 1: LB (Bs), lane 2: BA + SB (Bs), lane 3: LB (Bti), lane 4: BA + SB (Bti)

Fig. 7. SDS-PAGE of *Bacillus sphaericus* and *B. thuringiensis* serovar *israelensis* in various culture media.

Table 1. Toxicity of *Bacillus sphaericus* (SPH-88) produced from media formulated from biological waste materials against various mosquito species

Mosquito species	Intercept	Slope $\pm$ SE	LC ₅₀ (mg/l)	LC ₉₀ (mg/l)	χ^2 (df)
<i>Culex quinquefasciatus</i>					
A	6.54	0.76 $\pm$ 0.19	0.13 (0.19 – 0.09)*	0.72 (1.34 – 0.38)	1.17(6)
B	6.64	0.70 $\pm$ 0.18	0.09 (0.15 - 0.07)	0.60 (1.16 – 0.31)	0.97 (6)
C	6.75	0.83 $\pm$ 0.18	0.12 (0.17 - 0.08)	0.57 (0.99 - 0.32)	1.58 (6)
D	6.73	0.82 $\pm$ 0.18	0.12 (0.18 – 0.08)	0.58 (1.03 - 0.33)	1.89 (6)
<i>Anopheles stephensi</i>					
A	5.51	0.78 $\pm$ 0.19	0.52 (0.76 – 0.35)	2.68 (4.85 - 1.48)	2.64 (6)
B	5.37	0.69 $\pm$ 0.21	0.59 (0.88 – 0.39)	3.68 (7.10 – 1.91)	1.94 (6)
C	5.44	0.69 $\pm$ 0.21	0.53 (0.79 – 0.34)	3.28 (6.29 – 1.71)	1.10 (6)
D	5.26	0.74 $\pm$ 0.19	0.70 (1.03 – 0.48)	4.01 (7.55 – 2.13)	0.19 (6)
<i>Aedes aegypti</i>					
A	4.55	0.62 $\pm$ 0.23	2.06 (3.27 – 1.29)	16.18 (33.89 – 7.73)	2.64 (6)
B	4.19	0.80 $\pm$ 0.18	2.73 (3.94 – 1.89)	13.43 (24.19 – 7.46)	0.74 (6)
C	4.32	0.82 $\pm$ 0.18	2.27 (3.28 – 1.57)	10.78 (18.85 – 6.17)	1.57 (6)
D	4.64	0.68 $\pm$ 0.22	1.67 (2.66 – 1.08)	12.65 (21.55 – 5.70)	1.58 (6)

Bacillus sphaericus (SPH - 88) culture medium: A = LB; B = Bagases (BA); C = Soyabean (SB); D = BA + SB;

*95% Fiducial limits of upper and lower at LC₅₀ and LC₉₀ levels.

Table 2. Toxicity of *Bacillus thuringiensis* serovar *israelensis* (IPS-82) produced from media formulated from biological waste materials against various mosquito species

Mosquito species	Intercept	Slope $\pm$ SE	LC ₅₀ (mg/l)	LC ₉₀ (mg/l)	χ^2 (df)
<i>Culex quinquefasciatus</i>					
A	7.56	0.73 $\pm$ 0.19	0.03 (0.04 – 0.02)*	0.17 (0.33 – 0.09)	0.81(6)
B	8.15	0.84 $\pm$ 0.17	0.02 (0.03 - 0.01)	0.11 (0.18 – 0.06)	1.56 (6)
C	8.15	0.86 $\pm$ 0.18	0.02 (0.03 - 0.01)	0.11 (0.19 - 0.06)	1.19 (6)
D	8.07	0.83 $\pm$ 0.18	0.02 (0.03 – 0.01)	0.12 (0.21 - 0.06)	0.74 (6)
<i>Anopheles stephensi</i>					
A	5.79	0.74 $\pm$ 0.19	0.34 (0.50 – 0.23)	1.92 (3.74 - 0.98)	0.92 (6)
B	6.13	0.83 $\pm$ 0.18	0.25 (0.37 – 0.18)	1.19 (2.1 – 0.68)	0.74 (6)
C	6.05	0.83 $\pm$ 0.18	0.28 (0.40 – 0.19)	1.31 (2.32 – 0.75)	1.19 (6)
D	5.91	0.88 $\pm$ 0.17	0.36 (0.49 – 0.25)	1.50 (2.56 – 0.88)	0.73 (6)
<i>Aedes aegypti</i>					
A	4.90	0.70 $\pm$ 0.21	1.14 (1.71 – 0.76)	7.02 (13.78 – 3.57)	2.10 (6)
B	5.03	0.78 $\pm$ 0.19	0.96 (1.40 – 0.65)	4.99 (9.11 – 2.79)	0.42 (6)
C	5.09	0.79 $\pm$ 0.19	0.89 (1.30 – 0.61)	4.45 (8.01 – 2.47)	1.29 (6)
D	4.99	0.95 $\pm$ 0.16	1.00 (1.39 – 0.72)	3.87 (6.47 – 2.32)	2.22 (6)

Bacillus thuringiensis serovar *israelensis* (IPS-82) culture medium: A = LB; B = Bagases (BA); C = Soyabean (SB); D = BA + SB;

*95% Fiducial limits of upper and lower at LC₅₀ and LC₉₀ levels.

