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CONTENTS

SHORT COMMUNICATIONS

Journal of Parasitic Diseases
Vol. 27 (2) Dec. 2003, pp. 69-75

Review

Helminth Parasites of Public Health Importance - Trematodes

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Parasitic zoonoses are now well recognized as important public health problems throughout the world, both in developing and developed countries. There is a need to analyse the five components of zoonoses, which are the parasite, reservoir host, intermediate host, potential hosts and environment. The risk of human beings acquiring parasitic zoonoses seems to be greatly dependent on behaviour and food habits. Avoidable potential diseases have come to stay due to lack of awareness on the one hand and complacence on the other. The accumulation and improper disposal of animal waste and effluents including infected offal from abattoir is just another threat. With the knowledge of various studies made on different aspects of Helminths, particularly the Trematodes, prevention and control can be affected by breaking the chain of transmission at its epidemiologically weakest link. Keeping this in view the trematode infections of public health importance are considered here.

Key words : Helminths**,** Public health importance**,** Trematodes.

A great many parasites of animals are capable of inciting clinical disease in man, in their adult or larval stages. (Anantharaman, 1967). The three recognized subclasses of Digenea (diagenetic trematodes) produce infection in man and higher animals. Of the various trematode infections blood flukes have been dealt separately. Other trematode infections of public health importance are considered here. (Faust *et al.*, 1975)

Mostly Digenea (digenetic Trematodes) produce infections in man and animals of the various fluke infections. Intestinal, Amphistome, Echinostome, Heterophyid, Hepatic, Pulmonary and Blood flukes are considered to be more important and responsible for environmental pollution. Pathak. (1991) reviewed the fundamentals of parasitic zoonoses.

INTESTINAL FLUKES

Faciolopsis buski

The giant intestinal fluke was observed by Busk in the duodenum of Laskar sailor at autopsy in London. It occurs in the small intestine of man and pig in South Eastern parts of Asia. Dogs are occasionally infected, although they appear to be partially resistant to infection. Important snail intermediate hosts are *Segmentina trochideus* in Assam *S. hemisphaerula* in China, Formosa and Japan : *Hippeutis cantori (S. Schnackeri)* in China and *Gyraulus convexiculus* in Indo China, Philippines, Indonesia and India. Cercariae after emergence from the snails encyst on the plants / Vegetation / seed/Pods etc. The common plant hosts are water chestnuts namely *Trapa bicornis* in India, *T. bisoinosa* in Formosa, *Eliocharis tuberosa* in South China and Formosa. The water calatrop *Trapanatans* in canton, China; water bamboo Elizania aquatica or water hyacinth *Echhornia crassipes* in Formosa. Transmission occurs by the ingestion of Metacercariae in fresh or uncooked edible water plants etc., use of human excreta containing the eggs of *F. buski* to fertilize fields or aquatic plants provides a major source of inoculum for the molluscan stages of the lifecycle. Children are likely to have heavy infection. The infection is common due to peeling of the parasitised plants with the teeth. After encysting in the duodenum the young flukes become attached to the nearby intestinal wall and develop into adult worms in about three months. (Parija 1990)

The damage produced by these large fleshly worms is mechanical, obstructive and toxic. At each site of attachment a mucosal ulcer is produced. A few worms may not cause serious intestinal symptoms but more

number of parasites will be encountered in an infection. These embrass digestion and at times cause acute obstruction. Toxic metabolites cause edema of face, of the abdomen and lower extremities. There will be characteristic notable eosinophilia. There is often leucocytoses with eosinophilia upto 35%. Occasionally there may be leucopenia with lymphocytosis. (Hafeez 1993)

AMPHISTOMATE FLUKES

In domestic animals large number of species of amphistomes have been described from the rumen reticulum and bile duct of Ruminants and some species occur in the large intestine of equines, pigs and man. (Faust *et al* 1975, Hafeez 1993)

Gastrodiscoides hominis

It is relatively common human parasite, which was recorded from the intestine of pig and man by various workers. It has been incriminated that *Helicorbis coenosus* to be the intermediate host of this fluke. Cercariae probably encyst as metacercariae in aquatic vegetations after emerging from the snails as in case of *F. buski.* Exposure of the definite host results from consumption of infested grass/ vegetation or other plants. (Faust *et al* 1975)

Besides the damage caused by the immature flukes after encystment in small intestine , the worms inhabit the caecum and ascending colon, where it also cause mucous diarrhoea in man.

Dutt and Srivastava (1966) studied the life cycle of *G. hominis* - They have incriminated *Helicorbis coenosus* to be the intermediate host of this fluke. The different larval stages in the snail has been studied. Cercariae probably encyst as metacercariae in aquatic vegetations after emerging from the snails as in case of *F. buski.* Exposure of the definitive host results from consumption of infested grass/vegetation or other plant vectors.

Watsonius Watsoni

It has been obtained only once from man, a West African Negro who died of severe diarrhoea. At necropsy many worms were found attached to the duodenal and jejunal mucosa and free in the large bowel. Monkeys are also considered to be the natural hosts. Infection of the final host is by ingestion of the metacercariae with herbage. Outbreaks of Paramphistomiasis generally occur in the drier months. The snail population becomes concentrated around area of natural water, and these areas in the dry months, also have the most palatable grazing and thus there is a concentration of cattle, snails and metacercariae over a small area leading to heavy infections. Previous infection and the age of the host afford some protection against reinfection and hence acute disease is usually seen in young animals while older animals, capable of withstanding massive exposure, seed the pastures with eggs.

ECHINOSTOMATE FLUKES

Echinoparyphium paraulum

It occurs in the small intestine of duck, the pigeon and man. The 1st Intermediate Host is the snail and some authors suspect fish and snails as 2nd int. host.

Echinochasmus ilocanum

It occurs in the small intestine of man. It has also been found in the dog and Norway rat; the latter serving as a reservoir host. The 1st Intermediate Hosts are snails of *Gyraulus convexiculus, G.prashadhi* and *Hippeutis umbillicali* Cercariae encyst on almost any fresh water mollusc but *Pila luzomica, P .conica* and *Viviparus javanicum* are especially important (as 2nd I.H.), because they are regarded as a delicacy and are eaten raw or at the most with the sprinkling of salt. According to Anantaraman (1967), *Indoplanorbis* and *Lymnea spp.* may function as the first and *Pila* (or any fresh water fish) as the second int. host. (Faust *et al* 1975, Pathak 1991)

Artifechinostomum sufrartyfex

Occurs in man, pig, dog, and rat in India. *Indoplanorbis exustus* acts as 1st and the same snail may act at II nd Int. host. *Indoplanorbis* and *Lymnea* spp. may function as the first and pila (or any fresh water fish) as the second, I.H.

HETEROPHYID FLUKES

Heterophyes heterophyes

It is common parasite found in the small intestine of man, dog, fox, cat etc. They also act as reservoir hosts. The 1st intermediate host are the snails ***spirenella conica*** in Egypt and ***Cerithidia cingulata*** in Japan.

The second I.H. is a fish ***(Mugil cephalus, Tilapia nilotica, Aphanius fasciatus*** and ***Acanthogobius*** (sps). The cercariae which escape from the mollusc encyst superficially in fishes which constitute the source of infection for man and other mammals. Infection takes place through eating infected raw fish. (Faust *et al* 1975, Hafeez, 1993)

These parasites in the nucosal crypts at the duodenum and jejunum produce superficial irritation of the gonads, with excess secretion of mucus and superficial necrosis of the mucosa. In heavy infections this may be accompanied by Colicy pains and mucous diarrhoea. More serious is the occasional deep penetration of the worms into the mucous coat of the intestine , so that their minute eggs get into mesenteric venules (or) lymphatics and are carried to the heart, brain or spinal cord where they may stimulate granulomatous reactions with symptoms related to these lesions.

Metagonimus yokogawai

It is probably the most common heterophyid fluke in the USSR, Northern Siberia, Korea, Japan, Formosa, Egypt and Bukan states. It was discovered by yokogawa in 1911 in the gills, scales and muscles of fresh water fish in Formosa. These flukes usually occur in the small intestine of man and other definitive hosts like pigs, dogs, cats, etc. The first I.H. is the snail *Semisulcospira libertina* and related species, the 2nd I.H. are several sps. of fresh water fishes (the *Trout Necoglosscus altivelis, Salmoperri udontobutis* and *Leuciscus sps.)*. The cercariae encyst under the scales or in the tissues of the gills, fins or tails and the final host gets infection itself by eating these raw fishes.

These minute worms attach to the cell in the mucosal crypts, usually at the duodenal and jejunal walls of the small intestine, causing excess secretion of mucus, superficial erosion of the mucosal and granulomatous infiltration aroung egg deposits in the stomal tissue. The worms have also been demonstrated deep in the mucosal layer where they remain until they die but with out host tissue encapsulation. The symptoms consists of mild to moderate mucous diarrhoea of a persistant type. Similar observations were also noticed in man and domestic animals infected with *M. yokogawai* in USSR.

HEAPATIC FLUKES

Fasciola hepatica

F hepatica was the first trematode to be described and was likewise the first one on which the complete lifecycle was elucidated, by Leuckart in Germany in 1882 and by Thomas in England in 1883. It is particularly prevalent in sheep raising areas. In several countries human infection is an increasing clinical and public health problem. The parasite is cosmopolitan in distribution and occurs in the liver of sheep, goat, cattle and man.

The intermediate hosts are snails of the genus *Lymnea*. The infection is contracted by ingesting vegetation on which the cercariae of *F. hepatica* have encysted. The metacercariae encyst in the duodenum migrate through the intestinal wall into the peritoneal cavity, penetrate the capsules of the livers traverse its parenchyma and ultimately settle in the biliary passage. They begin to liberate eggs in about 3 to 4 months.

The migrating immature flukes cause both traumatic damage and toxic irritation with necrosis of tissues along their pathway. In the larger bile passages they produce hyperplasia of the biliary epithelium with leucocytic infiltration and development of a fibrous capsule around the ducts.

In sheep it causes a fatal disease called "Liver rot" with enlargement of the bile duct, cirrhosis of the liver and ascites. Early symptoms in human infections consists of right upper quadrant abdominal pain, fever, and hepatomegaly, biliary colic with coughing and vomiting, marked Jaundice, generalized abdominal rigidity, diarrhoea, irregular fever, profuse sweating. Urticaria, significant eosoniphilia and Loeffler's syndrome may appear. The mature or adolescent worms have been found in abscess pockets in blood vessels, lungs, subcutaneous tissues, ventricles of the brain and orbit, often associated with mature worms in the bile passage. It was also recorded from a breast abscess.

Fasciola gigantica

The giant liver fluke, differs from *F. hepatica* in its greater length, more attenuate shape, shorter anterior

cone, slightly larger acetabulum, more anterior position of the testes and large size of the eggs. The natural hosts are cattle, buffaloes, sheep and other herbivorous mammals. The life cycle parallels that of F. *hepatica*, including Lymnaeid snails as 1st I.H. Clinical aspects of this infection are essentially the same as in fasciolisis due to *F. hepatica*. Human infections have been reported from Senegambia, (West Africa) Vietnam, Tashkant, Iraq and Hawai.

Dicrocoelium dendriticum

D. dendriticum is a common parasite of bile passage of sheep and other herbivorous mammals in Europe, North Africa, Northern Asia and to a lesser extent in North and South America. Numerous cases of human infection have been reported from the USSR and else where. Most of these are cases of false parasites is resulting from consumption of infected livers with the evacuation of eggs of *D. dendriticum* in the consumer's faeces, but genuine human cases have been diagnosed from Europe, Asia and Africa. The adult worm is of lancet shaped and resides in the bile ducts of sheep, goats, cattle, pigs, horse, dog and rarely in man. The infection is endemic in hilly areas in India. The 1st I.H. are terrestrial gastropods *(Macrochlamys* species in India, Labrina, *Hellicella* and *Cionella* species in other countries 2nd I.H. is ant *(Formica fusca)* Cercariae (Xiphido type) clump together in masses by sticky substance (Slime balls) in the pulmonary chamber of the snails and are expelled to adhere the vegetation. These are swallowed by ants. Metacercariae develop in the ants. The 2nd I.H. is necessary for the completion of the lifecycle. Definitive host gets infection by ingesting infected ants. Immature flukes after excystment penetrate into the intestinal wall of the final host, pass by the portal circulation to the liver and eventually enter the bile ducts.

The young flukes develop in the smaller bile ducts, the old ones go to the longer bile ducts. They are fully grown after 7 weeks and the first eggs are laid 4 weeks later.

Distomiasis is not so pathogenic as Fasciola but in advanced infection there is extensive cirrhosis and bile ducts are markedly distended due to large number of flukes. The clinical picture in severe cases consists of anaemia, oedema and emaciation. (Hafeez 1993)

Clonorchis sinensis

C.sinensis, the Chinese liver fluke; was first reported from the bile passages of a Chinese carpenter who was antopsied in Calcutta. It was reported from Japan in 1883 and from South China in 1908. The endemic enzootic area of C.*sinensis* extends from Japan to Vietnam. The incidence is related to eating raw fish. In South China eggs of C. *sinensis* are found in the stools of over 10 percent of the population. In central China 75 to 100% of cats and dogs are infected, but the incidence of human infection is low. In Japan human infection was 6 to 40 percent. Adult parasites are found in a biliary tract of the man, cat, pig, rat, mouse, camel, etc. 1st snail I.H.: *Parafossarulus manchourius* in China, Formosa, Indo-China, Korea and Japan, Bulinus (Bithynia) *Fuchsianus* in S. China, *Alocinma longicomis* in China. 2nd I.H.: Fresh water fish. The cercariae released from the snail encyst on fresh water fishes. The infected uncooked fish on being eaten, the metacercariae excyst in the duodenum, migrate through the biliary passage make their way into ampulla of water to the smaller bile radicals and finally to the distal biliary passages, settle soon in liver and mature in one month. Each worm passes 2400 eggs daily.

Mature C. *sinensis* in the bile passages provoke marked hyperplasia of the biliary epithelium with subsequent dense fibrosis and encapsulation of the duct. As the number of worms increase, there is increase in lumial diameter of all the terminal bile ducts resulting in fibrous thickening of the walls and pressure necrosis of adjacent hepatic parenchyma.

The clinical onset may be gradual, or sudden with chills and fever. The liver becomes large and tender. In some cases there may be congestive splenomegaly followed by hepatitis and eosinophilia of 10-40 percent.

Opisthorchis felineus

0. felineus (synonym of *0. tenuicollis)* was originally described from a cat in Italy and a few years later from a man in Siberia. It has a wide distribution in Eastern and South Eastern Europe and Asiatic USSR. It is reported commonly in Vietnam and has been recovered from humans in Japan and India. It inhabits the distal biliary and pancreatic tracts of cat, dog, fox,

pig and man. According to Chandler 60 % of cats were found to be infected in India and human cases were also found in India. 1st Intermediate Host; *Bulinus tentaculatae* and *Bithynia leachi* IInd Intermediate Host : Fresh water cyprionoid fish like *Cyprinus carpio, Idns melatous* and *Tinea tinoa*. Transmission occurs by eating infected uncooked fish.

The clinical features are same as that of Clonorchiasis. The symptoms in man include diarrhoea followed by severe ascites, and other symptoms resulting in cirrhosis of the liver and derangement of portal circulation.

PULMONARY FLUKES

Paragonimus westermanii

This oriental lung fluke was discovered in the lungs of two Bengal tigers which died in Hamburg and Amsterdam. The next year a Portugese resident of Formosa was found to have a pulmonary worm. In 1880 monson found eggs of this fluke in rusty - brown sputum of a Chinese patients. Baelz found similar eggs in the bloody sputum of a native Japanese and three years later discovered the flukes in lungs of Japanese subjects. The most heavily endemic regions of oriental paragonimiasis are in central China, Korea, Japan, the Phillipines and Taiwan (Formosa). It occasionally has been reported from Manchuria, Nepal and Thailand. It is known to be present mostly in animals in Eastern USSR, Ceylon, India, Indonesia, scattered areas of Malaysia and elsewhere in South East Asia. Other species of paragonimus infecting man in the orient are *P. skrajabini* in North East China and P. *heterotremus* in Thiland and Laos. The adult is usually found in the cystic dilatations of the lungs of bronchi of man, dog, cat, pig and occasionally in the brain, liver of intestinal canal of man.

In India isolated foci of paragonimiasis exist in Madras, West Bengal, Assam and Manipur. A total of 39 cases of pulmonary paragonimiasis caused by *P.westermanii* have been reported from Manipur during the year 1981 (Singh et al., 1986).

1st Intermediate Host : fresh water snails of certain species of *Thiaridae* like *Semisulcospira libertina* and other species in China, Formosa, Korea, *Thiara gramifera* and *Brotia oblique granosa* in Formosa (Brotia and Melania are synonyms *of Semisulcospira).*

2nd I.H.is a crayfish or a crab. Eggs may be found in the sputum, which has characteristic rusty colour. But animals swallow mucus so that eggs are found in faeces. Eggs develop in 2-7 weeks, miracidium penetrates into snail - sporocyst- redia - cercaria - (Microcercous) penetrate crayfish or crab. Inside crustacean host, metacercariae are found. They become encysted in the liver, heart and gills of crab but in crayfish in the muscles and viscera. Transmission in man occurs by taking infected crab or cray fish. They are often uncooked or pickled.

Worms have been discovered in many ectopic locations, including the liver, intestinal wall, mesenteric lymphnodes, peritoneum, visceral and somatic muscles, myocardium, testes, pleura and brain. Paragonimiasis of lungs is usually insidepleura and brain. Paragonimiasis of lungs is usually insidious in its onset and mildly chronic in its course. There may be no symptoms other than the occasional coughing up of rusty sputum. However, dyspnoea, fever, malaise, easy fatigability and anorexia have been observed in case of extensive pulmonary involvement. Paragonimiasis at ectopic locations usually causes significant symptoms. In the pleura there may be a thick Purulent effusion and aspirated material may contain abundance of the fluke eggs. In the brain granulomatous pathology is common characteristically producing a Jacksonian type of epilepsy. Numerous cases of cerebral Paragonimiasis have been reported in the medical literature from Japan and Korea. Cerebral paragonimiasis is often accompanied by intracranial calcification which will be revealed in skull radiographs. Opthalmological signs including blurring and impaired vision are common.

BLOOD FLUKES

The group of digenetic trematodes which inhabit the blood steam of vertebrate hosts is commonly referred to as blood flukes or Schistosomes - so called because of the "split body" on the ventral side of the male, in which the female is held during insemination and egg laying. All blood flukes of man are dioecious. Human infection with blood flukes is often referred to as bilharziasis in honour of Theodor Bilharz, an Assistant Professor in the Cairo (Egypt) School of Medicine, who in 1851 discovered the causative agent *(Schistosoma haematobium)* during the postmortem of

a patient who died in that city.

Family: Schistosomatidae

G:- Schistosoma

Sps : *S.bovis* (cattle, sheep, goat) -portal & mesentric Veins.

S. mattheei (Domestic and wild ruminants) - portal and mesenteric veins as well as in the veins of the urogenital tract and stomach.