Table 3. Comparative costing for producing biopesticides from conventional and experimental culture medium

Culture medium	Main constituent of the medium	Quantity for culture preparation (gm/l) (dry weight)	Cost of quantum used (Rs)	Total cost for culture medium used (Rs)
LB	Peptone + Yeast extract + NaCl	20 + 10 + 20	45 + 94 + 2	141
BA	Bagasses	20	Nil	Nil
SB	Soya bean	20	0.50	0.50

LB = Luria Bertani (conventional); BA = Bagases (experimental); SB = Soya bean (experimental)

which is of negligible value, as they are sugarcane waste products (Table 3). Interestingly, this combination medium (BA + SB) is most economical, compared to any other media. Moreover, it is easily available globally as an environmental, bio-organic and industrial waste. On the other hand, preparation of one liter of LB involves a cost of Indian Rupees 141.

Discussion

Mosquito-borne diseases like, filariasis, malaria, dengue and Japanese encephalitis, remain a serious public health problem, all over the world. The control of these important mosquito borne diseases relies heavily on the extensive use of chemical insecticides, although, they are very expensive and toxic to non-target organisms. Owing to these constraints, biological control agents like, *Bacillus sphaericus* (Bs) and *B. thuringiensis* serovar israelensis (Bti) have been used widely and effectively in mosquito control programme. But, for operational purposes, there is an urgent need to produce these bacteria, utilizing cheap and commonly available biological waste materials, through simple fermentation biotechnology.

Everyday, enormous quantities of bio-organic wastes are discharged from factories, fisheries, poultries and food processing industries. Within the food processing sectors, various liquid, sludge, and solid biological and organic wastes require remediation and alternative disposal methods are increasingly being investigated. Degrading or handling these wastes as unused disposals without acquiring additional benefits has led to an idea to develop a suitable technology to utilize bio-organic wastes by means of simple fermentation technology into cost-effective vector control agents.

The by-products of rice milling are used for a variety of purposes. Rice bran is the most valuable by-product of rice milling industry. It is obtained from the outer layers of the brown rice. Generally, rice bran consists of pericarp, aleurone layer, germ and a part of endosperm. Bran removal amounts to 4 to 9 per cent of the weight of paddy milled and is abundant in oil (Martin, 1994). Rice bran is nutritionally rich with 16-22% lipid, 12-16% protein, 8-12% crude fibre and high levels of other vitamins and minerals (Saunders, 1990). It has been utilized for the extraction of edible oil and the remaining waste material is being discarded as de-oiled rice bran waste from oil mill industries. The de-oiled bran contains 20-22% protein, 16% crude fibre, 10% moisture, 0.5-1% fat and vitamins (Vitamins A and E). It is rich in amino acids like methionine, cystine, lysine and phosphorus (Ravinder *et al.*, 2003).

Carbon and nitrogen sources are very essential for the good growth of bacteria. Chicken feathers have also been discarded in bulk as waste from poultry processing industries, poultry farms and shops globally. They normally accumulate structural proteins (keratins) that are resistant to biodegradation. Keratinase produced from *Bacillus licheniformis* degrades the keratin and the resultant material was used for the production of cattle feed and fertilizer (Lin *et al.*, 1997, 1999; Cheng *et al.*, 1995; Zaghoul *et al.*, 1998; Riffel and Brandelli, 2002; Ramnani and Gupta, 2004; Wang *et al.*, 2005). Keratins consist of long chains of amino acids, mainly, alpha and beta-keratins (Lin *et al.*, 1996; Kim *et al.*, 2002; Takahashi *et al.*, 2004; Gupta and Ramnani, 2006). The present study focuses on the utilization of proteins and carbohydrates from bird feathers and de-oiled rice bran respectively, by *Bacillus*

sphaericus (Bs) and *B. thuringiensis* serovar israelensis (Bti), for the production of mosquitocidal endotoxins.

Biochemical studies have indicated that the mosquitocidal spore/crystal toxins produced from the experimental culture medium (BA + SB, ratio 1:1) were similar to that of conventional medium (LB). These toxins, were bioassayed against the mosquito vectors (*Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*), and it was found that the larvicidal effect of both the media was equally good. The present study indicated that a judicious combination of both these terms of, biomass and toxin production. Earlier, many Bs and Bti formulations produced from conventional media (Luria Bertani) have been tested in the field for mosquito control. Subsequently, cost-effective formulations were utilized for biopesticide production. Obeta and Okafor (1984) cultured Bti on five formulated media from seeds of legumes (groundnut cake, cow pea of white and black varieties, soya bean, and Bambara beans), dried cow blood and mineral salts. The other workers have also used potatoes, coconuts, fish meal and corn steep liquor for the production of biopesticides (Saalma *et al.*, 1983a, b; Ventosilla and Guerra, 1997; Poopathi and Anup Kumar, 2003; Kuppusamy, 1990). Our fermentation studies with mosquitocidal bacteria produced from agro-waste indicated that the growth and production of endotoxins were appreciable and comparable with that of conventional medium.

Bioassays of Bs and Bti toxins against mosquito larvae showed considerable toxicity. The toxicity is due to the binding of active toxins (crystals) to specific receptors present in the midgut brush border membrane (MBBM) (Charles *et al.*, 1997). The crystal toxins from spore/crystal complex are ingested along with the food material, by the mosquito larvae and after solubilization and proteolytic cleavage, the activated toxin interacts with the midgut epithelium, leading to the death of the larvae (Poopathi *et al.*, 2002b). Hence, these bioassay studies suggest that the toxins produced from BA + SB, are equally efficient as that of LB, for mosquito control.

Cost-effective analysis also indicated that production of this new culture medium (BA + SB) is very cheap, in comparison with that of the conventional medium, as both bagases and soyabean powder are abundantly available as industrial bio-organic wastes.

From the foregoing study, it can be concluded that- i) the use of bagase and soya bean, as an effective bacterial culture medium, is highly economical for the industrial production of these mosquito pathogenic bacilli, in terms of easy availability, cost-effectiveness, efficacy, environmental safety, and bio-waste remediation; ii) the mosquitocidal spore/crystal toxins produced from the experimental culture medium (BA + SB) are higher than that of conventional medium (LB); iii) the protein profiles of Bs and Bti spore/crystal toxins produced from BA + SB are similar to that of LB, by biochemical studies; iv) the entomotoxicity studies with different mosquito species showed that the efficacy of Bs and Bti toxins produced from experimental and conventional media were comparable; and v) the use of BA + SB culture medium is highly economical for the industrial production of these mosquito pathogenic bacilli.

This study is, therefore, important from the point of recycling of environmental waste, as it possesses the dual benefits of effective utilization of environmental bio-organic waste and efficient production of mosquitocidal biopesticides, as well.

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Compatibility of chemical pesticides with locally isolated entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from Meghalaya, Northeast India

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Abstract

The paper reports the compatibility of infective juveniles (IJs) of *Heterorhabditis indica*, *Steinernema thermophilum*, and *S. glaseri*, the three locally isolated entomopathogenic nematodes (EPNs) in Meghalaya (India), with conventionally used chemical pesticides viz. Carbaryl, Nimbecidine, Endosulfan, Quinolphos, Fenvalerate, Mancozeb and Carbofuran. The compatibility of pesticides was tested at two concentrations, one higher and other lower of their recommended field rate in terms of survival and infectivity of IJs of EPNs. Of all the pesticides tested, exposure to carbofuran showed significantly low survival and infectivity of the three tested EPN species. The IJs of *S. glaseri* was found to be compatible with all the tested pesticides, except Nimbecidine. Mancozeb was found to be compatible with *H. indica* and *S. glaseri* but not with *S. thermophilum*. The findings suggest that the EPNs with better compatibility with the tested pesticides may serve as viable candidates for integrated pest management (IPM), however the doses of pesticides be kept as low as possible to achieve the better results.

Keywords

Biological control; Biopesticides; Entomopathogenic nematodes; *Heterorhabditis indica*; *Steinernema thermophilum*; *Steinernema glaseri*

Introduction

With the increasing concern over pesticide resistance in insects and residues in environment, Biointensive Integrated Pest Management (BIPM) has emerged out as an important strategy for the control of insect pests. In the present scenario, none of the strategies can be used as a sole component for successful management of insect pests. Use of chemical pesticides and biocontrol agents in BIPM received much interest in the recent years (Ignacimuthu and Jayaraj, 2003). The entomopathogenic nematodes (EPNs) as highly competitive biocontrol agents have been used successfully to suppress the insect pests in several crops (Grewal *et al.*, 2005). Rapidly increasing knowledge on host range and epidemiology of EPNs has laid

groundwork for the eventual use of these nematodes as effective biological control agents worldwide. In developed countries like USA, Australia and Europe, commercial nematode-based products are available and are being utilized for biological control of several insect pests (Grewal *et al.*, 2005). Attributes making these nematodes as ideal biological insecticides include their broad host range, high virulence, safety for non-target organisms and high efficacy in favorable habitats.

For any biocontrol agent to fit into the BIPM strategy, it is expected that it should be compatible with other methods of control. It is in this context that while developing an ideal BIPM strategy involving the use of EPNs, it is important to ascertain the degree to which these nematodes may be affected by chemical pesticides. In the present study, we report our findings on the effects of some conventionally used pesticides on the survival and infectivity of infective juveniles of three locally isolated EPN strains viz. *Heterorhabditis indica*, *Steinernema thermophilum*, and *S. glaseri* in Meghalaya, north-east India.

Materials and methods

The chemical pesticides used in the study, with their lower and higher concentrations given in parenthesis, include Carbaryl (1 g/500; 2 g/500 ml), Nimbecidine (1 ml/500 ml; 2 ml/500 ml), Endosulfan (0.07%; 0.15%), Quinolphos (0.05%; 0.1%), Fenvalerate (0.05%; 0.1%), Mancozeb (1 g/500 ml; 2 g/500 ml) and Carbofuran (1 g/500 ml; 2 g/500 ml). All the pesticides were tested at 2 concentrations (one higher and other lower of their recommended field rates) (Pathak *et al.*, 2001). Stock solutions of pesticides were prepared in distilled water (D.W.) at double strength of the required concentration. About 1500 IJs of nematodes in 2.5 ml of D.W. were mixed with 2.5 ml of each chemical stock solution in a petridish that was covered with a black paper and incubated at $25 \pm 2^{\circ}\text{C}$ for 72 h in an incubator. One petri dish filled with about 1500 IJs in 5 ml of D.W. but without any pesticide served as control. There were 8 replicates for each pesticide.

Following 72 h of incubation, nematode suspensions from each pesticide and concentrations were centrifuged at 600 rpm for 3 min separately. The resultant supernatant was decanted and replaced by D.W. This procedure was repeated several times. The numbers of IJs survived were counted. Nematodes that did not move even after prodding were considered dead (Hussaini *et al.*, 2001). In order to assess the infectivity potentials of IJs, the standard Petridish assay was employed and 100 IJs/larva against *Galleria mellonella* larva (8 replications) were investigated. Following 3 days of exposure to the larvae, the IJs were recovered and rinsed in D.W. and subjected to pepsin digestion to record the numbers of IJs established per larva.

Statistical Analysis

The data were analyzed statistically and are represented as mean $\pm$ standard error of mean (SEM). Student's *t*-test was used to determine the level of significance of results. The values were considered significant when $p < 0.05$.