S. intercalatum (wild and domestic ruminants , equines and man) - portal and mescentric veins).

*S'. *spindale* (Ruminants & dogs) - mesentric veins.

S. indicum (Ruminants, equines and camels) - portal and mesentric veins.

S. japonicum (Man & animals) - portal & mesentric veins).

S. mekongi (Dog & Man) - mesenteric veins.

S. nasalis (Buffaloes, cattle, sheep , goats and horses) - veins of nasal mucosa.

The eggs in faeces or urine hatch in fresh water and the free swimming miracidia are released. If appropriate snails are found, the miracidia attack the snails soft tissues, penetrate and transform into first generation sporocysts. Within each sporocyst several second - generation sporocysts are developed and on escape from the parent sporocyst migrate further into the snail's tissues. Broad forktailed cercariae are produced over a period of several weeks. On maturation these larvae escape from the second generation sporocysts, break out of the snail and swim about in the water. Infection of the definitive host is through active skin penetration of the cercariae although cercariae may penetrate the wall of the lumen when swallowed with water. Skin penetration is assisted by the secretions of the cephalic glands, which digest the tissues. Cattle and horses may become infected when standing in the shallow water of dams, rivers, etc., during the heat of the day. These and other animals are also infected orally when water tanks and other source of drinking water become infected with snails and contaminated with faecal material. The infection is most common in man and animals where the rainfall is highest; there is a marked increase in incidence with increasing water conservation (the building of small and large dams) and with irrigation and with increase in the population density of animals. The usual methods of human exposure consist in wading, swimming, bathing or washing cloths in shallow fresh water near the infected snail hosts. Excreta of man and reservoir hosts containing viable Schistosoma eggs provide the inoculum which initiates the extrinsic phase of the life cycle of the parasite. *S. japonicum* has many mammalian reservoirs, yet the use of human faeces as fertilizer for crops in endemic areas probably constitutes the most important source of infection. In certain foci in Africa baboons have been found to maintain infection with *S. mansoni* with out involvement of humans (Faust et al., 1975).

Pathogenic changes resulting from blood fluke infection in a susceptible host are delivered into three consecutive periods (1) Pre-patent period, from skin penetration until the mature worms have arrived in the venules of the intestine or urinary bladder and the females are already to oviposit; (2) acute stage, which is one of active egg deposition and extrusion and (3) Chronic stage, stable egg output, tissue proliferation and repair.

Adult parasites cause phelibitis in the mesenteric veins and there is a proliferation of the tunica intima with portal or complete occlusion of the lumen. As the infection progresses the adult parasites shift away from their predilection site in the posterior small intestine and anterior large intestines and lesions will be seen in other parts of the intestine as well as in other organs. In addition with S. mattheei lesions in the bladder are striking and common. Pathology of bladder is seen as linear granulomata or granular patches with associated haemorrhages. Adults of *S. mattheei* and *S. indicum* may in heavy infections, be found in the pulmonary arteries.

The hepatic syndrome is an immunological disease resulting from the host's cell -mediated immune response to Schistosome egg in the liver.

In heavy infections the development and healing of large numbers of these egg granulomas cause massive fibrosis in the portal triads of the liver and the appearance of "Claypipestem" fibrosis.

S. nasalis causes nasal granuloma (snoring disease) of cattle, goats and horses in India. I.H. are *Lym. Luteola*, *L. accuminata* and *Indoplanorbis exustus*. The arasites

Parasites develop in the veins of the nasal mucosa, causing marked rhinitis with mucopurulent discharge. The mucosa is studded with small abscesses which contain the eggs of the worms and later shows much fibrous tissue and proliferating epithelium. Clinical signs of coryza with sneezing are succeeded by dyspnoea and snoring (Soulsby, 1986).

CERCARIAL DERMATITIS

In 1928 Cort demonstrated that cercariae of certain non-human blood flukes were the causal agents of an aggrevating form of dermatitis. Intensive investigations in many geographical regions have demonstrated that this skin infection usually occurs during the warm months and is due to skin penetration of the Schistosome cercariae; that non-operculate snails in bodies of fresh water serve as intermediate hosts for these blood flukes, the adults of which are usually parasitic in migratory birds; that transient birds pollute the water with their excreta from the eggs initiated infection in the snails. Schistosomes which are principally responsible for cercarial dermatitis belong to the genera *Trichobilharzia*, *Ornithobilharzia* and *Gigatobilherzia*. A third epidemiological type of Schistosome dermatitis is due to invasion of the human skin with non-human mammalian Schis tosomes including the cattle blood flukes - *S. spindale* and *S. bovis*, the Schistosome of rodents *Schistosomatium douthitti* and of the racoon and dog, *Heterobilharzia americana*, *Oreientobilharzia harinasutai*, a blood fluke of the water buffalo in Thailand, has not been associated with naturally occurring dermatitis but it is of special interest because its lateral spined-eggs is indistinguishable from that of *S. mansoni*. It is possible that the cercariae of mammalian and avian Schistosomes are all capable of penetrating the skin of man. Cercarial dermatitis, a worldwide problem is caused by the cercariae of a number of Schistosome species. Exposure to the cercaria result in sensitization which, on renewed contact with cercaria and their penetration of the dermis, produces according to the degree of sensitization - an initial burning sensation, then itching, followed by a papular or pustular eruption and later by a severe dermatitis which may persist for several days. The most severe forms result from sensitization with Schistosomes which survive for short periods only in the skin of man. The condition is commonly known as swimmer's itch, clamdigger's itch, hunter's itch etc., and may constitute a serious occupational disease and economic problem in areas where the work force comes into contact with water as in paddy fields, and a matter of public health concern in recreational areas, such as bathing beaches and lakes frequented by widfoul and used for hunting, sailing and swimming (WHO,1979).

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Review

Parasitic infections in travelers visiting tropical countries

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International travel for tourism, military operations, exploration and adventure is on the increase. About 20% of the world population harbors parasites and the resulting morbidity and mortality from such diseases are most prevalent in tropical and subtropical countries, which are visited every year by millions from the developed nations. A non-immune traveler from a non-endemic region is thus exposed to a multitude of infections including parasitic diseases, from enhanced contact in a changed ecological milieu. The travel related major parasitic infections in the Indian subcontinent could be classified as food and water borne (amoebiasis, giardiasis, intestinal coccidiosis), vector borne (malaria, filariasis, visceral leishmaniasis), and soil transmitted (geohelminthic infections like ascariasis, hookworm infestation, trichuriasis, strongyloidiasis). Lack of hygiene and sanitation in large parts of the tropical world, increased vector density and man-vector interaction in exotic environmental niches, and lack of adequate personal protective measures from ignorance or complacency have contributed to increased cases of parasitic infection among travelers, visiting the tropical countries. The resistance of parasites and vectors to chemotherapy and insecticides respectively has compounded the problem. As effective vaccines have not yet been developed in parasitic diseases, personal protective measures in the form of health education, pre-travel counseling, and chemotherapy remains the only effective approach to prevent and treat parasitic infections among travelers. Prophylaxis, diagnosis, and therapy of these infections represent an opportunity and challenge to the health care professionals in developed nations.

Key Words : Parasitic diseases, tropical countries, travel medicine, chemotherapy

INTRODUCTION

"For with long travel I am stiff and weary."

-William Shakespeare

People travel to many different places, for many different reasons, with many different interests, in many different ways. Man's movement from place to place either as a struggle for existence or as a source of psychological sustenance has paralleled his evolution from a primitive creature to a modern human. Today man travels for many reasons. A search for the unknown, a destination for leisure, a tour for business, and a voyage for a war, are some reasons, which make man move from one place to another. The evolution of the transport systems from sailing ships and caravans to jet planes and underwater tunnels has revolutionized the way we move between countries and continents. However, the globalization of man has had its own side effects. One of them is the plethora of infections to which a non-immune traveler from a non-endemic zone is exposed while visiting an endemic one. Tourists, seamen, business entrepreneurs, military personnel, the adventurer, and the explorer from developed nations visiting the tropics are subjected to a wide variety of infectious agents during the course of their travel. The disease that they acquire is either manifested during their travel, or remains asymptomatic initially, and get overt later on after the incubation period is over. In both instances however, the visiting traveler or the returning travelers' health is at stake.

Parasitic infections are one of the commonest causes

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of morbidity and mortality among visiting and returning travelers around the wortd. History is witness to the numerous outbreaks of parasitic diseases like malaria and sleeping sickness among laborers and soldiers. Ancient caravanners recognized the signs of sleeping sickness that had often been observed in travels to southern kingdoms of Africa. In the summer of 1828 "swamp fever" (or malaria) broke out along the construction route of the Rideau Canal in Canada. The "malaria", according to most experts was not native to North America but had been introduced by infected British soldiers who had returned from India. During the American Civil War (1861-65), one half of the white troops and 80% of the black soldiers of the Union Army got malaria annually. Therefore, it is clear from the lessons of history that the hazards of traveling have taken its toll from early times. Newer innovations in mass transport system, changes in humanity's way of life have altered the dynamics of man's interaction with the environment leading to an enhanced exposure of various diseases, including parasitic diseases (Desowitz, 1996).

Travelers visiting tropical countries: facts and figures

In recent years, international travel has increased enormously. With greater movement of people, there has been an increased encounter with a wide variety of parasitic diseases. The jet age travel, globalization of food and agricultural market, and the incubation period being longer than the travel period, the diseases may not be identified as travel-related ones. There is a greater need of information for travel related medical care to meet the challenges of travel-associated sickness. Worldwide statistics on travelers indicate that 1.4 million people cross international borders everyday (Freedman, 1999). Each year 12 million-35 million persons travel from an industrialized country to a developing country in the tropics or subtropics (Black, 1990; Lima, 2001). In a study of almost 8000 travelers to developing countries, Steffen and colleagues have reported that 15% of them had health problems, 8% consulted a doctor and 3% were unable to work for an average of 15 days. (Steffen, 1987) They also reported that the problems were more common in younger individuals, those who had significant contact with people especially in rural areas and those who had a prolonged stay. In another study, it has been documented that of 1000 travelers to developing countries, 15% traveled for business, 12% for teaching or study and 3% for missionary activity. The most popular countries visited were Kenya (27%), India (19%), Nepal (13%), Thailand (10%), Hong Kong, Tanzania or Peru (9%) and the people's republic of China (7%). The median duration of travel was 21 days while 5% had longer than 1-year travel period (Hill, 1988).

In a study of 171 cases at the tropical disease unit in Spain it was found that the commonest diagnoses were malaria (34 cases), amoebiasis (8 cases), giardiasis (6 cases), and cutaneous larva migrans (5 cases). The origin areas were Africa (84 cases), Central America (49 cases), South America (23 cases) and Asia (15 cases). Among the patients, 72 were tourists and 49 were immigrants (Zubero Sulibarria et al, 2000).The steady growth of intercontinental travel has caused an increasing incidence of tropical diseases in many parts of Europe, including continental Europe. Malaria and amebiasis deserve special attention, due to their frequency and potential fatality (Hassig, 1999).

Travelers' diarrhea is the most frequent health problem encountered by travelers in the tropics (Nettleman, 1996). Twenty to 50% of the 35 million people who travel from industrialized countries each year are affected by traveler's diarrhea (Lima, 2001). Travelers experience a high rate of diarrhea caused by a wide variety of enteric pathogens acquired by ingestton of contaminated food or water. A single or a combination of palhogen(s) can be found in the stool of a majority of ill individuals. Important risk factors regarding tropical diarrhea in travelers include the point of origin and destination of the traveler, host factors, and exposure to contaminated food and water. The most common parasitic causes of traveler's diarrhea in adults in developing countries include infection with *Entamoeba histolytica, Giardia lamblia, Cryptosporidium parvum* and *Cyclospora cayetanensis* (Black, 1990; Lima, 2001). Although intestinal protozoa account for a minority of cases of acute traveler's diarrhea, but they are the common pathogens in travelers who experience protracted diarrhea during or after travel (Okhuysen' 2001). It was observed that diarrhea is not only the most common health hazard during travel in the tropics but also the most frequent condition which prompts

returning travelers to see a physician (Markwalder, 1986). A retrospective study based on interviews conducted between 1975 and 1964 with 20,000 European travelers returning from climatic zones in 15 destinations showed that the incidence of travelers' diarrhea varied from 4% to 51%, depending on the destination. Persons younger than 30 years, adventurous travelers, and travelers with preexisting gastrointestinal illnesses where identified as high-risk groups. The clinical course of travelers' diarrhea was generally short and mild. Since, there are only minor differences in chronology and symptomatology of travelers' diarrhea due to various pathogens laboratory confirmation of various etiologic agents assumes significance (Steffen, 1986).

The causes of persistent diarrhea in the returned traveler are many. Careful evaluation is important and requires an understanding of the destination of the traveler, the time of his travel, and a detailed history of diarrheal illness, medications taken, and knowledge of the patients' other medical problems. Protozoan parasites like G. *lamblia*. *C. parvum*, and C. *cayetanensis,* are among the more commonly identified agents. If the patient is immuno-compromized, microsporidia and *Isospora belli* are more likely. Occasionally, helminths, which establish close contact with the intestinal mucosa, may also cause prolonged diarrhea (Thielman, 1998).

Vector borne parasitic infections

In recent years, there has been resurgence of several long-known vector-borne diseases. Malaria, filariasis, leishmaniasis are spreading geographically and their incidences are increasing. These diseases have resurged in numerous foci, in some cases where they were thought to be under effective control. The resultant large-scale alteration in ecology due to dam construction, irrigation, urbanization, deforestation and other development projects has all contributed to the changes in vector population. Greatly increased human travel has spread infectious agents, introducing them into areas in which they had been hitherto rare or unknown (Gratz, 1999).

Malaria, which is transmitted through the bite of the female *Anopheles* mosquito, is a global problem. There are about 300-500 million cases of malaria every year in tropics and subtropics leading to 1.5-2.7 million deaths every year (Tracy and Webster, 2001). It remains a major cause of morbidity and mortality throughout the developing world especially in sub-Saharan Africa (Baird, 2000; Murray and Lopez, 1994). About 50% of world population is at risk of malaria. Of the four species of *Plasmodium* that infect humans, *Plasmodium falciparum* is the most prevalent and results in the most serious illness. Severe complications of *P. falciparum* infection may lead to cerebral malaria, black water fever, severe anemia, hypoglyoemia or renal failure, which may have a poor prognosis. Recently, there has been an increase in the transmission of malaria in many countries where it had been eradicated (Baird, 2000; Baird and Hoffman, 1999). Population migration, military deployment, inadequate vector control measures and poor sanitation in tropical countries have increased the problem of malaria throughout the world. Further, it poses a diagnostic challenge to the physicians who do not have the experience in managing the disease. The compromised immune status and development of resistance to commonly used drugs may further complicate the treatment of malaria. A new dimension has been added to the existing problems due to substandard or fake drugs available in the local market. The key antimalarial drug, artesunate, used for resistant malaria in South-east Asia, is found to be fake in 38% of samples (Po, 2001; Newton, 2001). An earlier study conducted in Cambodia has reported that counterfeit artesunate and mefloquine preparations are sold widely (Rozendaal, 2001).

Increasing numbers of imported cases of malaria are being reported from several parts of the developed countries. Almost 80% of imported P. *falciparum* malaria is acquired in Africa while *P. vivax* infections are acquired most commonly in the Indian subcontinent (Suh et al, 1999).To cite one study in Norway, analysis of the data obtained from the Norwegian Surveillance System for Communicable Diseases from 1989 to 1998, was carried out to know the incidence of imported malaria and the most important risk groups for acquiring the disease in endemic areas. Results of the study showed that since 1992, P. *falciparum* has been the most common parasite being imported from other countries. Fifty-eight percent of the cases were among people of non-Norwegian origin, and 41% were among people whose

origin was Norway. Immigrants from the Indian subcontinent including India, Pakistan and Sri Lanka constituted 26% of all imported cases. Some of the travelers developed falciparum malaria after visiting sub-Saharan Africa. The study emphasized on the need to avoid exposure to mosquitoes while in endemic areas. Mefloquine was recommended as prophylaxis to the majority of travelers visiting regions with chloroquine-resistant malaria (BIystad, 2000).

In a study from Calcutta, India, a group of 452 subjects traveling to the Indian subcontinent from other countries were interviewed for compliance of chemoprophylactic drugs by travelers while visiting India (Chatteriee, 1999). This study was carried out by directly administered questionnaire at two selected locations in Calcutta during October 1992. Full compliance of chemoprophylactic drugs was defined as the uninterrupted use of drugs, as per the World Health Organization (WHO) schedule, during travel up to the date of interview. In this study, it was found out that female, first time visitors and long-term travelers were less compliant. The reasons for non-compliance in the study were stated to the inadequacy in pre-travel advice, discontinuation due to side effects due to drugs, and non-adherence to advice. Past travel experience, concern for side effects, perceived uselessness and confusion arising from alternative regimens were also found to have influenced the decision making among travelers (Chatterjee, 1999).

Filarial nematodes are metazoan parasites, which are residents of the lymphatic system, connective tissue, and body cavities like the peritoneal cavity. These parasites are cosmopolitan in distribution and found all over the world. Different species of filarial nematodes predominate in different geographical locations. In the present article focus has been given on the lymphatic filariasis, which is found, in and around the Indian subcontinent.. Lymphatic filariasis caused by *Wuchereria bancrofti* is found in 90 million people all over the world mostly affecting human populations in Tropical Africa, South America, India and Southern China, whereas rest of lymphatic filariasis is caused by *Brugia malayi*. Hypersensitivity reaction of the host to microfilarial antigen can manifest as occult filariasis, which can take the form of tropical pulmonary eosinophilia (TPE), arthritis, and dermatitis (Parija, 1996).

TPE is commonly found in the people living in the tropics, especially those in the Southeast Asia, India, and certain parts of China and Africa. This condition is increasingly seen in the developed countries due to the increased frequency of worldwide travel and the migration between continents. The disease is more likely to occur in non-immune travelers to endemic regions. TPE presents as paroxysmal fever, cough, wheezing and dyspnea and is clinically confused with that of bronchial asthma. Diagnosis of the condition in the laboratory is made by demonstration of high titers of antifilarial antibodies, increased serum total lgE>1,000 U/ml, and a positive response to diethylcarbamazine (DEC) (6-12 mg/kg body weight for 21 days). Characteristically, microfilariae will be absent in the peripheral blood smear .The condition if left untreated may lead to chronic bronchitis, pulmonary fibrosis or chronic respiratory failure (Ong and Doyle, 1998). In Switzerland, TPE has been reported to occur in persons who have resided in the countries of South East Asia endemic for lymphatic filariasis (Sturchler et al, 1978).