Results

The results of the effects of different pesticides on survival and infectivity of IJs of three entomopathogenic nematode species are presented in Figs. 1-3. The survival of IJs of *H. indica* and *S. glaseri* when exposed to lower and higher concentration of carbaryl was observed to be significantly lower ($p<0.05$) as compared to control which also resulted into their decreased infectivity to *G. mellonella* larva. Though, there was no significant difference noticed on survival of IJs of *S. thermophilum* exposed to carbaryl as compared to control, the infectivity of survived IJs were significantly low at both lower and higher concentration of pesticide than control. Nimbecidine did not show any adverse effects on survival and infectivity of *H. indica* and *S. thermophilum*; however, exposure of *S. glaseri* IJs to the higher concentration of nimbecidine revealed a significantly lower ($p<0.05$) infectivity of IJs compared to control. Exposure to endosulfan resulted in significantly lower ($p<0.05$) survival of *H. indica* and *S. thermophilum* IJs as compared to control; in contrast, no significant differences were observed in the survival of *S. glaseri* IJs at both concentrations of pesticide. The infectivity of *H. indica* IJs after exposure to lower and higher concentrations of endosulfan was significantly lower ($p<0.05$ and $p<0.01$, respectively) than control. However, the infectivity of *S. thermophilum* IJs at lower concentration of this pesticide did not show any significant difference except that it was significantly lower ($p<0.05$) at higher concentration than control. The infectivity of *S. glaseri* IJs at endosulfan's lower concentration ($33.9 \pm 1.71\%$) did not revealed any significant difference as compared to control. However, its IJs infectivity was significantly lower ($p<0.05$) at the higher concentration of pesticide.

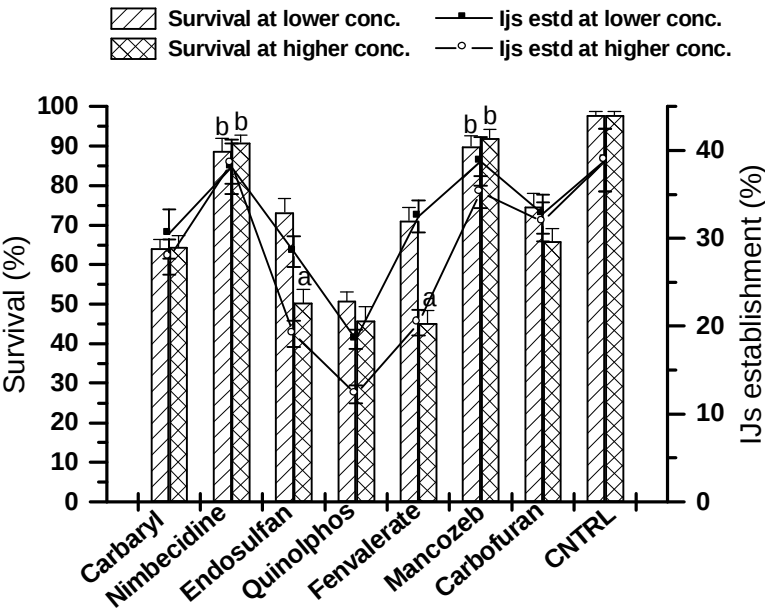


Fig. 1. Effects of various pesticides on survival and pathogenicity of infective juveniles (IJs) of *Heterorhabditis indica*, ^a Significant from lower concentration, ^b Not significant from control.

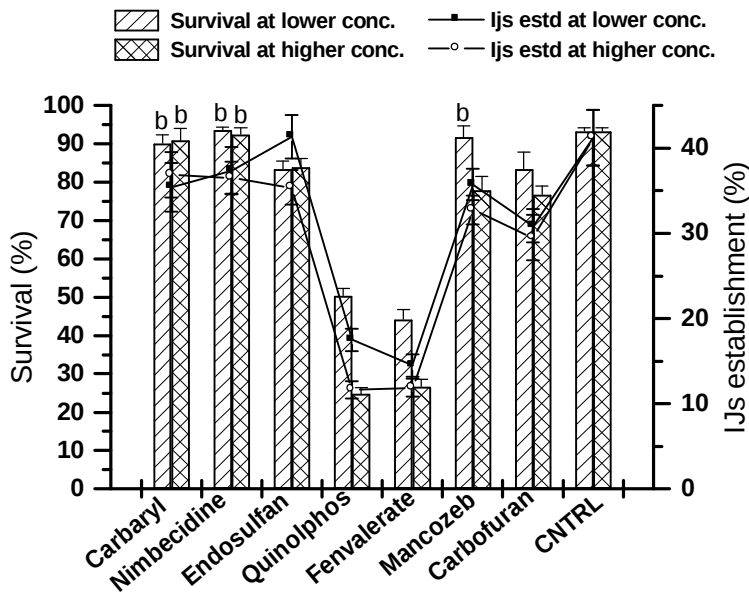


Fig. 2. Effects of various pesticides on survival and pathogenicity of infective juveniles (IJs) of *Steinernema thermophilum*, ^bNot significant from control.

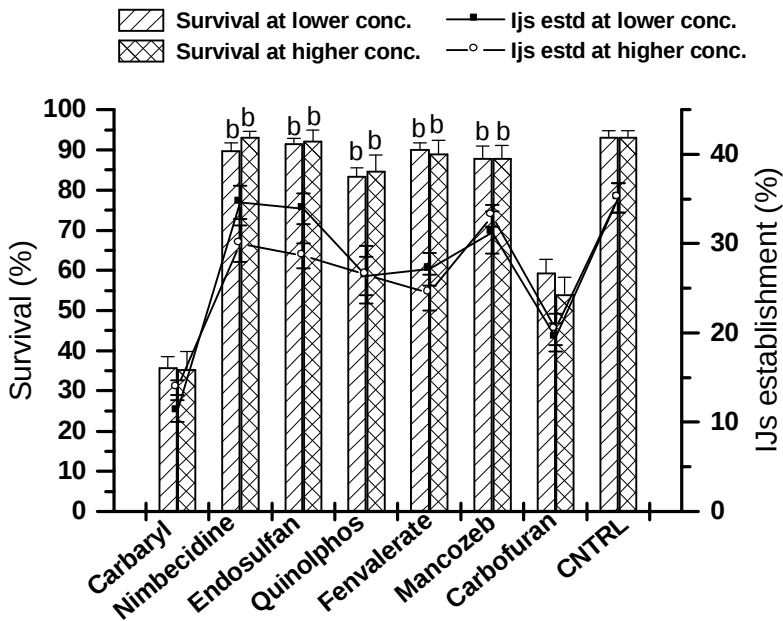


Fig. 3. Effects of various pesticides on survival and pathogenicity of infective juveniles (IJs) of *Steinernema glaseri*, ^bNot significant from control.

The survival of *H. indica* and *S. thermophilum* IJs when exposed to quinolphos was significantly lower ($p<0.01$) than control. In contrast, no significant

differences were observed between survivals of IJs of *S. glaseri* at both lower and higher concentrations of pesticide as compared to control. Further, it was observed that quinolphos lowers the infectivity of all the tested nematodes. Exposure to fenvalerate resulted in significantly lower ($p < 0.05$ and 0.01 , respectively) survival of *H. indica* and *S. thermophilum* IJs as compared to the control. In contrast, no significant differences were observed between survivals of IJs of *S. glaseri* at both lower and concentrations of this pesticide. Fenvalerate revealed a more marked effect on the infectivity of *H. indica* and *S. thermophilum* IJs. In case of *S. glaseri*, though, fenvalerate did not show any significant effects on survival of nematode, the infectivity of IJs at both the lower as well as higher concentrations was observed to be significantly lower ($p < 0.05$) than control.

Mancozeb was noted to be compatible at both its lower as well as higher concentrations with all the species of EPNs, except for IJs of *S. thermophilum*. In the latter case its higher concentration showed a low survival and infectivity ($p < 0.05$) of *S. thermophilum* IJs as compared to control. The survivals of IJs of the three tested EPNs when exposed to lower and higher concentrations of carbofuran were significantly lower ($p < 0.05$) than control. The low survival of IJs also reflected the infectivity of IJs of both *H. indica* and *S. thermophilum*. Carbofuran had also marked effects on the IJs of *S. glaseri*, the infectivity of its IJs at lower ($19.5 \pm 1.59\%$) and higher ($20.3 \pm 1.74\%$) concentrations was significantly lower ($p < 0.05$) than control ($35.1 \pm 1.66\%$).

Discussion

Chemical pesticides and EPNs offer different but potentially compatible approaches to suppress insect populations (Nishimatsu and Jackson, 1998). The EPNs may seek out the host in inaccessible areas where pesticides may not act. It is therefore advocated that efforts should be made to utilize the EPNs with existing IPM practices. Though, compatibility of nematodes with chemicals have been investigated by undertaking laboratory bioassays with direct exposure of nematodes to insecticides (Rovesti *et al.*, 1988; Rovesti and Deseo, 1990; Zhang *et al.*, 1994; Gordon *et al.*, 1996; Head *et al.*, 2000; Garcia del Pino and Jove, 2005), it is still necessary to test the adverse effects of these pesticides on nematodes, because formulations of different insecticides may vary in toxicity to various nematodes due to the use of different surfactants. Further, nematodes strains belonging to different areas may also differ in sensitivity to different formulations of the same insecticide (Rovesti and Deseo, 1990; Krishnayya and Grewal, 2002). Studies made in the recent past have found several species of EPNs to be relatively insensitive to a wide range of pesticides (Alumani and Grewal, 2004; Bednarak *et al.*, 2004), although some organophosphate and carbamate compounds have been found to be detrimental to IJs of *S. carpocapsae* (Head *et al.*, 2000).

In the present study, no significant differences were observed on survival of IJs of *H. indica* following exposure to mancozeb and nimbecidine at both lower and higher concentrations after 72 h, whereas other insecticides, particularly quinolphos, affected the survival and infectivity of *H. indica*. Hussaini *et al.* (2001) also reported that pesticides such as endosulfan, carbofuran and malathion have deleterious effects on IJs of *H. indica*. A reduction in the movement of IJs of *Steinernema carpocapsae* and *S. feltiae* following exposure to endosulfan has also been reported by Rovesti