Leishmaniasis affects 350 million people in 88 countries around the wortd (Martinez, 2000). It is a vector borne disease transmitted by sandflies (e.g. *Phlebotomus argentipes)* to man. The disease may manifest either as visceral, cutaneous or muco-cutaneous leishmaniasis. The visceral form of the disease, also known as kala-azar, caused by *Leishmania donovani* presents as irregular fever, hepato-splenomegaly, anaemia, lymphadenopathy and hyper gamma globulinaemia. Cutaneous leishmaniasis or oriental sore is caused by L. *tropica* and mucocutaneous leishmaniasis is caused by *L braziliensis or L mexicana* complex .The visceral leishmaniasis (VL) is distributed in Ethiopia, Sudan, Kenya and in the Indian subcontinent, including Nepal. VL is considered as a differential diagnoses when a patient returning from endemic regions with clinical presentations stated earlier. Because of the long incubation time, VL may be encountered also in travelers who had lived in endemic regions. In addition, the diagnosis of VL should also be considered in immunocompromized individuals. A review shows that in the last 50 years, a total of 8 cases of imported VL from East Africa, Croatia, and

southern Italy were registered in the Czech Republic, The diagnosis in these cases was confirmed by the microscopic finding of amastigotes in bone marrow, liver, spleen and, also, by serology. All these patients were successfully treated with amphotericin B. (Sundar S, 2002). Such cases of imported VL have also been documented in travelers from Austria (Stoiser et at, 1998).

Compromised travelers represent a diverse and challenging group of individuals. They include HIV-infected patients, functionally or anatomically asplenic and patients who are iatrogenically immunosuppressed from medications utilized for solid organ transplantation, chemotherapy, or treatment of malignancies. These patients during travel may encounter leishmaniasis and other unfamiliar opportunistic and classical tropical infections, whose pathogenesis can be enhanced by the presence of prior HIV infection (Mileno and Bia, 1998). Leishmaniasis and HIV co-infection has resulted in the spread of leishmaniasis from rural to urban areas and the spread of HIV outward from cities (Martinez, 2000). In addition to these, intravenous drug uses have compounded the problem of leishmaniasis. *Leishmania* species have been detected in 52% of the discarded syringes of the drug users in Madrid, Spain (Cruz et al, 2002).

Geohelminthic infections

Geohelminthic infections are acquired by humans as a result of his contact with soil. The infective form of the parasites are present in the soil, and man acquires the infection from the soil, during ingestion of food contaminated with infective forms, walking bare foot, gardening activities, and other activities which entails contact with the soil. Ascariasis, ancytostomiasis, trichiuriasis, and strongyloidiasis are the principal geohelminthic infections to affect man. Roundworm infestation occurs in 70-90% people in tropical regions due to *Ascaris lumbricoides.* It affects 1.4 billion people in the world. Hookworm affects 1.3 billion people worldwide. Trichuriasis affects 800 million people globally, and is widespread in South East Asia, including India. *Strongyloides stercoralis* affect 200 million people world wide and prevalent in tropics with hot and humid climate. It is also common in travelers', immigrants and military personnel who live in the endemic area. Many of these diseases are responsible for considerable morbidity among travelers to the tropics (Parija, 1996).

A study was carried out to assess helminth infestations in non-European travelers coming from tropical countries to Leipzig (East Germany), in 1985. In the study, 52% of the individuals were found to be infected with a variety of helminthes. Infestation rate was 38% for T. *trichiura,* 21% for "Ancylostomatidae" and 17% for *A. lumbricoides.* Travelers from South Asia and Southeast Asia showed the highest rate of infestation, 93% on average (Vietnamese: 98%). The smallest rate of infestation (12%) was found with persons from Central and East Asia (Mongolians: 0.4%). Multi-infestations were frequent (Laos 67.4%, Vietnam 65.3% of the examined persons) (Krause, 1985). In another study, the majority of intestinal parasites isolated in Switzerland were found to be imported by refugees and travelers. Nematodes were more frequently isolated among refugees, whereas *G.lamblia* and S. *stercoralis* predominated in travelers. Hypereosinophilia occurred frequently with some parasites (Landry and van Saanen, 1997).

Nine species of parasites were found in 64.9% of Thai laborers working in Taiwan. These induced *Opisthorchis viverrini,* hookworm, S. *stercoralis, G. lamblia, T. trichiura, Fasciolopsis buski, Taenia sp, Echinostoma sp, Entamoeba coli.* These findings suggest that the migration of foreign workers from developing regions to developed countries may potentially lead to transmission of intestinal parasitic infections (Cheng and Shieh, 2000). A retrospective study of clinical features in expatriates returning to Britain from the tropics with diagnosis of ascariasis, trichuriasis, hookworm, and strongyloidiasis showed that one half of all patients with intestinal helminthiasis were asymptomatic. The presence of eosinophilia was not a reliable indicator of infection. This study suggests that it may be worthwhile performing stool microscopy in expatriates returning from the tropics even if they are asymptomatic (Fryatt et al, 1990).

Cutaneous larva migrans (CLM) is a condition caused by skin-penetrating larvae of nematodes, mainly of the hookworm *Ancylostoma braziliense* and other nematodes of the family *Ancylostomatoideae.* Travelers to tropical and subtropical countries are mainly affected. Jelinek et al, 1994, in their study on

CLM, has shown that the efflorescences typically were found on the lower extremities (73.4% of all locations). The buttocks and anogenital region were affected in 12.6% of all locations, and the trunk and upper extremities each were affected in 7.1%. Only a minority of patients presented with eosinophilia or an elevated serum level of IgE. Therapy with topical thiabendazole was successful for 98% of the patients. Systemic antihelmintic therapy was necessary in two cases because of disseminated, extensive infection (Jelinek et al, 1997). Nakamura-Uchiyam have suggested that *A. braziliense* and *A. caninum* be considered as possible causative agents for creeping eruption, especially when the patients have a history of traveling overseas (Nakamura-Uchiyama et al, 2002).

Occasional reports regarding the occurrence of rare intestinal parasitic diseases in travelers include visceral larva migrans due to Toxocara species (Lund-Tonnesen, 1996) and syndrome pulmonary infiltration with eosinophilia (PIE) caused by *Ascaris suum* (Matsuyama et al, 1998). An imported human case of rectal hookworm infection with worms in the rectum has been reported in South Korea in a patient returning after a travel to South -East Asia (Yong et al, 1992).

Future prospects with parasitic diseases due to international travel

Tropical countries have a favourable warm and humid climate, which flourishes the parasitic diseases, and is transmitted by various ways to travelers. International travel exposes the travelers in tropics to infectious diseases, insect vectors, and contaminated food and water.

The risk of infection is an intrinsic part of tourism, especially tropical tourism. Reasons are many which increase susceptibility of travelers in tropical countries to many parasitic infections (Box I). The alteration of the mode of living caused by the holiday, and the frequent development of a false sense of confidence and safety in the hygienic conditions in tourist resorts in tropical countries favor the infection. Factors which are less dependent on the behavior of the tourists, are the infections produced by insect bites, such as malaria, filariasis, or leishmaniases, many of which may end fatally for lack of recognition. Hence, a better enlightenment of the travelers, the use of prophylactic agents and improvement of diagnosis is a must to prevent morbidity and mortality from travel related infections (Weise, 1976).

The parasitic diseases pose two major problems. The first is the problem of the disease itself. The other is associated with the development of drug resistance (Sundar, 2001) treatment failures (El Hassan and Khalil, 2001) co-existence of other diseases or interacting drugs which cause immune suppression (corticosteroid therapy, HIV infection) (Martinez, 2000). Chemotherapy is the most efficient strategy. Improvement in sanitation, vector and reservoir control, and development of suitable vaccines are other alternative approaches. The spectrum of drug resistance mandates search for new drugs, combination of existing drugs, and understanding the biology of parasitic infection like ivermectin modulated chloride channel receptors (Tracy and Webster, 2001).

The traveler returning from the tropics in clinical practice poses a definitive problem before the clinicians. Sometimes travelers returning from the tropics consult a physician even if they have no actual symptoms. However, physical check-ups in asymptomatic travelers rarely detect any specific parasitic infection. If no diagnostically suggestive symptoms are apparent a systematic, step-by-step workup based on the laboratory investigations are essential. Laboratory investigations may include stool microscopy for intestinal parasites, serology, and special methods to demonstrate parasites in blood or tissues (like Millipore filtration of blood to detect microfilaria, and bone marrow aspiration to detect LD bodies in cases of PUO). The diagnostic approach of the clinician should be to consider first all those systemic parasitic infections, which are potentially life threatening and can be cured by specific therapy such as falciparum malaria, extra intestinal amoebiasis, and African trypanosomiasis (Markwalder et al, 1995).

An increased awareness of family physicians for recognition of unusual diseases in patients who have returned from foreign travel is crucial in proper diagnosis and management of the parasitic diseases acquired by patients during travel. For example, an increased awareness is essential about the possibility of intestinal coccidian parasitic infections in patients with prolonged diarrhea who have traveled to endemic

areas. These infections are associated with a protracted course, especially in immunocompromized individuals. Moreover, debility in the form of weight loss is severe in patients with a prolonged illness. Increased awareness, leading to high index of clinical suspicion, timely diagnosis, and appropriate therapy can reduce morbidity and mortality to a great extent.

Weekly chemo-prophylaxis against malaria, protective clothing and insecticide-treated bed nets, application of insect repellants, adherence to safe sex guidelines and selection of safe food and water may protect the travelers from these infections. Traveling to underdeveloped countries necessitates the implementation of several important preventive measures, such as vaccination and bringing water boiling apparatus (Box II). It is recommended that after arriving in the underdeveloped country, travelers must avoid unheated water, ice, beverages containing ice, raw vegetables, fruits cut by local people. However, many travelers do not pay much attention to these points, resulting in increasing numbers of tropical orally contracted intestinal diseases. In addition to the education of travelers about preventive measures, doctors who will see patients returning from underdeveloped countries must be able to recognize and treat tropical diseases. Unfortunately, this is often not the case in developed countries, resulting in delays in diagnosis and treatment, it is imperative to diagnose communicable diseases as quickly as possible to avoid complications and unnecessary secondary infections. Thus, for a traveler to the developing world accumulating knowledge on the current hygienic conditions in the tropical country must also be one of the necessary preparations (Sakaue, 1997).

Questionnaires can be an effective tool in the assessment of health-risk associated with short-term (less than three months) travel to developing countries from developed nations. Steffen and colleagues in a study has shown the usefulness of questionnaires to assess health risk associated with travel to tropics, In their study, questionnaires designed to elict information regarding pre-travel vaccinations, malaria prophylaxis, and health problems during and after their journey in 10,524 travelers (with an answer rate of 73.8%) showed that the incidence of infection per month abroad was 7/1000 for giardiasis, 4/1000 for amebiasis, and 1/1000 for malaria. Such information was extremely valuable in identifying the major threats to the health of a traveler, so that appropriate prophylactic measures could be undertaken (Steffen et at, 1987).

To conclude, travel will continue to be source of human enlightenment, rejuvenation, and betterment only when the traveler is safe, and is protected from the vices of nature. Increased knowledge and understanding of the epidemiology of tropical parasites is one of the significant steps in the achievement of this much-desired objective.

Box I

Reasons for the increased susceptibility of travelers to parasitic infections

1. False sense of security and confidence among travelers about ecological, environmental, health, and hygienic measures in different tourist resorts.
2. Lack of protective immunity among travelers from non-endemic region visiting a country with high endemicity.
3. Absence or inadequate pro-travel advice or counseling.
4. Adventure tourism in tropical countries with poor sanitation and high vector density.
5. Consumption of unboiled local water, beverages, ice, and ice containing beverages, fruit juices, and salads by travelers.
6. Inadequate personal protection like non-use or irregular use of mosquito nets, long sleeved garments, shoes.
7. Bathing in unclean water.
8. Non-compliance to chemoprophylaxis.

Box II

Dos and don'ts for travelers visiting tropical countries

1. Wear protective clothing and shoes to avoid insect bites and exposure of the feet to soil.
2. Avoid bathing in unclean water.
3. Never drink unbottled water.
4. Drink bottled mineral water from a reputed company.
5. Avoid consumption of ice, ice containing beverages, fruit juices, salads.
6. Consult advice of counselors and physicians about prophylactic measures to be adopted during or before travel.
7. Take chemoprophylactic drugs in adequate dosages as recommended by a qualified physician.
8. Maintain strict compliance with chemopro-phylactic regimens.
9. Take drugs in serious diseases under supervision.
10. Take prophylaxis for malaria 2 weeks before journey and continue for 4 weeks after return from endemic region.
11. Discourage children and pregnant women from traveling to endemic zones. The disease becomes more severe and may even to lead to fatality in such cases. Incase, already affected, they should be shifted to the nearest hospital where special care can be given.
12. Carry the necessary itineraries like bed nets, personal prophylactic drugs and mosquito repellants right from beginning of the journey.

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Induction of colony-stimulating factors by *Leishmania donovani* amastigote soluble antigens

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***Leishmania donovani* amastigote antigens soluble in culture medium (LDAA; 0.01-10 mg/kg), following intravenous injection in BALB/c mice, induced the production of serum colony-stimulating factors (CSFs); 1 mg/kg LDAA induced maximum response (137 ± 18 colonies). *In vitro,* LDAA (0.01-1 mg/ml) induced mouse macrophages (MØs) to elaborate CSFs in the conditioned medium (CM); 0.1 mg/ml LDAA induced maximum production by peritoneal (73 ± 9 colonies), splenic (69 ± 10 colonies) and bone marrow-derived MØs (77 ± 10 colonies). Both *in vivo* and *in vitro,* the CSF production could be observed as early as 6 h, reached maximum by 24 h and then levelled-off to background levels by 72 h. Pre-treatment of LDAA with rabbit anti-LDAA polyclonal antibody significantly ($p<0.05$) reduced the LDAA-induced CSF production by MØs, *in vitro.* Polymyxin B and goat anti-mouse tumor necrosis factor-α (TNF-α) immunoglobulin G treatment, separately, lacked any effect on the LDAA-induced CSF production by MØs, *in vitro,* suggesting it to be both lipopolysaccharide- and TNF-α-independent. Furthermore, LDAA-induced CSF production appeared to be *de novo* as it was completely inhibited by cycloheximide. The proportions of *in vitro* induction of granulocyte (G), MØ (M) and GM colony formation by LDAA-induced CSFs, and their selective neutralization with specific anti-mouse G-, M- or GM-CSF antibodies, both in the serum and CM, indicated that GM-CSF was the major activity (>60%). The colony forming unit-GM counts in the spleen and femur of LDAA-treated mice showed a maximum increase of 2.7- and 2.4-fold, respectively. These data, apparently for the first time, suggest that *L. donovani* amastigote soluble antigens can induce the production of CSFs.**

Key Words : Amastigotes, Colony-stimulating factors, *Leishmania donovani*, Macrophages, Soluble antigens.

INTRODUCTION

Leishmania sp. are dimorphic protozoan parasites that cause a wide range of human diseases called leishmaniases, which include self-healing cutaneous lesions, localized or diffuse mucosal lesions and fatal visceral infections. In the mammalian hosts, the amastigote stages of *Leishmania* parasite are obligatorily intracellular as they reside and multiply in the phagolysosomal compartments of macrophages (MØs) and dendritic cells (Bogdan and Rollinghoff, 1998). *Leishmania donovani,* the causative agent of human visceral leishmaniasis (VL), causes VL in BALB/c mice also (Cotterell *et al.*, 2000a).

The colony-stimulating factors (CSFs; mol. wt. 18-90 kDa) are a group of glycoprotein hormones, which regulate the differentiation and proliferation programme of the committed progenitor cells by binding to their specific surface receptors, *in vitro* (Metcalf, 1989; Metcalf, 1991); *in vivo* they stimulate hematopoiesis (Donahue *et al.*, 1986). The lineage specific CSFs i. e. granulocyte (G)-CSF (G-CSF) and MØ (M)-CSF (M-CSF), stimulate G and M colony formation, respectively. GM-CSF, on the other hand, supports the formation of colonies consisting mainly of G, M and eosinophils, whereas multi-CSF (interleukin-3; IL-3) induces colonies containing cells of different lineages. The genes for mouse and human CSFs have been cloned, and large quantities of recombinant CSFs can now be produced (Metcalf, 1991). Structurally, M-CSF is a homodimer, whereas G, GM- and multi-CSFs consist of a single polypeptide chain. The CSFs are active at picomolar

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concentrations, and their constitutive levels are very low; however, during infections, the concentrations of CSFs are rapidly elevated (Cheers *et al.,* 1988). Functionally, CSFs augment several immune parameters which include adherence, chemotaxis, respiratory burst activity, cytotoxicity, and increased synthesis of various cytokines like interferon, tumor necrosis factor-α (TNF-α) and IL-1 (Grabstein *et al.*, 1986; Warren and Ralph, 1986; Heidenreich *et al.*, 1989; Metcalf, 1991). CSFs are known to boost host defence against infections (Freund and Kleine, 1992; Armitage, 1998; Buchsel *et al.,* 2002; Hubel *et al.*, 2002).

GM-CSF has been reported to activate MØs for the killing of *Leishmania* parasites, *in vitro* (Handman and Burgess, 1979; Weiser *et al.,* 1987; Ho *et al.,* 1990). *In vivo* treatment with recombinant mouse GM-CSF (rmGM-CSF) has beneficial effects, whereas treatment with rabbit anti-mouse GM-CSF antibodies has detrimental effects on the course of *L. donovani* infection in mice (Murray *et al.,* 1995). Injection of BALB/c mice with GM-CSF-activated MØs previously incubated with *L. major* antigens has also been reported to protect against subsequent homologous challenge (Doherty and Coffman, 1993). Increased hematopoiesis has been reported in murine leishmaniasis, which is probably driven by CSFs (Mirkovich *et al.,* 1986; Lelchuk *et al.,* 1988; Cotterell *et al.,* 2000a) as evidenced by the enhanced expression of GM-CSF mRNA both *in vitro* (Cotterell *et al.,* 2000b) and *in vivo* (Murray *et al.,* 1995). Herein, apparently for the first time, we report the CSF-induction potential of *L. donovani* amastigote antigens soluble in culture medium (LDAA), both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals and parasite: BALB/c mice (18-20 g; male) were obtained from the Central Animal Facility of the institute, and maintained at 22-24°C with food and water provided *ad libitum*. All studies were carried out in accordance with the guidelines for Care and Use of Animals in Scientific Research, Indian National Science Academy, New Delhi, as adapted and promulgated by the Institutional Animal Ethics Committee. *L. donovani* (MHCM/IN/80/Dd8 strain) was maintained by cryopreservation and serial passage in golden hamsters *(Mesocrisetus auratus),* intracardially (i.c.). Full-blown infection (150-200 amastigotes/100 spleen cell nuclei) was observed after 30-40 days, which was assessed by the microscopic examination of Giemsa-stained spleen touch prints (Singal *et al.,* 2003).

Harvesting of amastigotes and preparation of LDAA: Amastigotes were isolated from the spleen homogenates of heavily-infected hamsters, and treated with rbc-lysis buffer (Sigma-Aldrich, USA). The amastigotes were washed (x2) with and suspended in sterile Dulbecco's modified Eagle medium (DMEM; Gibco, USA). For LDAA, the amastigotes were freeze-thawed twice, sonicated for 30 s at 4°C (x3), and then centrifuged at 3000 *g* for 20 min at 4°C. The clear LDAA-containing supernatant (0.41 mg protein/ml) was harvested aseptically, filter-sterilized (0.2 μ) and stored at -70°C. Control antigen was prepared similarly by using splenocytes from normal male hamsters. A single preparation of LDAA was used in these studies.