and Deseo (1990). In case of *S. thermophilum* the study revealed that barring quinolphos, fenvalerate and mancozeb which showed deleterious effects on nematodes, the other pesticides did not show significant effects on survival and pathogenicity of its IJs. As compared to the other two EPN species, the IJs of *S. glaseri* showed a high rate of survival when exposed to nimbecidine, endosulfan, quinolphos, fenvalerate and mancozeb. However, carbaryl and carbofuran also had significant effects on survival and infectivity of IJs of *S. glaseri*. It is relevant to mention here that Hussaini *et al.* (2001) also reported that carbofuran impairs the activity of steinernematids. The authors however, found *S. glaseri* to be compatible with quinolphos. The chemical groups most toxic to steinernematids and heterorhabditids are considered to be organophosphates and carbamates (Rovesti and Deseo, 1990; Gordon *et al.*, 1996). However, it has been suggested that in some cases simultaneous use of nematodes and insecticides may improve insect pest control (Ishibashi, 1992). Though, we found the nimbecidine and mancozeb to be compatible with all the tested nematode species in this study, however, Krishnayya and Grewal (2002) reported that neem formulation, nimbecidine, cause 14-17% mortality of *S. feltiae* IJs after exposure of 24 h. Similarly, the water extract of neem seed kernel has also been reported to be toxic to *S. carpocapsae* by Rovesti and Deseo (1989). The differing effects of different pesticides observed in the present study may be due to differences in the chemical groups and/or mode of action of these pesticides. While pesticides such as carbofuran and carbaryl are considered to be acetylcholinesterase inhibitors, fenvalerate acts through peripheral and central nervous system.

It may therefore be concluded from the present study that only IJs of selected EPN species show compatibility with the tested pesticides, most others show detrimental effects on these EPNs. The EPNs with better compatibility may be considered as viable candidates for Biointensive Integrated Pest Management.

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Author Index

- Ahmad F, 147
 Arumugam Padmini, 33
 Bagai U, 203
 Bavani Mitra Zare, 95
 Brinesh R, 169
 Chakrabarti Satadal, 81
 Chandel S, 203
 Chatterjee A, 153
 Chishti MZ, 147
 D'Souza Placid Eugene, 101
 Desai Dattatraya V, 239
 Goswami LM, 153
 Govind Hare, 163
 Gupta RK, 13
 Gupta S, 213
 Hoti SL, 1, 219, 227
 Janardanan KP, 169
 Jyrwa DB, 113, 137
 Kamal S, 193
 Kaur Harpreet, 75
 Krishnamoorthy K, 219
 Lalramliana, 261
 Lyngdoh RD, 137
 Madhavi R, 21
 Mahanta HC, 193
 Majumdar Barnali, 109
 Malsawmtluangi C, 127
 Manna Buddhadeb, 81
 Mary K Athisaya, 219
 Mathur Aruna Swaroop, 109
 Mirhadi Fateme, 95
 Nagalakshmi K, 227
 Pandey CM, 13
 Parsani HR, 61
 Peerzada MY, 175
 Phanithi Prakash Babu, 33
 Poopathi Subbiah, 245
 Prasad A, 13
 Prasad KN, 13
 Prasad PK, 153
 Rajan A, 203
 Rajeswari G, 245
 Rao Archana V, 239
 Rao JR, 55
 Rezaeian Mostafa, 95
 Sabhyata, 163
 Sanjeevamurthy Mamatha
 Gowribidanur, 101
 Shukla Meena, 33
 Singh Ranjeet, 75
 Singh Veer, 61
 Sinha Anant Kumar, 187
 Sinha Chitra, 187
 Sivapragasam G, 1
 Sripada Anand, 33
 Srivastava AK, 213
 Suryanarayana Veluvorthy Venkata
 Sanyasi, 101
 Tandon V, 113, 127, 137, 153
 Tariq KA, 147
 Tewari AK, 55
 Thapa S, 113
 Vastya Stuti, 65
 Vasuki V, 227
 Verma A, 13
 Yadav Arun K, 261
 Yadav CL, 65

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20th National Congress of Parasitology

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Congress Summary

20th National Congress of Parasitology

Focal theme “Food-borne Zoonoses of Parasitic Origin: Molecular Taxonomy and Epidemiology”

**Organized by
Department of Zoology
North-Eastern Hill University
Shillong, India**

**In collaboration with
The Indian Society for Parasitology**

Sponsored by

Department of Science & Technology GOI, Department of Biotechnology GOI, Department of Ocean Development GOI, North-Eastern Council, Indian Council of Medical Research, Council of Scientific & Industrial Research, National Academy of Sciences, India, Indian National Science Academy, AICOPTAX (MOEF) & NEHU

The 20th National Congress of Parasitology with the focal theme **“Food-borne Zoonoses of Parasitic Origin: Molecular Taxonomy and Epidemiology”** was inaugurated on November 3, 2008 at the Multi-use Convention Centre, North-Eastern Hill University, Shillong by His Excellency, the Governor of Meghalaya, Shri R. S. Mooshahary. Dr. A. Pariong, Hon’ble Minister of Health and Family Welfare, Govt. of Meghalaya was the Guest of Honor and the program was chaired by Prof. P. Tandon, Hon’ble Vice-Chancellor, North-Eastern Hill University (NEHU). In his welcome address Prof. Tandon introduced NEHU to the delegates and briefed about various academic and research activities and achievements of University to the delegates. Dr. J. K. Saxena, Secretary, Indian Society for Parasitology (ISP) & Scientist at Central Drug Research Institute, Lucknow, then presented a brief account of various activities undertaken by ISP in the recent past. Prof. V. Tandon, Convener of the Congress and President, ISP, in her presidential address apprised delegates of the economic losses parasites cause to the society and opined that future wellbeing of the mankind depends on the progress and breakthroughs that would emerge in the health care fields; and this could only be achieved through committed efforts of researchers in the fields of life sciences. In his talk, Dr. A. Pariong emphasized on the need of study of Parasitology for the welfare of man and animals. His Excellency, the Governor of Meghalaya, Shri R.S. Mooshahary, in his inaugural address, welcomed all the delegates to this beautiful city of Shillong and wished that the Congress would come out with useful conclusions to control the incidence of parasitic diseases. Vote of thanks was proposed by the Local Organizing Secretary of the Congress, Dr. A. K. Yadav.