Generation of rabbit anti-LDAA serum: LDAA (400 μg) in complete Freund's adjuvant (FA; 1:1) was injected in a rabbit at four different subcutaneous (s.c.) sites, followed by a booster of 200 μg of LDAA in incomplete FA, s.c., four weeks later. A second booster was given one week later, with 100 μg of LDAA, intravenously (i.v.), and the rabbit was finally bled for anti-LDAA serum, three days after the last boost. The serum was heat-inactivated (HI; 56°C; 30 min) and stored at -20°C. The serum had an antibody titre 1:2048 as determined by enzyme-linked immunosorbent assay.

Macrophages: For peritoneal MØs, thioglycollate-injected (4% wt/vol; 0.5 ml/mouse; 96 h) normal mice peritoneal exudates cell suspension was centrifuged (700 *g;* 7 min; 4°C), and the cell pellet was resuspended ($1x10^6$ cells/ml) in 10 ml DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), 0.01 M HEPES (Gibco), $1x10^{-4}$ M 2-mercaptoethanol (2-ME, Sigma) and 40 μg/ml gentamicin (Gibco; CDMEM), and cell culture-tested polymyxin B sulfate (5 μg/ml; Sigma). The splenic MØs were obtained from splenocyte suspensions made by using a 20 μ nylon sieve, and the cells were then washed by centrifugation (700 *g;* 12

min; 4°C). Erythrocytes were lysed with rbc-lysis buffer, and the cells were suspended in CDMEM. For bone marrow (BM)-derived MØs, mouse femurs were flushed with chilled DMEM using a 24 G needle, and washed (x2) and suspended in CDMEM. The adherent MØs from these three cell suspensions were harvested, separately, by allowing them to attach with the plastic surface of T-25 culture flasks at 37°C for 3 h in 5% CO_2-air atmosphere, and were then further incubated for 30 min in an equal volume of CDMEM containing 2 μg/ml indomethacin (Sigma). The MØs were detached by using a sterile rubber scraper, washed (x3) and resuspended in 5 ml chilled polymyxin B-free Hanks' balanced salt solution (HBSS, Gibco). T-cells from these MØs were eliminated by rabbit anti-mouse T-cell serum (1:20) treatment for 1 h at 4°C followed by HBSS wash (xl), and incubation with rabbit complement (1 h; 37°C). The DMEM and HBSS contained <0.1 ng/ml endotoxin as determined by chromogenic *Limulus* amoebocyte lysate assay. MØs were >96% pure as determined by morphologic, phagocytic and non-specific esterase staining criteria, and were >98% viable as judged by Trypan Blue exclusion.

Generation of CSFs: A single injection of LDAA (0.01, 0.1, 1, 5 and 10 mg/kg) was administered in mice (i.v.; 6 mice/dose), and their blood samples were collected aseptically, after 2, 6, 12, 24, 48 and 72 h. Sera from these blood samples were separated and pooled for each time-point, separately and used to estimate serum CSFs. For controls, pooled sera obtained from mice injected with muramyl dipeptide (MDP; 25 μg/kg), DMEM (vehicle) and HI-LDAA (70°C; 1 h; pH 7.0) were used. *In vitro,* different concentrations of LDAA (0.01-1 mg/ml) were added to the cultured peritoneal, splenic and BM-derived MØs ($1x10^4$ -$1x10^6$ cells/ml; 3 ml/dish) and their CM were collected aseptically after various time intervals (2-72 h), centrifuged (1000 *g;* 10 min; 4°C) and filter-sterilized (0.2 μ). For controls, CM of MØs treated with MDP (1 μg/ml), CDMEM only or HI-LDAA were used. All the sera and CM were stored at -20°C until use.

Measurement of CSF activity: CSFs were measured in terms of their colony-stimulating activity (Nanno *et al.*, 1988; Singh and Dutta, 1991). Briefly, mononuclear cells were obtained by density gradient centrifugation of normal mouse femur BM cells. The cells were washed (x3) and suspended ($5xl0^6$ cells/ml) in CDMEM. The non-adherent cells were separated by adherence/depletion processes, and resuspended ($2xl0^4$cells/ml) in CDMEM without FBS but containing 30% HI-horse serum (Gibco), 0.9% methylcellulose (Sigma), 0.9% deionized bovine serum albumin (Sigma) and $1xl0^{-4}$ M 2-ME. The test and control serum and CM samples were added to this cell suspension at 5% and 10% concentrations, respectively. One ml cultures of this cell suspension were then established in sterile 35 mm plastic dishes and incubated at 37°C in humid 5% CO_2-air atmosphere for 14 days. Following incubation, the number of colonies with 50 or more cells was counted under a dark-field inverted microscope (40x). The colonies were fixed on the glass slides and stained with May-Grünwald-Giemsa solution for morphological identification. Further, to determine the predominant type of CSF produced, the serum and CM samples were incubated (37°C; 30 min) with excess amounts of neutralizing concentrations of anti-mouse G-CSF-, M-CSF- or GM-CSF-specific goat polyclonal IgG (R & D Systems, Inc., USA), and then assayed for the residual CSF activity.

GM colony forming units (CFU-GM) assay: The CFU-GM count in single cell suspensions prepared from the spleens and BM of LDAA (0.01-10.0 mg/kg)-treated mice were determined in colony-forming assays, performed in semi-solid cultures (Yap and Stevenson, 1992; Riopel *et al.*, 2001). Briefly, spleen cells, obtained by mincing the spleens and passing through a sterile nylon mesh (20 μ), were suspended in 15 ml of DMEM containing 10% FBS, 2% HEPES and 40 μg/ml gentamicin and centrifuged (700 *g;* 12 min; 4°C). Erythrocytes were lysed with rbc-lysis buffer, the cells were washed with DMEM, and erythrocyte ghosts were removed by filtering cell suspensions through sterile gauze. BM cells were flushed out from the mice femurs with 1 ml of cold Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 5% FBS, 40 μg/ml gentamicin and 2 mM L-glutamine. The spleen and BM cell suspensions were washed (x3) and suspended at a concentration of 4 x 10^6 cells/ml in IMDM. The cells were >96% viable as determined by Trypan Blue exclusion. The CFU-GM

assay medium consisted of 0.8% methylcellulose, 30% FBS, 10% pokeweed mitogen (Sigma)-stimulated spleen cell-CM, 2 mM glutamine and 5 x 10^{-5} M 2-ME. The spleen and BM cells were resuspended in CFU-GM assay medium and plated in sterile 35 mm petri dishes at 2 x 10^5 and 3 x 10^5 cells/dish, respectively. The number of CFU-GM was determined according to colony morphology after 7 days of incubation in humidified 5% CO_2-air atmosphere at 37°C. Based on the total spleen or BM cell numbers, the final CFU-GM counts were expressed/spleen or femur.

Statistical analysis: All experiments were run in triplicate, three-times, separately. Student's t-test was used for statistical analysis, and $p<0.05$ was considered significant.

RESULTS

Induction of serum CSFs by LDAA: LDAA (0.01-10 mg/kg), in a dose-dependent manner, induced the production of serum CSFs in mice (Fig. 1). Whereas, a single injection of as low as 0.01 mg/kg LDAA could induce detectable increase in serum CSF activity, a 1 mg/kg dose induced maximum response (137 ± 18 colonies); at further higher doses the response became static. The LDAA-induced augmented serum CSF production was observed as early as 6 h after administration, reached its maximum after 24 h, and then gradually returned to normal levels within 72 h. Control antigen and HI-LDAA (10 mg/kg each), and only,DMEM did not induce the production of CSFs.

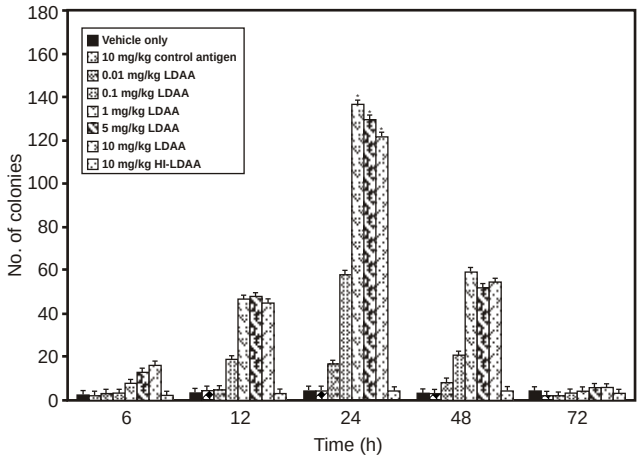

Fig 1. LDAA-induced serum CSF production in mice. Mice (6/group) were injected with LDAA (0.01-10 mg/kg x1), i.v., on day 0. Blood samples from each mice were collected aseptically at time indicated, and the sera were separated. The pooled serum samples were heat-inactivated (56°C; 30 min). Vehicle, control antigen and HI-LDAA were used as controls. Data are the mean number of colonies + SD of three separate experiments, run in triplicate. *Significantly ($p<0.05$) different from vehicle/ control antigen- and HI-LDAA-treated groups.

LDAA-induced CSF production by macrophages, *in vitro:* LDAA (0.01-1 mg/ml), in a concentration-dependent manner, induced CSF elaboration by elicited peritoneal MØs ($3x10^{-4}$-10^{-6} cells/dish; Table I). Whereas, a low concentration of 0.01 mg/ml LDAA could induce detectable levels of CSFs in the CM, the maximum response (73 ± 9 colonies) occurred at 0.1 mg/ml; at higher concentrations the response became static. The CSF activity was detectable in the CM after 6 h, peaked at 24 h and declined to background levels by 72 h. Control antigen- and HI-LDAA (1 mg/ml each)-induced CSF production was comparable to that of vehicle only. Similarly, splenic and BM-derived MØs also, following interaction with LDAA,

Table I: LDAA-induced CSF elaboration by mouse elicted peritoneal MØs, *in vitro*

MØs[a]	LDAA	CSF activity after (h)[b]					
(no./ml)	(mg/ml)	2	6	12	24	48	72
Ixl0^4	-	1±1	2±1	4 ±1	2±2	3±1	2±1
	0.01	2±1	2±1	5±1	16±2	4±1	3±1
	0.1	2±1	3±1	12±2	38±5	17±3	2±1
	1.0	3±1	6±1	16±3	36±5	18±2	3±1
1xl0^5	-	2±1	3±1	4±1	3±1	2±1	2±1
	0.01	2±1	2±1	11±2	29±4	8±1	2±1
	0.1	3±1	5±1	21±2	54±8	34±4	3±1
	1.0	4±2	9±2	20±3	46±6	38±5	6 ±2
1xl0^6	-	2±1	2±1	3±1	2±2	2±1	3±1
	Control antigen	2+1	3±1	4±1	5±2	3±1	2±1
	0.01	3±1	3±1	16±2	30±5	17±2	2±1
	0.1	3±2	10±2	25±3	73±9*	47±6	4±1
	1.0	5±2	15±2	33+4	69±8*	43±6	3±1
	HI-LDAA	2±1	2±1	3±1	5±1	4±1	3±1
	MDP[c]	5±1	8±1	78±11	47±6	23±3	5±1

[a]MØs (3 ml cultures) were incubated with LDAA at 37°C in 5% CO_2-air atmosphere for the durations indicated. Only CDMEM, and control antigen and HI-LDAA (1 mg/ml each) were used for controls.

[b]The CSF activity was determined in the LDAA-treated MØ CM collected at times indicated. Data are the mean number of colonies ± SD of three separate experiments, run in triplicate.

[c]MDP (1µg/ml) in CDMEM was used as positive control.

*Significantly ($p<0.05$) different from vehicle, control antigen - and HI-LDAA-treated groups.

elaborated CSFs (Table II); here again 0.1 mg/ml LDAA induced maximum CSFs (69 ± 10 and 77 ± 10 colonies by splenic and BM-derived MØs, respectively) after 24 h. Furthermore, LDAA (0.01-1 mg/ml) did not directly induce the colony formation (data not shown). Neutralization of LDAA with polyclonal rabbit anti-LDAA antibody significantly ($p<0.05$) diminished the CSF-induction potential of LDAA (Table III). Treatment of MØs with polymyxin B (25 μg/ml), an antibiotic that can neutralize the biological activities of LPS, did not inhibit the LDAA-induced production of CSFs (Table IV). Further, polymyxin B had no effect on the basal production of CSFs by unstimulated MØs or on the responsiveness of the committed progenitor BM cells to CSFs (data not shown). LDAA induced *de novo* CSF production, as MØs co-treated with LDAA and cycloheximide (50 μg/ml) did not elaborate CSFs (Table V). Additionally, to rule out the possibility that TNF-α produced by LDAA-treated MØs was responsible for the induction of CSF production, MØs were stimulated with LDAA

Table II: LDAA-induced CSF elaboration by mouse splenic and BM-derived MØs, *in vitro*

MØs[a]	LDAA concentration (mg/ml)	CSF activity after (h)[b] 12	24	48
Splenic	-	2±1	5±1	3±1
	0.01	12±2	37±5	24±3
	0.1	18±3	69±10*	38±5
	1.0	21±3	62±9*	36±4
BM-derived	-	2±1	3±1	3±1
	0.01	9±2	32±5	16±2
	0.1	16±3	77±10*	31±1
	1.0	19±3	71±9*	29±4

[a]MØs ($1x10^6$ cells/ml; 3 ml) were incubated with LDAA at 37°C in 5% CO_2-air atmosphere for the durations indicated. Only CDMEM was used for controls.

[b]The CSF activity was determined in the LDAA-treated MØ CM collected at times indicated.

Data are the mean number of colonies ± SD of three separate experiments, run in triplicate.

*Significantly ($p<0.05$) different from vehicle treated groups.

Table III: Effect of rabbit anti-LDAA antibody on the LDAA-induced production of CSFs elaboration by mouse peritoneal MØs, *in vitro*

Inducer[a] (0.1 mg/ml)	Pre-immune rabbit control serum[b]	Rabbit anti-LDAA ploYclonal antibody[b]	CSF activity (at 24 h)[c]
-	-	-	3±1
LDAA	-	-	75±10
-	+	-	2±1
-	-	+	4±1
LDAA	+	-	72±9
LDAA	-	+	5±2*

[a]MØs ($1x10^6$ cells/ml; 3 ml) were incubated with LDAA at 37°C in 5% CO_2-air atmosphere for 24 h. Controls received only CDMEM

[b]Neutralizing (100%) concentration of rabbit anti-LDAA polyclonal antibody was mixed with LDAA just before MØ treatment. Pre-immune rabbit serum was used as negative control.

[c] The CSF activity was determined in the CM. Data are mean number of colonies ±SD of three separate experiments, run in triplicate.

*Significantly ($p<0.05$) different from those mixed with pre-immune control serum.

Table IV: Effect of polymyxin B on CSF elaboration by LDAA-stimulated mouse peritoneal MØs, *in vitro*[a]

Polymyxin B (25 μg/ml)	LDAA (0.1 mg/ml)	CSF activity after (h)[b] 24	48
-	-	2±1	2±1
-	+	71±9	42±6
+	-	3±1	1±1
+	+	69±9	41±6

[a]MØs ($1x10^6$ cells/ml; 3 ml) were incubated with LDAA in the presence (+) or absence (-) of polymyxin B sulfate at 37°C in 5% CO_2-air atmosphere for the durations indicated. Only CDMEM was used for controls.

[b]The CSF activity was determined in the CM. Data are mean number of colonies±SD of three separate experiments, run in triplicate

Table V: Effect of cycloheximide on the LDAA-induced CSF production by mouse peritoneal MØs, *in vitro*[a]

Inducer[a]	Cycloheximide[b]	Dialysis[c]	CSF activity (at 24 h)[d]
CDMEM	-	-	2±1
LDAA	-	-	69±9
	-	+	72±10
	+	+	0
MDP	-	-	52±8
	-	+	48±7
	+	+	0

[a]MØs ($1x10^6$ cells/ml; 3 ml) were cultured with or without LDAA or MDP for 24 h at 37°C in 5% CO_2-air atmosphere. Only CDMEM was used for controls.

[b]MØs were cultured with (+) or without (-) cycloheximide at 50 μg/ml.

[c]The CM were dialyzed (+) or not dialyzed (-) against DMEM.

[d]The CSF activity was determined in the CM. Data are mean number of colonies±SD of three separate experiments, run in triplicate.

in the presence of goat anti-mouse TNF-α polyclonal immunoglobulin G (IgG; R & D Systems). Data in Table VI show that anti-mouse TNF-α antibody did not block LDAA-induced CSF production. To determine the functional properties of LDAA-induced CSFs in serum and CM, the types of colonies induced by them were examined. Fig. 2 shows that CSFs from both the sources formed G, M and GM colonies in the same proportion of colony types; the GM colonies were maximum (>60%). To further confirm, as shown

Table VI: Effect of goat anti-mouse TNF-α polyclonal antibody on the LDAA-induced production of CSFs by mouse peritoneal MØs, *in vitro*

LDAA[a] (0.1µg/ml)	TNF-α[a] (5 µg/ml)	Normal goat IgG[b]	Goat anti-mouse TNF-α polyclonal IgG[b]	CSF activity (at 24 h)[c]
-	-	-	-	3±1
+	-	-	-	71±9
-	+	-	-	83±11
-	-	+	-	2±1
-	-	-	+	4±1
+	-	+	-	68±9
+	-	-	+	62±8
-	+	-	+	3±1

[a]MØs (1x10[6] cells/ml; 3 ml) were incubated with LDAA at 37°C in 5% CO_2-air atmosphere for 24 h. Controls received only CDMEM

[b]Neutralizing (100%) concentration of goat anti-mouse TNF-α polyclonal IgG was added to LDAA or TNF-α just before MØ treatment. Normal goat IgG was used as negative control.

[c] The CSF activity was determined in the CM. Data are mean number of colonies±SD of three separate experiments, run in triplicate.

Table VII: Neutralization of CSF activity in LDAA-treated mice serum and MØ CM with specific IgG[a]

Source	CSF activity (% inhibition) after treatment with[b]			
	Medium	Anti-G-CSF IgG	Anti-M-CSF IgG	Anti-GM-CSF IgG
Serum	140±18	130±16 (7.14)	100±13 (28.57)	50±8 (64.30)
CM	75±10	70±9 (6.66)	54±8 (28.00)	26±4 (65.44)

[a]Serum/CM samples were incubated (37°C; 30 min) with an excess amount of indicated IgG and then assessed for residual CSF activity. % inhibition was calculated as (no. of colonies pre-neutralization - no. of colonies post-neutralization/no. of colonies pre-neutralization) x 100.

[b] The CSF activity was determined in the CM. Data are mean number of colonies±SD of three separate experiments, run in triplicate.

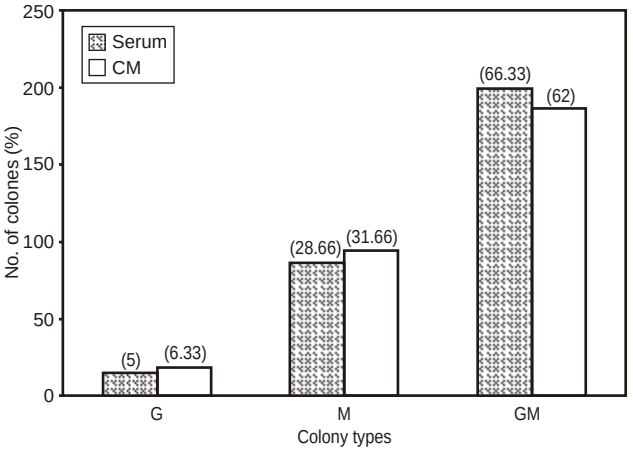

Fig 2. Composition of the colonies formed in response to LDAA-induced CSFs. Serum or CM CSF supported colonies were fixed on glass slides and stained with May-Grünwald-Giemsa solution for identification. G, granulocyte; M, M0; GM, granulocyte-MØ. Data are based on an examination of 300 colonies.

in Table VII, neutralization of the serum and CM samples with goat anti-mouse GM-CSF IgG led to 64.3 and 65.33% inhibition of the colony formation, respectively. Similarly, selective neutralization of G-CSF, in the serum and CM**,** with goat anti-mouse G-CSF IgG led to 7.14 and 6.66% inhibition, respectively, whereas, neutralization with anti-mouse M-CSF IgG resulted in 28% inhibition of the colony formation induced by CSFs from both the sources.

LDAA-induced hematopoietic activity in the spleen and BM of mice: The CFU-GM counts in the spleen and BM exhibited a maximum of 2.7- and 2.4-fold increase, respectively, after 24 h of LDAA (0.01-10 mg/kg) administration, compared to those given control antigen, HI-LDAA or vehicle only (Table VIII).

DISCUSSION

Our laboratory is engaged into the research in the molecular mechanisms of the pathogenesis of VL, especially at the stimulus/response coupling level of MØ-amastigote interaction. The results of this study, apparently for the first time, demonstrate that LDAA can induce the synthesis and secretion of CSFs both *in vivo* and *in vitro*. Further, a >2-fold increase in the CFU-GM counts, both in the spleen and BM, of LDAA-treated mice, suggest the induction of CSFs in these organs that are the primary sites of *L. donovani*

Table VIII: CFU-GM counts in spleen and BM of LDAA-treated mice at different time intervals[a]

LDAA (mg/kg)	CFU-GM/spleen[b] (x 10^3)			CFU-GM/femur[b] (x 10^3)		
	0 h	24 h	48 h	0 h	24 h	48 h
-	1.1±0.3	1.0±0.2	1.1±0.2	1.1±0.2	1.2±0.1	1.1±0.1
Control antigen	1.0±0.1	1.0±0.2	1.1±0.3	1.1±0.2	1.1±0.3	1.0±0.1
0.01	1.1±0.1	1.3±0.2	1.1±0.2	1.0±0.2	1.4±0.3	1.1±0.2
0.10	1.1±0.2	2.3±0.6	1.8±0.4	1.2±0.2	1.8±0.3	1.7±0.2
1.00	1.0±0.2	2.7±0.8*	1.8±0.6	1.1±0.1	2.9±0.8*	1.5±0.2
5.00	1.1±0.3	2.7±0.7*	1.6±0.5	1.0±0.2	2.8±0.8*	1.5±0.2
10.00	1.0±0.3	2.4±0.6*	1.5±0.4	1.1±0.1	2.6±0.5*	1.6±0.3
HI-LDAA	1.0±0.2	1.1±0.1	1.1±0.2	1.2±0.3	1.1±0.2	1.0±0.2

[a]Spleen and BM scells ($2x10^5$ and $3x10^5$ cells/dish, respectively) from LDAA-, HI-LDAA- or control antigen (10 mg/kg)-treated mice were cultured in CFU-GM medium (1 ml) for 48 h at 37°C in 5% CO_2-air atmosphere.

[b]After 7 days, CFU-GM colonies were counted. Data are the mean number of colonies ± SD of three separate experiments, run in triplicate.

*Significantly ($p<0.05$) different from vehicle, control antigen - and HI-LDAA-treated groups.

multiplication in infected animal and human hosts (Cotterell *et al.*, 2000a). These observations, therefore, appear to support the earlier reports, which indicate enhanced hematopoietic activity in spleen and BM and upregulated expression of mRNAs for CSFs during *L. donovani* infection in mice (Murray *et al.*, 1995; Cotterell *et al.*, 2000a; Cotterell *et al.*, 2000b). Increased myelopoiesis has also been observed in later stages of *L. major* infection in mice (Mirkovich *et al.*, 1986). However, apparently, no report has appeared so far suggesting the leishmanial components-induced enhanced CSF production and secretion.

LDAA induced a dose/concentration-dependent increase in the CSF production within the dose/concentration limits studied. Although, the lowest single LDAA stimulus of 0.01 mg/kg *in vivo* and 0.01 mg/ml *in vitro* could induce detectable CSF levels, the optimal levels were obtained with 1 mg/kg and 0.1 mg/ml, respectively. At further higher doses the response plateaued indicating the saturation of the induction mechanisms. Further, the *in vivo* induction kinetics of CSF elaboration almost paralleled the *in vitro* kinetics. The gradual decrease in the LDAA-induced CSF production after 24 h may be explained due to the "turning-off" of the stimulatory signal through decay and/or the initiation of the "negative feed-back loop," possibly mediated by IL-10, that is known to have cytokine inhibitory functions. Nonetheless, these MØs were fully responsive to a fresh LDAA stimulus after another 48 h in culture in fresh medium (data not shown).

In this study, we have used MØ populations from three different anatomical sites. Whereas, the splenic and peritoneal MØs represent the terminally differentiated and mature population of MØs, the BM-derived MØs are relatively immature and thus may differ in their various immune functions. Further, splenic MØs are immunologically more competent, as compared to the peritoneal MØs, as they are constantly interacting with B- and T-cells present in their microenvironment. We, therefore, expected that these three different MØ populations might differ in their ability to elaborate CSFs following interaction with LDAA. Surprisingly, our results very clearly showed that these three different MØ populations responded almost similarly to LDAA stimulation indicating that LDAA interaction with these MØs populations, at least in terms of CSF production, was similar.

The mechanism(s) of LDAA interaction with MØs remains unclear. Fc-receptors for IgG are known to play major role during MØ infection by amastigotes, besides CR3 and mannose receptor (Peters *et al.*, 1995). Our results demonstrating that LDAA-induced CSF production was saturated by both dose and time and was specifically inhibited by rabbit anti-LDAA antibody also suggest it to be a ligand-receptor interaction, and thus appear to be in line with the earlier reports (Handman and Bullen, 2002).

LPS, a potential contaminant of all biological materials, can induce CSF production both *in vivo* and *in vitro* (Sullivan *et al.*, 1983). The antibiotic polymyxin B is known to neutralize the biological functions of LPS (Morrison and Jacobs, 1976). Therefore, our results indicating the lack of any effect of polymyxin B treatment on LDAA-induced CSF elaboration suggest that LPS, which might have been present in the LDAA preparation in traces, may not be responsible for the observed CSF induction. Cycloheximide, an inhibitor of *de novo* protein synthesis, completely inhibited the LDAA-induced production of CSFs by MØs, which suggests that the CSFs elaborated were not preformed but rather freshly synthesized following LDAA stimulation. Our results

also indicate a high order of functional and molecular similarity between the LDAA-induced CSFs in the serum and CM as evidenced by the similar proportion of G, M, and GM colonies formed under the influence of CSFs generated from both these sources, as well as by the selective neutralization of individual CSF. These observations also suggest a common induction mechanism of CSF production, *in vivo* and *in vitro*. Additionally, GM-CSF appeared to be the major activity both in the serum and CM.

Both IL-1 (Lovhaug *et al.,* 1986) and TNF-α (Munker *et al.*, 1986) are reported to induce the macrophages to produce CSFs. Since peritoneal MØs obtained from *L. donovani*-infected. mice do not produce IL-1, *in vitro* (Reiner, 1987), it may not account for the CSF induction observed in this study. On the other hand, increased TNF-α levels are reported to occur in the CM of *L. donovani*-infected MØs (Moore and Matlashewski, 1994) and in the liver of *L. donovani*-infected mice (Tumang *et al.*, 1994). Therefore, to exclude the possible role of TNF-α in mediating the LDAA-induced production of CSFs, MØs were co-treated with LDAA and neutralizing concentrations of anti-mouse TNF-α antibody, and the lack of any effect on the CSF production clearly suggested that the LDAA-induced production of CSFs was not mediated by TNF-α.

The biological role(s) of CSFs in leishmaniasis remains largely unclear. Various reports have indicated that GM-CSF has a host protective role - enhancing the intracellular inactivation of the parasite (Handman and Burgess, 1979; Weiser *et al.*, 1987; Doherty and Coffman, 1993). Nevertheless, the precise role(s) of CSFs in various disease states are yet to be defined. Our results demonstrating LDAA-induced CSF production may have two potential implications: that the CSFs induced may merely serve to increase the number of phagocytes, which are the host cells for parasite expansion "safe targets", as well as for increasing the phagocytic uptake of the parasite, and the CSFs produced may augment the leishmanicidal activity of the MØs. Therefore, CSFs may play important role(s) in the pathogenesis of VL, and detailed studies along these lines will greatly increase our understanding of the molecular basis of the disease processes in VL.

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Study of humoral immune response to Helminth infection in some fishes of Kashmir

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The humoral immune response of *Schizothorax niger* and *Cyprinus carpio* to helminth infection was studied. Precipitating antibodies to *Adenoscolex oreini* (Cestode) and *Pomphorhynchus kashmirensis* (Acanthocephala) were demonstrated using agar gel diffusion technique. Immunochemical investigations of these antibodies indicated that they were macroglobulins resembling IgM of mammals. The fishes infected with helminth parasites responded well with the production of precipitating antibody to the antigens of the parasite *in vivo*. The experiments were designed to investigate the effect of helminth parasite infection on the humoral immune response of some fishes which were reared in a pond under controlled conditions and the same experiments were performed on the sera of infected fishes of Wular lake and the results were compared .It was observed that with the seasonal variation in the helminth infection in these fishes, the antibody titre changed considerably. It was also observed that due to fall in temperature during winter months, the antibody level against helminth parasites went down, therefore it was not detected by immunodiffusion test. The rate and magnitude of antibody production by the host was determined by the helminth infection with respect to temperature.

Key Words: Acanthocephala, cestode, *Cyprinus carpio*, Humoral immune response, *Schizothorax niger*.

INTRODUCTION

Serological and haematological studies of fishes have been used as a probe for the diagnosis of fish diseases in connection with pollution and other environmental factors. Although, now much information is available on the fundamental principles of fish immunology, review on the subject has been presented by Clem and Leslie (1969) and Corbel (1975). There has been little emphasis on the role of immunity in parasitic diseases, particular report of Nigrell and Breder (1934) is available who demonstrated acquired protective immunity in fish to parasitic antigens and that of Harris (1972) who characterized both the antigen and antibody involved in the fish host-parasite system. In the present instance, two helminth parasites, namely, *Pomphorhynchus kashmirensis* (Acanthocephala) and *Adenoscolex oreini* (Cestode) were selected with an aim to conduct immunological studies in *Cyprinus carpio* and *Schizothorax niger* inhabiting the fresh water of world famous Wular lake of Kashmir. Both the parasites *(P. kashmirensis* and *A. orienti)* occur in the gastro-intestinal tract of fishes. The approach of analysis of fish humoral immune response to these parasites was largely dictated by the availability of each parasite. Experimental immunization was however, only conducted in case of *C. carpio* which was reared in a controlled condition in a pond. The immune-diffusion results obtained from immunized C. *carpio* with PBS antigen of *P. kashmirensis* were correlated with results obtained from naturally infected C. *carpio* with the same parasite.

The present investigation gave the evidence that adult *C.carpio* and *S.niger* produce specific immuno-globulins in response to both the parasites. These immunoglobulins would seem to closely resemble the macroglobulins of mammals and their production determined by ambient temperature.

MATERIALS AND METHODS

Collection of fish:- The live fishes were collected from Wular lake by nets and were transferred into buckets filled with water. Sometimes the blood from the fishes was collected on the spot. The fishes were

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brought to the laboratory for investigation of general conditions and parasitic infection.

Rearing of fish:- Some fishes collected from lake were brought to the laboratory in buckets filled with cold and fresh water. The water was changed thrice during transportation. Two species of fish, viz, *Cyprinus carpio* and *Schizothorax niger* were selected. In the laboratory, the fishes were examined and then transferred into artificial ponds. In the ponds, the quality of water was kept under control and fishes were fed with artificial feed. However, *Schizothorax niger* failed to survive in the controlled conditions. Thus *C. carpio* was alone selected.

Sampling of blood:- The blood was collected from the fishes by giving incision near the caudal peduncle. Blood was also drawn out with a syringe from the heart by stabbing a needle through the ventral body wall exactly in mid line from the posterior margin of opercular cover and directed dorsocaudally at an angle of 45°.

Preparation of PBS antigen and immunization of fish:- whole worm antigen *of P. kashmirensis* and *A. orieni* intestinal fish parasites was prepared separately and 100 ml of the phosphate buffer saline having pH 7.2 was added to 2 gm net weight worm homogenate of parasites crushed in homogenizer. The solution was kept in refrigerator overnight and centrifuged at 6000 rpm for 30 minutes. The clear supernatant was collected in small tubes as a purified PBS antigen.

The total protein content of PBS antigen of *A. orieni* and *P. kashmirensis* was 3.6 mg/ml and 4.5 mg/ml respectively. 1.5 ml of the antigen was intraperitonially injected into the fishes with Fraund's complete adjuvant at an interval of 3 days.

Detection of antibody to parasites:- The blood samples were randomly collected from fishes of pond (control) and Wular lake(naturally infected). The fishes infected with parasites other than *Pomphorhynchus* and *Adenoscolex* infection were discarded. Blood was allowed to clot and left overnight at 4°C. The clot was removed and the serum was centrifuged at 6000 rpm for 30 minutes. The serum was then decanted and tested for antibody activity using a modification of original immunodiffusion technique of Ouchterlony (1958) and Molnar and Berczi (1965). Test sera were allowed to diffuse against parasite antigen in 1% ion agar (Difco pH 7.2) in a sterile petri dish.

RESULTS

Initial tests on the sera of C. *carpio* indicated that a restricted number had detectable precipitins to *Pomphorhynchus kashmirensis* antigen, but from the months of March to July, most of the infected fishes had detectable amount of antibodies in their serum as indicated in Table I and II. The highest number of infected fishes positive for the precipitating antibodies was recorded in the month of June and this may be due to the fact that the increase in water temperature may have caused the highest antibody titre in the infected fishes. The adult fishes were preferred for the experiments as it was possible to screen them continuously for the presence of serum antibodies. Also, adult fishes provided sufficient immune serum for immunological assays. The naturally infected *S. niger* caught from the Wular lake were also subjected to immunological tests and positive immuno diffusion results were obtained during the summer months of the year 2000. None of the fishes during winter months when the temperature of the lake was recorded between 4°C to 7°C showed an antibody response to *P. kashmirensis* as well as to *A. oreini*. This may be due to the fact that the poikilotherm and hibernating animals have little antibody titre during the cold seasons and the antibody levels increase due to increase in the temperature of the lake. The elevated temperature perhaps shorte.ns the induction period between initial infection and the primary antibody response of the hosts. These results therefore confirm the influence of ambient temperature on the immune response of fishes. In case of P. *kashmireasis* and *A. oreini,* although the antibodies were produced by *C. carpio* and *S. niger* this could not be correlated with absolute protection against recurrent infection of the parasite. In the present study, variation in fish sera was recorded that could be associated with the presence of parasite. In adult *C. carpio* and *S. niger,* infected with large numbers of P. *kashmirensis* and A. *oreini,* there was an obvious rise in globulin levels in comparison with the uninfected controls. Immunoelectrophoresis of immune *C.carpio* fish serum followed by diffusion against the helminth antigens revealed a single arc of precipitation with the immunoglobulin migrating as a slow (β-or fast gamma-globulin. The same results

Table I

Humoral immune response of *Cyprinus carpio* to *Pomphorhynchus kashmirensis* infection with respect to temperature

Month	No. of fishes examined	No. of fishes infected	No. of parasites found	Average monthly temp of lake	Ouchterlony	
					No. tested	No. +ve
January	15	4	16	4.7	10	0
February	20	6	18	7.1	10	0
March	16	3	10	12.4	8	0
April	20	4	13	15.3	10	2
May	25	7	26	17.4	7	2
June	16	5	40	23.2	10	3
July	20	6	53	26.2	8	6
August	15	2	6	22.9	10	2
September	18	0	0	20.3	10	0
October	20	0	0	19.9	8	0
November	20	2	6	14.3	8	0
December	15	3	12	7.6	8	0

Table II

Humoral immune response of *Schizothorax niger* to *Adenoscolex oreini* infection with respect to temperature

Month	No. of fishes examined	No. of fishes infected	No. of parasites found	Average monthly temp of lake	Ouchterlony	
					No. tested	No. +ve
January	10	1	3	4.7	5	0
February	12	2	6	7.1	6	0
March	11	2	5	12.4	8	0
April	8	3	10	15.3	5	0
May	15	3	16	17.4	9	1
June	9	1	5	23.2	8	3
July	12	3	6	26.2	6	2
August	16	4	15	22.9	7	1
September	8	2	11	20.3	10	0
October	7	3	12	19.9	6	0
November	6	0	0	14.3	3	0
December	10	4	10	7.6	9	0

were obtained in *S. niger.* The appearance of precipitation patterns that were encountered in this analysis were the molecules that share an identical antigen structure and exhibited a pattern of complete coalescence of the precipitation lines, while those with partial antigen difference show crossing over of precipitin bands.

To conclude, *S. niger* and *C. carpio* infected with *A. orieni* and *P. kashmirensis* responded with the production of precipitating antibody to the antigens of these parasites. The low antibody titre may be partly explained by the relative insensitivity of the Ouchterlony tests. It is possible however that a large proportion of the serum antibody may be bound to the parasite, and this could be significant where very large numbers of helminths occur in the host. Hence it is possible that the parasites bound sufficient antibody so that very little is available for immunological assay.