There were more than **125 distinguished delegates** registered for the Congress. They represented about 40 reputed universities and research institutes in the country, such as Indian Institute of Science, Bangalore; ICGEB, New Delhi; CDRI, Lucknow; PGIMER, Chandigarh; SGPGIMS, Lucknow; VCRC, Puducherry; NIPER, Mohali; RMRIMS, Patna; CRDT (IIT-Delhi), University of Hyderabad;

Banaras Hindu University; Visva-Bharati; Andhra University; Allahabad University; University of Kashmir; Calicut University; IVRI, Izatnagar; G.B. Pant University of Agriculture & Technology, Pantnagar; to name a few. Besides, there was one delegate each from Mexico and Iran. In total **10 scientific sessions** were arranged besides 2 poster sessions and panel discussion during the 3-days program. There were **18 plenary lectures, 78 presentations (30 oral and 48 poster)**, besides **6 presentations under the Young Scientists' Award session**.

The first session started with a **Keynote Address by Dr. Kanury V. S. Rao**, an eminent scientist at ICGEB, New Delhi. The Session was chaired by **Dr. V. P. Sharma**, Meghnad Saha Distinguished Fellow of the National Academy of Sciences, India. Dr. Rao talked on **“Defining host-pathogen interactions through genome-wide screens: An alternate approach to drug target discovery”**. In his exciting talk Dr. Rao summarized the research findings of his research group and mentioned that the interaction of pathogen with the host cell is mediated by protein secreted by intracellular pathogen, which can be disrupted so as to provide alternative strategy for chemotherapy. His work extends the approach of ‘Systems Biology’ towards translation-oriented exercise that could be exploited in the advancement of systems pharmacology.

The first plenary lecture in **Session 2A** was delivered by **Prof. Nancy Malla** of PGIMER, Chandigarh on **“Excretory secretory antigens for the diagnosis of food-borne parasitic zoonoses”**. Prof. Malla discussed that the excretory-secretory (ES) products of helminths are considered to comprise immunogenic molecules of high diagnostic value and their use may expand the diagnostic perspectives in parasitic diseases. She also impressed upon the participants as to how ELISA with the use of ES antigens has significantly showed high sensitivity and specificity for the diagnosis of several helminthic infections, such as lymphatic filariasis, paragonomiasis, echinococcosis, etc. The second plenary lecture in this session, **“Fish-borne zoonotic haplorchine trematodes: the question of differentiation of species”**, was delivered by **Prof. R. Madhavi**, a senior parasitologist at Andhra University, Vishakapatnam. In her lecture Prof. Madhavi summarized the issues of ambiguity in the species differentiation of closely related species of zoonotic haplorchine trematodes, a group of fish-borne liver and intestinal trematodes. Her work revealed that these species may be differentiated with respect to their life cycle, behavior of larval stages, surface ultra-structure of adult flukes and alignment of chromosomes in the karyotype, besides conventional morphological taxonomic approach. **Prof. G. Salgado-Maldonado**, a leading fish parasitologist at the Universidad Nacional Autónoma de México, México spoke about **“Helminth parasites of freshwater fishes from Mexico and Central America”**. His study was a compilation of current knowledge of the taxonomic composition and distribution of the helminth parasites of freshwater fish in Central America.

The first plenary lecture in **session 2B** was delivered by **Dr. K. N. Prasad**, SGPGIMS, Lucknow, on **“Taenia solium taeniasis and neurocysticercosis (NCC): experience from North Indian pig farming community”**. His study showed very high prevalence of *T. solium*, taeniasis, neurocysticercosis, the common parasitic infection of the central nervous system, in the community where pigs are raised. He thus advocated that an interventional strategy be immediately adopted to reduce the disease burden in such societies. **Prof. Veer Singh** of S. D. Agricultural University,

Sardarkrushinagar in his talk on **“Animal trypanosomosis: *Trypanosoma evansi* - An emerging threat to human”** reported few case of occurrence of *T. evansi* in humans in Western parts of India and cautioned the medical and veterinary practioners to combat the newly emerging zoonotic trypanosome infection.

The **sessions 3 and 4** were dedicated to the physiology, cellular and molecular biochemistry, genomics & functional biology of parasites. **Dr. Pawan Malhotra** of ICGB, New Delhi was the first to speak on **“Tudor Domain” containing RNA binding protein family in *Plasmodium falciparum*, a human malaria parasite**”. He described the existence of RNA-binding protein family, the Tudor domain containing protein family, apart from the Puf RNA-binding protein family, which has already been identified in the protozoan parasite *P. falciparum*. **Dr. Amit Sharma**, of the same institute in his talk on **“Structural biology of malaria parasite proteins”**, elucidated the role of various malarial parasite proteins involved in erythrocyte invasion, hepatocyte invasion and gametocytogenesis. His work thus provided insights into the functions of these proteins that may be having potentials for the development of anti-malarials in the near future. The first lecture in session 4 was given by **Dr. Neena Goyal** of CDRI, Lucknow. The lecture, **“Molecular mechanisms of antimony resistance in field isolates of *Leishmania donovani*”**, unraveled the molecular mechanism of clinical resistance of the parasite, which could allow prevention and circumvention of resistance and rational drug design for the treatment of drug resistant *Leishmania*.