DISCUSSION

The study of humoral immune response gave a deep insight in understanding the impact of parasitism and ambient temperature on antibody production in the fresh water fishes of the Kashmir waters. Our results are in accordance with Alien and McDonial (1973) and Bisset (1948) who reported a great effect of temperature on the production of antibodies in fishes and other cold blooded animals. Cushing (1942) Clem and Sigal (1963) and Alien and McDonial (1973) suggested that the immune response of the fish is not as fully evolved as that of higher vertebrates. The single class of immunoglobulins in fishes compared with the several classes in mammals certainly supports this view, whether this lack of a wide range of immunoglobulin classes which results in deficiency of fish immune response to parasites is not yet known. Some examples of protective immunity have been given by Nigerlli and Breeder (1934) who noted that ability of marine fish to develop protection against the external parasite *Benedenia*. Other workers (Buchmann *et al.,* 2001) have also recorded increase in the serum globulins of fishes infected with the parasitic agents. As majority of teleost species have β-electrophoretic mobility (Fine and Drilhen 1961; Klontz *et al., 1966:* Evelyn, 1971 Marchalonis. 1971) it is consistent that the sera of infected *C.carpio* and *S. niger* should show increase in the globulins, especially in β fraction. A similar situation occurs in teleosts immunized with antigens, increases in the β-2 and gamma-1 globulins with fall in albumin levels were noted by Summerfelt (1966) in immunized gold fish. Our results are in accordance with the findings of Buchmann *et al,* (2001) who reported that both specific and nonspecific factors in humoral and cellular branches of the fish immune system are well developed, thus production of specific antibodies in the fish B-lymphoeyte against natural and synthetic antigens has been well characterized.

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Focus on red cell genetic defects and malaria

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The relative distribution and occurrence of certain red cell (erythrocyte) genetic defects viz., sickle cell haemoglobin (Hb-S), β-thalassaemia (β-thal.) and glucose-6-phosphate dehydrogenase deficiency (G^d) has been observed in 1914 malaria positive cases (1264 of *falciparum* and 650 of *vivax* malaria) as well as in 5903 apparently healthy and unrelated subjects (non-malarials or control group). The occurrence of these erythrocyte defective genes in malaria cases was found to be relatively low as compared to non-malarial subjects. Individuals having Hb-S trait, β-thal. minor and G^d showed comparatively low parasite counts of *Plasmodium* species *(falciparum* and *vivax)* as compared to that in patients with genetically normal erythrocytes. An association between these red cell genetic defects and lethal malaria has been discussed and also evaluated statistically.

Key words : β-Thalassaemia, G-6-PD deficiency, genetic defects, malaria, red cell, sickle cell Hb

INTRODUCTION

A high frequency of sickle cell haemoglobin (Hb-S), β-thalassaemia (β-thal.) and G-6-PD enzyme deficiency (G^d) genes in malaria endemic areas has been observed by several workers and it is considered as a genetic adaptibility against lethal malaria (Allision, 1954; Stamatoyannopoulos and Fessas, 1964; Siniscalo *et al.*, 1966; Luzzato, 1979; Bienzle *et al.*, 1981; Choubisa, 1985a,b, 1991). Later on this fact was popular in the form of "malaria hypothesis" which indicates that erythrocytes with any of these genetic defects or disorders are unable to sustain the multiplication of malarial parasite. Except few controversial findings or reports, it has been generally accepted that malaria (as bio-factor) exerts a selective pressure for maintenance of higher frequency of these red cell defective genes in trait forms. In India, a high incidence of these mutant genes has also been reported from different geographical regions where malaria is/ was hyperendemic. Such studies are mostly conducted in tribal populations (Bhatia and Rao, 1987), as they inhabit malarious environment. However, studies pertaining to prevalence or incidence of these erythrocyte defective genes in malaria positive cases (P. *falciparum* and *P. vivax)* and malaria parasite counts (parasitaemia) in individuals having any of these abnormal genes have been conducted rarely (Kruatrachue *et al.*, 1969). Therefore, the present study was undertaken to find out the prevalence of Hb-S, β-thal. and G^d genes in malaria positive cases *(P. falciparum* and *P. vivax).* Simultaneously, malaria parasite counts (density or parasitaemia) have also been done in those subjects who had any of these red cell defects.

MATERIAL AND METHODS

The present study was performed in the tribal areas of southern Rajasthan (Banswara, Dungarpur and Udaipur districts) where malaria is hyperendemic and genes of Hb-S, β-thal. and G^d are more prevalent in tribal populations (Jain *et al.*, 1983 and 1984; Choubisa *et al.*, 1984; Choubisa, 1985a, 1988).

Venous blood samples were collected in ACD vials from 3205 malaria suspected cases as well as from 5903 apparently healthy and unrelated individuals of either sex and considered them as normal or control group for the present study. For the identification and quantitative evaluation of abnormal haemoglobins (Hb-S, Hb-D, Hb-E etc.) and Hb-A_2 (for (β-thal. minor), horizontal paper (using Whatman filter paper No. 3) and cellulose acetate electrophoreses were performed (Choubisa *et al.*, 1984; Choubisa, 1985b). Identification of Hb-S from Hb-D variant was done by

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solubility test whereas fetal haemoglobin, Hb-F was confirmed by alkali denaturation technique as described elsewhere (Dacie and Lewis, 1986). Methaemoglobin reduction test (MRT) was followed for rapid screening of large number of blood samples for the evidence of G-6-PD enzyme deficiency (Brewer *et al.,* 1960). The number of blood samples studied for *P. falciparum, P. vivax* and normal (control group) individuals belonging to different ethnic groups viz., scheduled tribes (S.T.), scheduled castes (S.C.), and general castes (G.C.) has been depicted in Table I. The basic sources for sampling were General Hospitals, Primary Health Centres, and hostels located in the study areas.

During blood sampling, peripheral thin and thick blood smears of malarial and non-malaria (control) individuals were also prepared, stained by Giemsa stain and examined under high power and oil immersion lens of microscope for the evidence of any red cell (erythrocyte) abnormalities and malaria parasite *(Plasmodium* spp.). The initial parasite counts were also done by counting the number of parasitized cells per 1000 erythrocytes in the smears. The red blood cells count per mm^3 was also determined and the number of parasites per mm^3 were then calculated. The data of the present study was analysed statistically. A few individuals with Hb-D and Hb-E were excluded from the study.

RESULTS AND DISCUSSION

Out of 3205 malaria suspected individuals, 1914 (59.71%) were found to be infected with *Plasmodium* parasites with varying parasitaemia. Of these 1264 (66.03%) showed *P. falciparum* whereas 650 (33.96%) were infected with *P. vivax.* Amongst these malaria positive cases, the incidence of Hb-S, β-thal. and G^d genes was found to be 1.51% (29 cases), 2.24% (43 cases), and 5.64% (108 cases) respectively which is lower than that observed in non-malarials (3.50%, 5.08% and 10.45% respectively). However, subjects having *falciparum* malaria did not show the evidence of homozygous state of Hb-S and β-thal. genes (Table I). The relative distribution and occurrence of these red cell defective (mutant) genes in different types of malaria cases *(falciparum* and *vivax)* as well as in normal individuals belonging to different ethnic groups have been shown in Table I. Data pertaining to the occurrence of Hb-S, β-thal. and G^d genes in malarial and non-malarial subjects were also analysed statistically and found to be non-significant.

Plasmodium parasite density (per mm^3) was determined in the subjects who had heterozygous forms of Hb-S and β-thal. as well as in G-6-PD enzyme deficients and was found to be relatively low in these individuals as compared to normal subjects. However, *P. vivax* parasite counts were relatively higher than subjects infected with *P. falciparum.* Data pertaining to parasite density in subjects having these red cell genetic defects relation to different genetic defects have been analysed and found relatively highly significant in the case of *P. falciparum* (Table II) as compared to *P. vivax* (Table III).

It is obvious that in the malaria endemic provinces Hb-S, β-thal. and G^d genes are more prevalent. 'In fact, heterozygous forms of these red cell defective genes are reported to provide a selective advantage against *P. falciparum* infection. In the present study *falciparum* infection was not found in homozygotes of Hb-S and β-thal. but was found in heterozygotes with less severe parasitaemia (Table II). It has also been observed that heterozygotes can become infected with *P. falciparum* but high parasitaemia and subsequent mortality is relatively low amongst such individuals (Raper, 1956; Fleming *et al.,* 1979). These findings suggest that *Plasmodium* parasites can infect or invade the erythrocytes (red cell) with any of these defects (Hb-S, β-thal. and G^d) but their further development or multiplication is restricted or not enhanced. This is also supported by many *in vitro* studies which showed that *P. falciparum* does not easily invade and grow well in the erythrocytes with Hb-S, β-thal. and G^d. The suggested reasons for this include defective erythrocyte membranes, oxygen stress, increased sickling and low Hb content (Pasvol and Wilson, 1982; Roth *et al.,* 1983; Nagel and Roth, 1989). Therefore, it has been concluded that the abnormal erythrocytes of individuals with Hb-S, β-thal. and G^d genetic defects are less easily parasitised by *P. falciparum* than the genetically normal erythrocytes (normal subjects) and this provided a considerable degree of protection against lethal malaria (P. *falciparum)* which is also in support of Darwin theory of "natural selection".

Table I : Relative distribution and incidence of sickle cell Hb, β-thalassaemia and G-6-PD enzyme deficiency (G^d) in malarial and non-malarial (normal) subjects of each ethnic groups

Ethnic group	Type of Malaria	No. of tested	Red cell genetic defects				G-6-PD deficiency (G^d)
			Sickle cell Hb		β-thalassaemia		
			Hb-AS	Hb-SS	Minor (trait)	Major	
Scheduled Tribes (S.T.)	*falciparum*	584	11 (1.88)	- (0.00)	16 (2.74)	- (0.00)	47 (8.05)
	vivax	260	9 (3.46)	1 (0.38)	11 (4.23)	1 (0.38)	19 (7.31)
	Normal	3163	146 (4.62)	22 (0.70)	202 (6.39)	18 (0.57)	443 (14.00)
Scheduled Castes (S.C.)	*falciparum*	331	3 (0.91)	- (0.00)	5 (1.51)	- (0.00)	18 (5.43)
	vivax	180	2 (1.11)	- (0.00)	5 (2.77)	- (0.00)	15 (8.33)
	Normal	1155	20 (1.73)	2 (0.17)	40 (3.46)	2 (0.17)	95 (8.23)
General Castes (G.C.)	*falciparum*	349	2 (0.57)	- (0.00)	4 (1.15)	- (0.00)	9 (2.58)
	vivax	210	- (0.00)	1 (0.48)	- (0.00)	1 (0.48)	- (0.00)
	Normal	1585	17 (1.07)	- (0.00)	37 (2.33)	1 (0.06)	79 (4.98)
Total	*falciparum*	1264	16 (1.27)	- (0.00)	25 (1.98)	- (0.00)	74 (5.85)
	vivax	650	11 (1.69)	2 (0.31)	16 (2.46)	2 (0.31)	34 (5.23)
	Normal	5903	183 (3.10)	24 (0.41)	279 (4.73)	21 (0.36)	617 (10.45)

Figures in parentheses represent percentage

Differences between infected and controls was statistically non-significant ($p > 0.05$) for all genetic defects.

Table II : *Plasmodium falciparum* parasite density in subjects of red cell genetic defects

S. No.	Genetic defects	Incidence (%) of parasite density (per mm^3) Very mild (1-999)	Mild (1000-9999)	Moderate (10000-49999)	Severe (>50000)
1.	Sickle cell trait (Hb-AS)	7^d (43.75)	6^c (37.50)	3^a (18.75)	-^{a} (0.00)
2.	β-thalassaemia (trait or minor)	10 (40.00)	8 (32.00)	6^b (24.00)	1^b (4.00)
3.	G-6-PD deficiency (G^d)	28 (37.84)	23^c (31.08)	18^b (24.32)	5 (6.76)
4.	Control or Normal (without genetic defects)	18 (25.71)	17 (24.29)	22 (31.43)	13 (18.57)

Figures in parentheses represent percentage

Superscripted alphabets denote level of satistically significant differences of genetic defects values compared with the controls in the corresponding group. a, b, c, d = Significant at $p < 0.001$, $p < 0.005$, $p < 0.025$ and $p < 0.05$ respectively.

Table III : *Plasmodium vivax* parasite density in subjects of red cell genetic defects

S. No.	Genetic defects	Incidence (%) of parasite density (per mm^3) Very mild (1-999)	Mild (1000-9999)	Moderate (10000-49999)	χ^2 Severe (>50000)
1.	Sickle cell trait (Hb-AS)	3^a (27.27)	4^c (36.36)	3^a (27.27)	1^c (9.09)
2.	β-thalassaemia (trait or minor)	5^c (31.25)	5^d (31.25)	5^b (31.25)	1^c (6.25)
3.	G-6-PD deficiency (G^d)	10 (29.41)	10^d (29.41)	11 (32.35)	3 (8.82)
4.	Control or Normal (without genetic defects)	20 (28.57)	18 (25.71)	21 (30.00)	11 (15.71)

Figures in parentheses represent percentage

Superscripted alphabets denote level of statistically significant differences of genetic defects values compared with the controls in the corresponding group. a, b, c, d = Significant at $p < 0.001$, $p<0.005$, $p < 0.01$ and $p < 0.025$ respectively.

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Restriction Fragment Length Polymorphism analysis of Apical Membrane Antigen-1 and Duffy Binding Protein genes in North Indian field isolates of *Plasmodium vivax*

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Apical membrane antigen-1 (AMA-1) and Duffy binding protein (DBP) of *Plasmodium vivax* are potential vaccine candidate antigens. High degree of polymorphism in the candidate antigens may compromise the efficacy of an otherwise effective vaccine. Polymorphic regions of the genes encoding AMA-1 and DBP were analyzed by PCR-RFLP in 25 north Indian isolates *of Plasmodium vivax*. No size variation was seen in target segments of both the genes by PCR. Further analysis of PCR products of AMA-1 and DBP by RFLP using *Pvu-II* and *Eco R-I* endonuclease respectively, also failed to detect polymorphism in both the genes. The presence of low or no variation within these genes may be due to the functional constraints as both the proteins have important functions in the life cycle of the parasite.

Key words: AMA-1, DBP, Genetic polymorphism, PCR-RFLP, *Plasmodium vivax*.

INTRODUCTION

Malaria is an important tropical disease with an estimated global incidence of 300-500 million cases and around two million deaths every year. In India *P. vivax* is a predominant species. Its molecular analysis has been neglected, mainly because parasite can not be cultured, the parasite material available is limited to small quantities obtained from either infected human patients or experimentally infected primates.

Apical membrane antigen-1 (AMA-1) of plasmodia is a protein that first appears in the apical complex and then migrates to the merozoite surface. This protein has been suggested to be involved in merozoite release from erythrocytic schizont and subsequent erythrocyte invasion (Dvorak et al 1975, Perkins 1988). Several studies have revealed that antibodies against this antigen block invasion of the red cell by the parasites (Deans et al 1988, Collins et al 1994). Due to the importance of this antigen in the life cycle of parasite, it may be a promising blood stage malaria vaccine candidate antigen. AMA-1 has been cloned and sequenced in a number of *Plasmodium* species including *P. chabaudi* (Marshal et al 1994), *P. fragile* (Peterson et al 1990), *P. falciparum* (Oliveira et al 1996) and *P. vivax* (Cheng and Saul 1994). The protein consist of 558 to 662 amino acids in different plasmodial species (Cheng and Saul 1994) and contains conserved N' and C' terminal regions of non-repetitive sequences. In *P.vivax* AMA-1 has been reported to have 562 amino acids (Cheng and Saul 1994). The analysis of the sequence of this antigen allowed the division of the molecule into twelve blocks, which are conserved, semi-conserved or variable (Cheng and Saul 1994).

P. vivax merozoites require interaction with duffy blood group surface antigen (on duffy positive human erythrocytes) and cannot enter duffy negative erythrocytes (Miller et 1976, Mitchell et al 1986, Nichols 1987, Barnwell et 1989). Wertheimer and Barnwell (1989) identified a 140 kDa ligand on *P. vivax* merozoites which binds with human duffy protein and termed it as duffy binding protein. Duffy binding protein (DBP) on the parasite surface is a member of the protein family localized in the *Plasmodial* micronemes that binds to erythrocytes carrying the receptors. It is also known as microneme protein-1 (MP-1), because it was the first protein identified from the micronemes of merozoites (Camus 1985, Adams et al 1990). Recent molecular analysis of duffy binding ligands of *P.knowlesi*, *P. vivax* and

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erythrocyte binding protein of *P.falciparum* shows homology. (Sim et al 1994). The sequence analysis of duffy binding protein (DBP) of *P. vivax* allowed division of this gene into six domains named Region-I to Region-VI based on amino acid sequences (Adams et al 1992). Chitnis and Miller (1994) has identified the Region-II (N' terminal) as a critical ligand which binds to host erythrocytes (Singh et al 2001). Thus, Duffy binding protein is essential for the survival of *P.vivax*.

Due to the importance of AMA-1 and DBP in the life cycle of *P. vivax* both these antigens are strong vaccine candidates. As significant genetic diversity could compromise the efficacy of an otherwise effective vaccine (Crewther et al 1996), we investigated the genetic polymorphism by PCR-RFLP techniques in AMA-1 and DBP in North Indian isolates of *P.vivax*.

MATERIAL AND METHODS

The blood samples were collected from symptomatic malaria patients found positive for *Plasmodium vivax* by microscopy. The patients included were those attending Nehru Hospital of Postgraduate Institute of Medical Education & Research, Chandigarh or malaria clinics and hospitals in various regions of Northern India. The patients showing mixed infection (with *P. falciparum* and *P. vivax*) were excluded. 2-3 ml of blood was obtained from each patient by venepuncture in a citrated anticoagulant (Laserson et al 1994).

The DNA was prepared by a rapid method as describe by Foley et al (1994) with slightly modifications. Briefly, 50µl of parasitized blood was washed thrice with 1 ml of ice-cold 5 mM sodium phosphate (pH 8.0) by mixing well by vortexing. Finally the suspension was centrifuged at 10000 rpm for 10 minutes and 50 µl sterile distilled water was added to the pallet and after mixing was boiled for 10 minutes. The suspension was centrifuged at 10000 rpm. Ten µl of supernatant was used as DNA template in 50µl polymerase chain reaction (PCR) mixture.

All PCR reactions were carried out in a 50µl reaction volume in a thermocycler (Eppendorf master cycler gradient). Amplification was performed in 50 mM KC1, 10 mM Tris-Cl (pH 9.0), 1.5 mM $MgCl_2$ 0.01% gelatin, 0.2 mM each of dNTPs, 1.25 unit AmpliTaq DNA polymerase (GIBCO-BRL), 250 nM each of primers and 10 µl of parasite DNA as template. The Hypervariable region of AMA-1 and central polymorphic region of DBP gene (Including partial sequence of R-II and R-VI) were amplified by PCR using specific primers. The sense and anti sense primers used for AMA-1 were: 5' AGA ATT CCA GCT GGA AGA TGT -3' and antisense 5' TCC TAA ATT TTT ACG GGG GC-3. For DBP the sense and antisense primers were: 5' GAA GAT ATC AAT TAT GTA TG -3' and antisense 5' CTT CAA ATT CCT TTT TCA TG - 3. For AMA-1, thirty five cycles of 95°C for 1 min, 55°C for 50 sec and 72°C for 2 min, and for DBP, 30 cycles of 95° for 1 min, 48° C for 30 sec and 74° C for 3 min were performed. PCR products were electrophoresed on 1.5% agarose gel and the fragment size was determined by comparison with molecular weight marker (100 bp ladder plus, MBI Fermentas).

The amplified products of AMA-1 and DBP gene were digested with *Pvu-II* and *EcoR-I* endonuclease respectively. Digestion was performed in a 20µl reaction mixture utilizing 16 µl of PCR product, 1.5 µl sterile ddH_2O, 2µl (1 X) digestion buffer and 0.5 to l µl (5 units) of restriction enzyme. The digestion was carried out at 37^0C overnight. Finally, 10 µl of each digested PCR product was electrophoresed on a 2% agarose gel containing ethedium bromide and photographed in a UV transilluminator.

All the 25 clinical isolates were amplified. No PCR product was obtained in control samples which included the DNA extracted from *P.falcipamm* and blood samples from healthy individuals.

RESULTS

The PCR amplified products of AMA-1 and DBP gene produced a single band, the molecular weight of PCR products being approximately 500 bp and 2.3 kb respectively. No size polymorphism was observed on electrophoresis in both the genes. (Figure-1&2)

The RFLP patterns, (using *Pvu*-II and *EcoR*-l restriction enzymes for AMA-1 and DBP respectively) obtained did not show differences among these 25 clinical isolates. The restriction digestion of PCR products of AMA-1 gene from all the isolates resulted in two fragments of 280 and 220 bp (Figure -1). The restriction digestion of PCR product of DBP gene in all the 25 isolates resulted into two fragments of 1600 bp and 700 bp (Figure-2).

Thus, the PCR-RFLP analysis of the HVR region of AMA-1 gene and central polymorphic region of DBP gene revealed that the *P. vivax* isolates did not show variation in the target genes and probably these were conserved due to functional constraints.

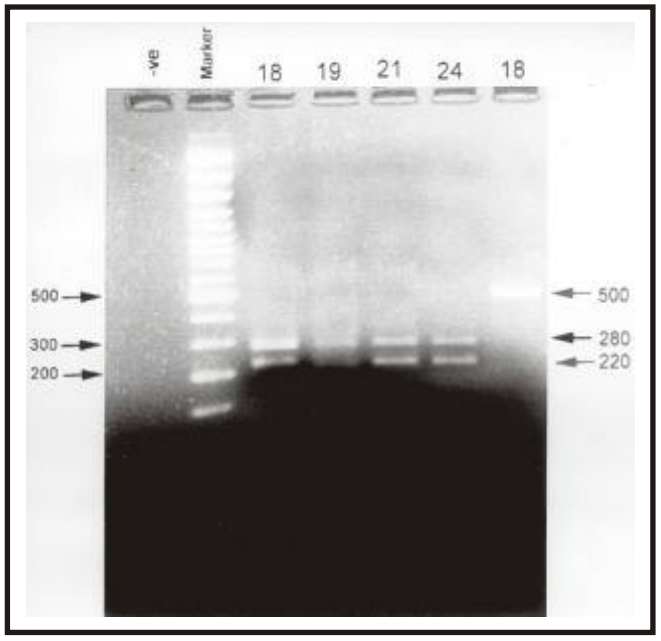

Figure-1: PCR product and RFLP patterns of AMA-1 (HVR) gene digested with *Pvu-II* restriction enzyme of few representative isolates of *P. vivax* collected from Northern region of India. (Lane-1 Negative control, Lane-2 DNA molecular weight Marker (100 bp ladder plus). Lane 3-6 RFLP patterns of representative isolates, Lane-7 PCR Product)

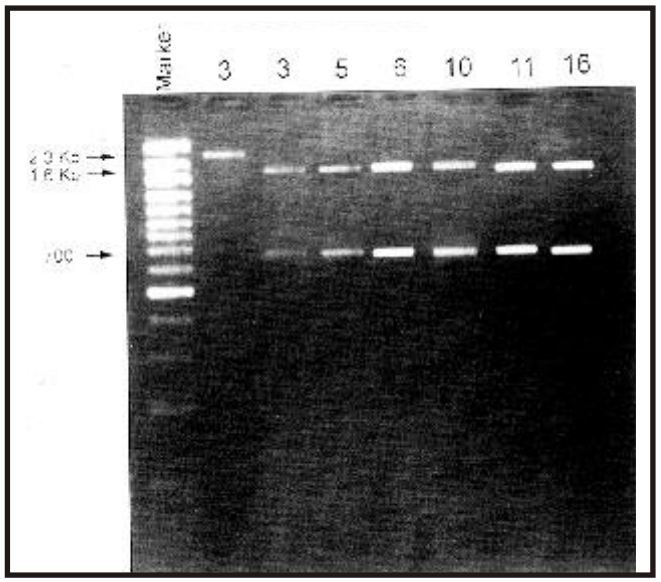

Figure-2: PCR product and RFLP patterns of DBP (Central Polymorphic region) gene digested with *Eco R*-1 restriction enzyme of few representative isolates of *P. vivax* collected from Northern region of India. (Lane-1 DNA molecular weight Marker {100 bp ladder}; Lane-2 PCR product of isolates 3; Lane 3-8 RFLP pattern of shown isolates.

DISCUSSION

The results of present study confirm the earlier observation (Oliveira et al 1996) that AMA-1 gene is largely conserved because of functional constraints of this gene in the biology of parasite. The absence of size variation in this segment of AMA-1 gene has also been observed in Philippines and Indonesian isolates of *P.vivax* by sequencing techniques (Cheng and Saul 1994). The sequencing of full length of AMA-1 gene also showed limited polymorphism (Oliveira et al 1996, Cheng and Saul 1994). Figtree et al (2000) in a study on isolates collected from different geographical regions including Africa, China, India, Indonesia, Philippines, Papua New Guinea, Solomon Island and Thailand also did not observe any size variation in 219 isolates in the same region of the gene. These investigators observed dimorphism in 4 nucleotides (680 - 684) in all isolates except those from Africa by sequencing analysis. However, Cheng and Saul (1994) has also shown the presence of polymorphism in variable region particularly at the 5' end of AMA-1 gene.

The MP-1 family of erythrocyte binding proteins of *Plasmodium* species has a highly conserved structure (Adams et al 1992). In the present study, no size variation was observed in DBP gene among these clinical isolates. This is in accordance with findings of Tsuboi et al (1994) who did not observed size variation among 50 clinical isolates collected from hyper endemic area of Papua New Guinea. Recently another study from Korea also reported no size variation among 30 clinical isolates (Kho et al 2001)

PCR amplified product was subjected to enzymatic digestion with *Eco-R1* to check the restriction fragment length polymorphism. Digestion resulted into two fragments of 1.6 Kb and 700 bp in all the isolates showing no difference in RFLP pattern of different isolates. However, in contrast to our study, Tsuboi et al (1994) studied the same region of DBP (RIV) and reported variation in RFLP patterns with *Eco-R 1*.

Further they observed the presence of three different types of insertions within R-IV on the basis of nucleotide sequence analysis i.e., 30 bp, 6 bp and both 30 bp and 6 bp. On the basis of these inserted nucleotide sequences, Tsuboi et al (1994) has categorized strains into three allelic types (group 1, 2 & 3). Several studies conducted in diverse geographical region have reported polymorphism in principal Duffy binding domain (R-II) of DBP gene.

All these observations are based on sequence analysis of this region (*Tsuboi et al* 1994, Ampudia et al 1996, Kho et al 2001, Xalini et al 2000). In the present study no variations in polymorphic region of DBP gene in clinical isolates collected from Northern region of India by PCR-RFLP technique was observed.

PCR or PCR-RFLP is not ideal method to study the genetic variation. The strains which show no variation by these techniques may show small nucleotide changes on the sequencing of the genes. Therefore, there is a need of further analysis of AMA-1 and DBP genes of *P. vivax* strains prevalent in Northern region of India by gene sequencing which is a powerful technique to study small variations. However, the functional constraints in both the proteins appear to limit the scope of significant variation in these genes.

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Human infesting ixodid ticks of Kerala

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Considering the increasing incidence of tick bites and associated problems among the tribals and forest dependent personnels, a study was carried out on the common tick species inhabiting some of the selected forest ecosystem in Kerala. Results of the study enabled to recover the various developmental stages of four species of ticks from the forest vegetation, forest grazing domestic animals and also from the body of forest dependent people. Of these, three species of ticks viz., *Haemaphysalis spinigera, H. turturis* and *Aponoma* sp. were collected from the body of human beings and also from forest vegetation where as four species including *B.decoloratus* were recovered from the body of forest grazing domestic animals. *H. spinigera* was identified as the most common and prevalent species in all the forest vegetation sites observed. The paper provides information on the nature of damage inflicted by ticks on human beings.

Key Words : Human beings, Ixodid ticks, Kerala.

INTRODUCTION

Ticks are well known to science because of the important roles they perform as notorious vectors of life-threatening diseases and distressing pests of domestic animals and man. Despite their biomedical importance, the study of ticks remained in dormancy, especially in India until Sharif (1928) published a key for the identification of ixodid ticks. Later studies by Sen (1938) and the checklist prepared by Jagannath et *al.* (1973) helped to enrich our knowledge on Indian ticks. Trapido *et al.* (1964) published a guide to the identification of all stages of haemaphysalid ticks of South India. The discovery that arthropods could serve as vectors of important human and animal diseases, elicited a revival of interest in the study of ticks leading to the formulation of a revised system of their classification. Demonstration of the role of ticks in the transmission of Kyasanur Forest Disease in Karnataka by Varma *et al* (1957) and the untiring efforts of NIV personnels like Dhanda (1996), Bhat (1985) and Geevargheese *et al.* (1997) further extended our knowledge on the tick fauna of India. In the present study, an attempt has been made to study the tick species inhabiting the forest ecosystems of Kerala and their impact on human beings. The study probably forms the first report on human biting ticks.

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MATERIALS AND METHODS

A survey on the tick species harbouring various forest ecosystems like the Muthanga, Kurichiyad and Sulthan Battery range of Wayanad Wildlife Sanctuary, Aralam forest area, Kannur and Chikkad forest area in the Kerala-Karnataka border was carried out during April-May months of *2002*. Ticks were collected from forest vegetation, temporary resting places of wild animals and directly from the body of forest grazing domestic animals. The unfed tick species that quested on vegetation were collected by sweeping with tick flags prepared by attaching large sheets of coarse weave fabric to a long handle. Larvae, nymphs and few adults of various tick species were recovered from the forgest vegetation, meadows and from the dry leaves accumulated at plantation area. Direct collection of ticks was also made from the clothing and body of trackers and tribal people with the help of a strong blunt forceps. For inducing easy detachment, cotton plugs soaked in ether or chloroform were pressed at sites of attachment. Adults and immature stages of ticks thus collected were preserved in 70% alcohol and were washed, dehydrated, cleaned and mounted in DPX or Hoyer's medium. Identification up to species level was made following relevant literature and consulting specialists. The foot prints and soil markings at the temporary resting places of wild animals were confirmed by consulting experts from the Kerala Forest Research Institute, Peechy. The

feeding symptoms and nature of damage induced on human beings were carefully observed and recorded. Additional data about tick bite symptoms were also collected from tribal and local people.

RESULTS

Results of the present study enabled to recover the various developmental stages of different species of ticks (Table 1) from most of the observed sites in the natural forest and plantation area. The number of ticks was more in the meadows adjacent to the stream where the animals were found frequently visiting for drinking water. Tick infestation could be evidenced on the body of tribal people and on all the forest grazing domestic animals like the cows and goats examined in the forest area. However, the rate of infestation appeared comparatively low in the case of buffaloes as only 25% of them were found infested with ticks. Generally, the number of ticks recovered during early morning hours appeared to be less.

All the species recovered during the study were recognised as members of the family Ixodidae. The species recovered from ground vegetation included *Haemaphysalis spinigera, H. turturis* and *Aponomma sp.* where as those collected directly from domestic animals were *H.bispinosa, H.turturis and B.decoloratus*. Of these, *H. spinigera* was identified as the most common and prevalent species in the forest vegetation. In no instance, the recovery of *H. spinigera* could be made from the domestic animals examined during the present study.

The larvae and nymphs of *H.spinigera, H. turturis* and *Apononomoma sp.* could be recovered from the clothing and directly from the body of trackers and tribal people. The most prevalent species infesting human beings as evidenced during the study was *H.spinigera* followed by *H.turturis*. Larvae of both the species could be collected in large numbers directly from the body of tribals. Seed ticks were found attracted by the host odour and body temperature. Questing height of the various species of ticks was found determined by the size of the host animals concerned. Seed ticks were found adhering to the lower extremities of the body of tribal people. Within short span of time, they managed to reach near to their shoulder or hip joint. On reaching the concealed preferred sites like the shoulder or hip joint and the lower abdominal area, the seed ticks initiated feeding activity (Plate I, Figs. 5 & 6)

The irritations caused by tick bite included the development of itching sensation followed by the appearance of coloured patches around the site of attack. Later, a white coloured fluid filled papule was developed at the site of attachment, imparting a swollen appearance (Plate I, Fig. 2 & 3). Swelling, oozing out of watery fluid, development of lesions etc. were the common symptoms recognised on the body of the host (Plate I, Fig 3, 4 & 5). The lesions thus developed were found spreading to adjacent area and in extreme cases were found leading to difficulties in clothing. Such lesions were found often lasting for more than 2 months. A round black scar left behind at the infested site was often found persisting for years, causing frequent itching as reported by the tribals (Plate I, Fig. 6).

DISCUSSION

The present study enabled to record the prevalence of Ixodid ticks in the forest range of Kerala. The various developmental stages of these ticks could be collected not only from ground vegetation but also directly from the body of man and forest grazing domestic animals. The prevalence of ixodid ticks on wild animals has already been noticed by Zieger *et al* (1998) in the Central Province of Zambia where mammals and birds constituted the major hosts. The current study further confirms the prevalence of ixodid ticks in the forest range of Kerala also and suggests their ability to inflict both domestic and wild animals.

The results of the study helped to locate various developmental stages of ixodid ticks belonging to five species categorised under three genera. Of these, *H. spinigera* was identified as the most common and prevalent species in the forest vegetation. The abundance of this species in the vegetation as well as on the body of domestic animals was reported by Varma *et al.* (1957) in the Kyasanur forest area of Karnataka. Contrary to this, in no instance the recovery of *H. spinigera* could be made from any of the domestic animals examined during the present study. Extensive surveys covering more forest ecosystems and distant localities of Kerala may probably reveal the presence of this species on the domestic animals of Kerala also.

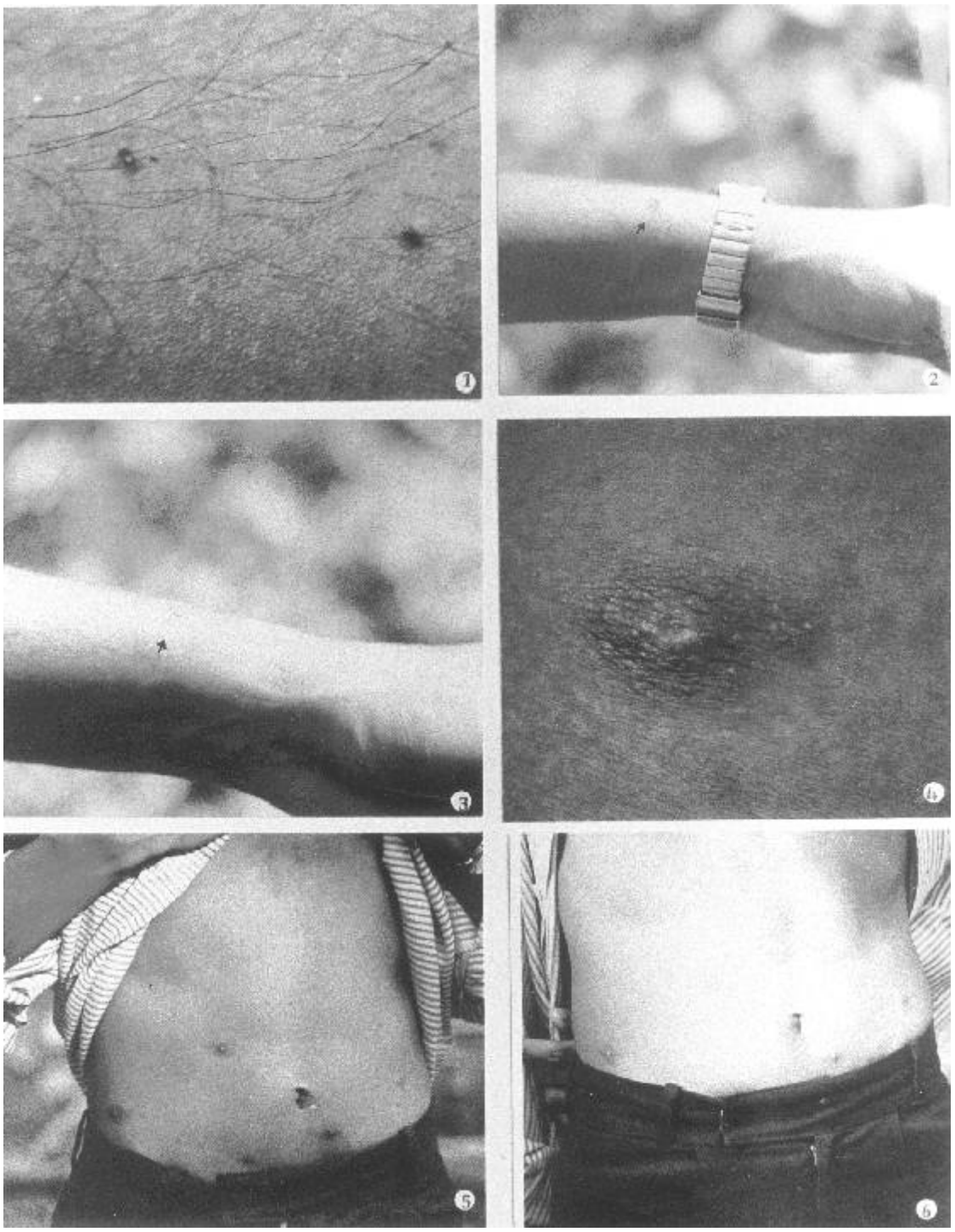

PLATE I

Fig. 1 : Ticks biting on human body.

Fig. 2 : Papule developed at the biting site

Fig. 3 : Development of lesion followed by oozing out of a fluid from the biting site.

Fig. 4 : Large lesion surrounded by the reddened area developed at the site of tick bite.

Fig. 5 : Round black scars left behind at the preferred sites of tick bite.

Fig. 6 : Scar persisting at the biting sites even after months.