The **sessions 5 and 6** were assigned to **“Water-borne diseases of parasitic origin”** and included 6 lectures by eminent parasitologists. **Dr. V. P. Sharma**, Meghnad Saha Distinguished Fellow of NASI at CRDT, IIT, Delhi, spoke on **“Urban malaria in India”**. He suggested that integrated malaria vector management in urban areas comprises source reduction, recurrent larval control measures, minor engineering methods, biological control, legislative measures and IRS in the peri-urban areas in India. **Prof. P. P. Singh** of NIPER, Chandigarh in his talk on **“Amoebiasis: an overview with special reference to the discovery of new antiamoebic drugs”** suggested that with the recently published genome of *Entamoeba histolytica*, the causative agent of amoebiasis, lots of information about its metabolism are available, which may allow the discovery of new targets and potential anti-amoebic agents. **Dr. J. K. Saxena** of CDRI, Lucknow in his lecture on **“Can Glycolytic pathway be utilized for development of anti-filarials?”** identified the hexokinase to be an important putative target for anti-filarial drug development. **Prof. P. Prakash Babu** of University of Hyderabad, in his lecture on **“Role of c-Jun N-terminal protein kinase family and stress activated proteins during cerebral malaria induced cell death in mice brain”**, suggested the crucial role of c-Jun N-terminal protein kinase family and stress activated proteins during cerebral malaria in mice model. **Prof. C. L. Yadav** of G.B. Pant Univ. of Agriculture & Technology, Pantnagar, in his deliberation on **“Development of vaccines against parasites: Current status and future directions”**, suggested that ‘Genomics’ and ‘Proteomics’ can be utilized in identifying noble target of parasites in silico using available genome sequences in data banks as can be the gene knockout RNA interference (RNAi) technique. The last lecture in the session was delivered by **Dr. Arun Ghosh** of Agartala Government Medical College on **“Study of intestinal protozoal carriers among food handlers in Agartala city (Tripura)”**. His study

revealed that the overall prevalence rate of protozoal infection among street food handlers in Agartala city is considerably high, which reflects on their poor hygiene.

The **session 7** began with an Oration Award (2007) lecture of ISP by **Prof. Baba Jadhav** of B.A.M. University, Aurangabad on “**Piscian tapeworms from Maharashtra**”. He suggested that there is a great need to study the tapeworm diversity and to make efforts to determine the weakest point in their life cycle, which could be exploited to tackle them more effectively. The lecture was followed by six ‘Young Scientists’ Presentations’ for ISP award.

Sessions 8 and 9 included deliberation on Chemotherapy, Bioprospection, Drug Resistance, Diagnosis, Immune Response and Vaccines/Vector-borne Parasitic Diseases and Vector Control. **Dr. S. L. Hoti** of VCRC, Puducherry, in his talk on “**Lipid binding nematode polyprotein allergens: potential targets for drug and immunoprophylactic development**” investigated the polymorphism of gp15/400 polyprotein molecule of lymphatic filarial parasite in modulating the host immune response. **Prof. Utpal Tatu** of IISc, Bangalore, in his lecture on “**System analysis of heat shock proteins in malaria**”, revealed the critical processes regulated by malaria parasite heat shock proteins and also shed light on their involvement in pathobiology of clinical malaria. **Dr. Asif Mohammed** of ICGB, New Delhi, spoke on “**Identification and molecular characterization of novel drug targets for malaria**”. He identified and characterized pJClpY, which may be exploited in the screening of new inhibitors to evaluate their anti-malarial activity. **Dr. A. K. Mishra** of IVRI, Izatnagar, in his talk on “**Babesiosis - A new challenge to human health**”, discussed babesiosis as an emerging life threatening disease to human beings where majority of the species described in domesticated and wild animals are capable of infecting human beings.

Awards Conferred during the Congress

Prof. M. B. Mirza Award, 2008 – Mr. Alok Ranjan Singh, CDRI, Lucknow
ISP Young Scientist Award – Ms. Ritu Berwal, ICGB, New Delhi
Young Scientist Award, 2008 - Ms. Shewta Joshi, CDRI, Lucknow

Best Oral Presentation Awards

Mr. L. M. Goswami, NEHU, Shillong; Mr. Tarun Kumar Bhatt, ICGB, New Delhi;
 Mr. Bijoy Kumar, CDRI, Lucknow; Ms. K. A. Mary, VCRC, Puducherry;
 Mr. E. Srikanth, BHU, Varanasi

Best Poster Presentation Awards

Mr. Rakesh Selva, Andhra University, Visakhapatnam; Ms. T. L. Tilari, Andhra University, Visakhapatnam; Ms. Temjenmongla, NEHU, Shillong; Ms. Reema Gupta, CDRI, Lucknow.

Ms. Neeshima Jaiswal, Allahabad University, Allahabad; Mrs. C. Malsawmtluangi, NEHU, Shillong; Mr. Rajeev Ranjan Kumar, GB Pant Univ. of Agric. & Tech., Pantnagar; Dr. Lalramliana, Pachhunga University College, Aizawl; Mr. Santosh Kumar, CDRI, Lucknow; Prof. Sushma Rathaur, BHU, Varanasi

Panel Discussion

It emerged from the panel discussion that porous borders in the neighborhood in the northeastern part of India allow movement of people and animals into our country and this migration poses a serious threat of parasitic infections in the mainland. It was suggested that the Regional Medical Research Centre (RMRC- ICMR), Dibrugarh may look into this aspect and develop collaborative research programmes with other institutes in the region. Dr. A. Ghosh of Govt. Medical College, Tripura emphasized an alarmingly high prevalence of water-borne diarrhoeal diseases in Tripura state and suggested that more attention should be paid to provide safe drinking water in the region.

The delegates lauded the efforts of Organizing Committee for the successful organization of the **20th National Congress of Parasitology**.