TABLE I : SPECIES OF TICKS RECOVERED WITH RESPECT TO HOST ANIMALS

Sl.No.	Name of tick species	Hosts animals recorded			Host animals recorded anew
		Wild animals	**Domestic animals**	**Others**	
1.	*Haemaphysalis spinigera* Neumann, 1897	Rodents (Saxena, 1997) Monkeys (Trapido *et al.*, 1964; Geevarghese, 1997) Wild birds, Porcupines, Wild boar, Deer, Hare (Hoogstraal, 1970, Geevarghese, 1997)	Cow (Sharif, 1925, Geevarghese, 1997) Sheep (Saxena, 1970) Buffalo (Varma *et al.*, 1957)	Man (Geevarghese 1997)	-
2.	*Haemaphysalis bispinosa* Neumann, 1897	Bison, Deer, Tiger (Sharif, 1928) Monkeys, Leopard, Hare Rodents (Geevarghese, 1997) Horse, Lion (Dhanda & Rao, 1964)	Cow, Goat, Sheep, Dog, Horse, Donkey, (Sharif, 1928, Miranpuri and Naithani 1978, Dhanda and Rao, 1964) Buffalo (Jagannath, *et al.*, 1986)	-	-
3.	*Haemaphysalis turturis* Nutall & Warburton, 1915	Nilgiri wild goat (Sharif, 1928) Monkeys, Deer, Small mammals, Birds (Geevarghese, 1997; Trapido *et al.*, 1964)	Dove (Sharif, 1928 Cow, Buffalo, Goat (Geevarghese, 1997)	-	Man
4.	*Boophilus decoloratus* Koch, 1844	Birds & Rodents (Zieger *et al.*, 1998)	Cow, Horse, Sheep (Arthur, 1960; Geevarghese, 1997) Dog (Horak *et al.*, 1995)	Man	-
5.	*Aponomma* sp.	Python, Indian monstar, Lizard, Cobra, Rat snakes (Geevarghese *et al.*, 1997) Kolonin 1995, Sharif, 1928)	-	-	Man

The members of the genus *Haemaphysalis* have been reported earlier as the common parasites of monkeys (Trapido *et al.*, 1964, rodents and sheep (Saxena, 1997) and also on cattle and buffaloes (Varma *et al.*, 1957). Recovery of the members of this genus from the forest vegetation surveyed during the current study suggests their ability to enjoy wide host range including the wild and domestic animals of Kerala.

The number of ticks recorded during early morning hours was comparatively low, particularly from. ground vegetation. Probably, the morning dew might have exerted an adverse effect on the climbing behaviour of ticks to surface vegetation. The wet nature of the flag also can be accounted for the lesser number of ticks recovered, as the efficiency of dry flags would always be greater than that of wet ones (Sonenshine, 1971).

Results of the present study also helped to correlate the questing height of the various species of ticks with the size of host animals concerned. This observation seems to support the earlier study of Fourie *et al* (1990) who correlated the questing height of ticks with the size of the host. The study further helped to trace active host searching ability of immature ticks during the dry part of the year, thereby substantiating the results of earlier studies of Varma *et al.* (1957).

The human infesting species collected during the study included the various developmental stages of *H. spinigera*, *H. turturis* and *Aponomma* sp., all of which were found feeding from concealed sites like shoulder and hip joints on human body. This observation clearly substantiates the anthrophilic nature of the above species of ticks. The scars left behind often were found lasting for years. The data obtained from tribals and forest personnels supplemented with the current observations made on ticks enabled to record ticks as the most annoying pests of wild and domestic animals and man, especially in the forest ecosystem surveyed during the study. More extensive studies are warranted to trace the vector role of these ticks and the pathogenic status of the various micro organisms harboured by them in the above area.

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Distribution of trace metals in different proglottid regions of *Avitellina lahorea* (Woodlands, 1927) and serum of sheep

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The distribution of trace metals in different proglottid regions of *Avitellina lahorea* (woodlands, 1927) (anoplocephalid cestode) and serum of sheep host was determined by the use of an atomic absorption spectrophotometer. The sodium content was found to be higher in the mature segments compared to both immature and gravid segments. The electrolyte potassium level showed minimal concentrations in all the three different regions. Calcium concentration showed consistent increase from immature to gravid regions. The concentration of zinc, cobalt and selenium remained higher in the immature and mature regions when compared to the gravid regions. Studies on selenium content in the parasite revealed that the parasitic somatic tissue contained higher selenium levels than did the host somatic tissue.

Key Words *: Avitellina lahorea;* Proglottid; Trace metals.

INTRODUCTION

Inorganic elements play a significant role in the physiology, metabolism and immuno tolerance of parasites. Thus an understanding about the trace metal elements in the distribution of the host body and that of the parasite is significant. It can give way to further research to ascertain growth of the parasites by elucidating specific mechanisms of poor or non withdrawal of the essential elements without which the parasite load will not persist in the host's body. m the recent past there have been a few attempts to identify and quantify certain inorganic elements in helminths (Goodchild *et al.,* (1962); Nadakal and Nair, 1982; Lal and Kumar, 1985; Riggs *et al.,* (1987); Pandey. and Chowdhry, 1989 and Sures *et al.,* 1997) or individual elements in different groups of helminths (Chowdhury and Singh, 1989, 1993 & 1995). Trace metals in broad fish tapeworm *Diphyllobothrium latum* and blood of carriers was analysed by Markkanen and Vuopala (1965). Riggs *et al.* (1987) described elevated selenium concentrations in the cestode *Bothriocephalus acheilognathi* in comparison with the tissues of two of its final host species. Certain parasites, particularly cestodes of fish, can accumulate heavy metals at concentrations that are orders of magnitude higher than those in the host tissues of the environment (Sures *et al.,* 1997).

Little information in available on the inorganic composition of anoplocephalid parasites it was felt that a quantitative study of Electrolytes (Na^+ , K^+ and Ca^{++}) and Trace elements (Co, Se and Zn) present in the different proglottid regions *adult A.lahorea* would be beneficial in supplementing other studies. In the present report an attempt has been made to analyse the probable role of certain elements during the differentiation of proglottid regions of *A.lahorea.*

MATERIALS AND METHODS

The tapeworms *Avitellina lahorea* (Woodland, 1927) were collected from the intestine of naturally infected sheep autopsied in the slaughterhouse at Chennai, Tamil Nadu, India. The sheep intestines were transported to the laboratory within half an hour of the collection. In the laboratory each intestine was carefully dissected and the tapeworms Were collected. Then the worms were washed in distilled water to render them free from intestinal contents and rinsed quickly 3-4 times in normal saline. The immature, mature and gravid proglottid region of the worm was separated.

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Tissue and Serum Electrolytes

100 mg of tissues from immature, mature and gravid proglottid regions were taken for the electrolyte analysis. The tissues were digested in 2 ml of concentrated HC1 and diluted with distilled water and made up to 10 ml for the estimation. The above was filtered and the filtrate was used for the analysis of sodium, potassium and calcium by using Atomic Emission Spectrophotometer (THE VARIAN TEACHTRON AA6 6D) at the range of 589.592nm for Na^{+}, 422.673nm for Ca^{++} and 766.49 Inm for K^{++}IIT, Madras). The host blood was collected in different test tubes without adding anticoagulant and brought to the laboratory for the analysis of serum. One ml of serum was made up to 3 ml with double distilled water and the above extract was used for the electrolyte analysis.

Determination of Cobalt, Selenium and Zinc

100 mg of immature, mature and gravid segments were digested with triple acid in the ratio of 10:3:l (concentrated nitric acid + 70% perchloric acid + concentrated sulphuric acid). Cobalt, Selenium and Zinc contents of the digests and 1 ml of serum with 2 ml double distilled water were determined using an Atomic Absorption Spectrophotometer (THE VARIAN TEACHTRON AA6 6D) with aqueous calibration standards.

RESULTS

The concentrations of electrolytes, sodium, potassium and calcium measured in the different strobilar regions of *A.lahorea* and host serum were given in the Table I and Figure I (A, B & C). Regarding sodium concentration, it was observed that mature region showed significantly higher value than the other regions. No significant difference was observed between the values of immature and gravid regions. Remarkably highest value of sodium concentration was noticed in host serum, than the strobilar regions of the parasite, which was found to be significant (P=0.01). The different strobilar regions viz., immature, mature and gravid of the parasite were found to contain lesser concentration of potassium than the host serum. This higher content of potassium in host serum was found to be statistically significant (P=0.01). The calcium content observed between gravid region of parasite and host serum and the highest concentration of calcium in the gravid region was found to be significant.

TABLE I

Concentration of sodium, potassium and calcium ions, in the proglottides of adult cestode *Avitellina lahorea* and host (sheep) serum (ppm/μg/ml)

	Immature	Mature	Gravid	Serum	F-value	P-value
Sodium	(a)	(b)	(a)	(b)		
Mean	101.769	162.960	104.707	2412.00	14513.36	0.000**
	±	±	±	±		
S.E.	4.101	3.653	3.634	17.827		
Potassium	(a)	(a)	(a)	(b)		
Mean	1.447	1.687	1.847	10.717	648.78	0.000**
	±	±	±	±		
S.E.	0.016	0.022	0.020	0.354		
Calcium	(a)	(a)	(b)	(b)		
Mean	14.736	17.758	25.501	22.642	28.42	0.000
	±	±	±	±		
S.E.	0.237	0.260	1.766	0.194		

Each value represents mean ±SE of six observations
** denote significant at 1% level
Concentration in order a<b<c

The concentrations of cobalt, selenium and zinc in the different strobilar regions of A.lahorea and host serum were summarized and given in Table II A & Figure I (D, E & F). The highest content of cobalt level was noticed in the metabolically active immature region and lowest level was observed in gravid region and host serum. A consistent decrease of cobalt level from the region of growth up to the region of senescence was markedly noticed which was found to be statistically significant (P=0.01). Similar to cobalt a steady significant decrease in the content of selenium was observed in the anterio posterior direction with the highest content in the anterior immature region and lowest in the posterior gravid region that was found to be statistically significant (P=0.01). The selenium and zinc content of the host serum was found to be higher than the parasite regions. Similar to selenium and cobalt concentration highest content of zinc was revealed in immature and lowest in the gravid region. The percent water content in the different regions of *A.lahorea* shows no marked variation (Table II B).

TABLE IIA

Concentration of cobalt, selenium and zinc in the proglottides of adult cestode *Avitellina lahorea* and host (sheep) serum (ppm/µg/ml)

	Immature	Mature	Gravid	Serum	F-value	P-value
Cobalt	(b)	(a)	(a)	(a)		
Mean	0.029	0.011	0.007	0.015	16.02	0.000**
	±	±	±	±		
S.E.	0.004	0.000	0.001	0.002		
Selenium	(c)	(b)	(a)	(d)		
Mean	0.379	0.280	0.188	0.526	106.19	0.000**
	±	±	±	±		
S.E.	0.017	0.017	0.006	0.014		
Zinc	(c)	(b)	(a)	(d)		
Mean	0.418	0.314	2.227	0.625	1760.03	0.000
	±	±	±	±		
S.E.	0.004	0.005	0.004	0.002		

Each value represents mean ± SE of six observations
** denote significant at 1% level
Concentration in order a<b<c<d

TABLE II B

Percentage of water content in the proglottides of adult cestode *Avitellina lahorea* and host (sheep) serum (ppm/µg/ml)

	Immature	Mature	Gravid	Serum	F-value	P-value
Water content	(d)	(c)	(a)	(b)		
Mean	92.500	90.133	88.000	88.825	154.23	0.000**
	±	±	±	±		
S.E.	0.146	0.206	0.106	0.158		

Each value represents mean ± SE of six observations
** denote significant at 1% level
Concentration in order a<b<c<d

DISCUSSION

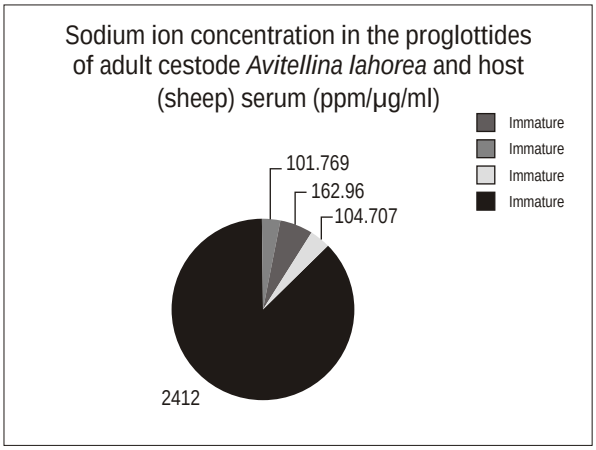
In the present study the higher level ofNa in the mature regions may infer its role in ATPase activity as well as in the transport of glucose necessary for the development. In this context several studies have revealed the glucose transport coupled to Na cations in cestodes like *H.diminuta*. (Read *et al.*, 1974, Pappas *et al.*, 1974 and Uglem, 1976). The Na levels in the different region of *A.lahorea* also imply their importance in the transport of aminoacids and glucose by the above symports viz., Na/glucose and Na/aminoacids (Thomborough, 1995). There is no marked variation in the percent water content in the different regions of *A.lahorea*. The above finding also suggests that the cell electrolyte Na may be involved in the cell volume regulation against the osmotic changes in the luminal environment. The influx of Na along with cations inside the segments may increase their osmolarity and prevent the water loss in case, fluidity of the lumen changes.

The electrolyte potassium level showed minimal concentrations in all the three different regions. Such hypokalemic condition confirms to the documented concept that the living organisms will exhibit hypematremia and hypokalemia in the normal physiological condition (von Brand, 1966).

The increasing gradient of calcium concentration towards the posterior segments agrees with the report of others (Chowdhury and DeRycke, 1977; Chowdhury and Singh, 1978; Farooq and Farooqi, 1984 and Chowdhury and Singh, 1993). This condition confirm the manifold role of Ca inside the cell, as its presence regulates the sodium level, maintenance of inter cellular ionic bridges, neuro-motor functions and several other changes inside the cells (Gingell- *et al*, 1970). The inclusions of inorganic elements in the corpuscles also include calcium. Hence the increase of Ca along anterio-posterior gradient in *A.lahorea* observed in the present study may imply that the Ca may be meant for organization and reorganization processes of adult cestodes as suggested by Chowdhury and DeRycke (1977) and Chowdhury and Singh (1978).

Among helminths the distribution of cobalt was found to be highest in cestodes. In the present study, the cobalt concentration remains highest in the immature regions and significantly decreased in the mature and gravid regions. Similar to zinc, cobalt also maintains a higher concentration than that found in the host serum. The above higher concentration may be derived from both the host tissues as well as from the luminal contents of absorption. As the ruminants (sheep host) contain vitamin 812 in which cobalt being a part, the high level of cobalt in the parasite might have been derived from the host vitamins.

Studies on selenium content in the cestodes revealed



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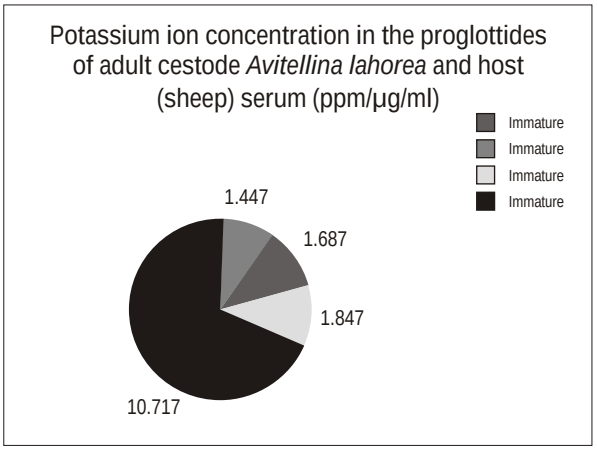

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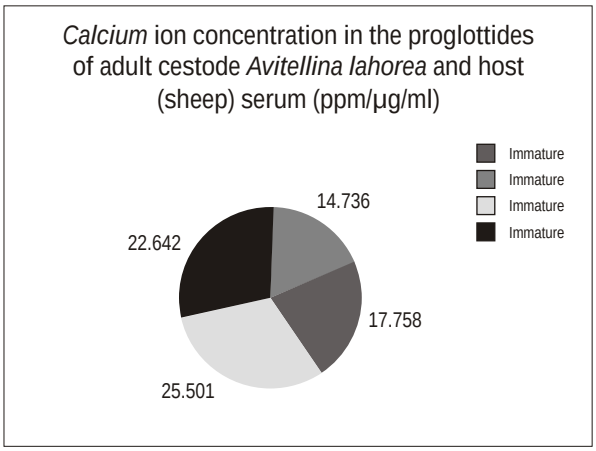

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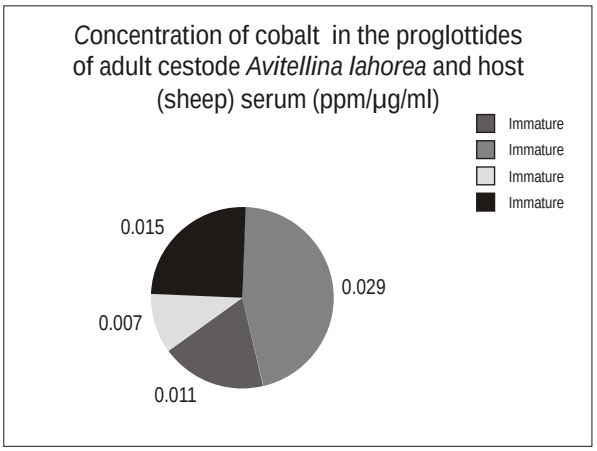

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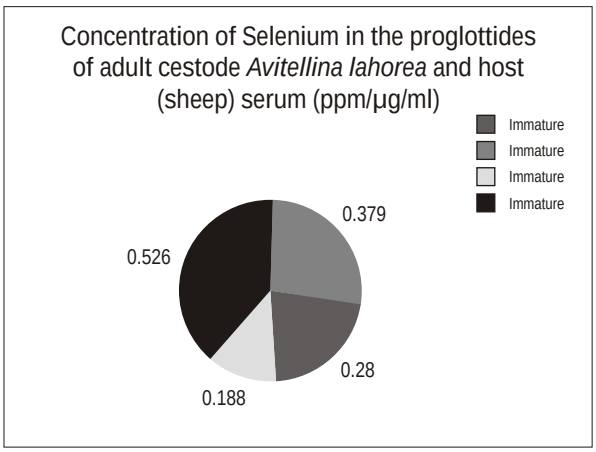

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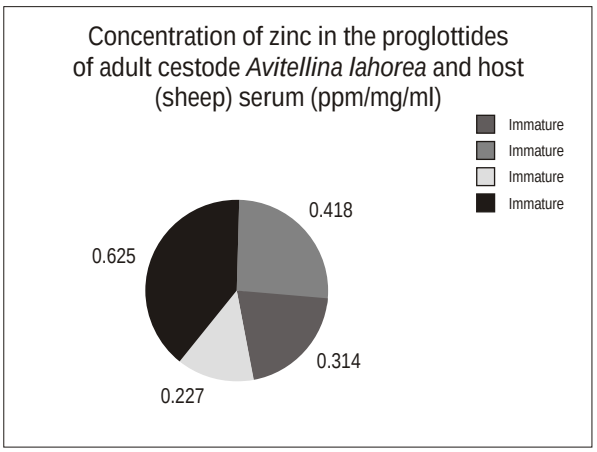

F

Figure 1

that the parasitic somatic tissue contained slightly lesser selenium levels than did the host somatic tissues (Riggs *et al.,* 1987). But Sures *et al.* (1997) also revealed the higher selenium deposition in the gravid proglottides than immature regions. The present result on the selenium content *A.lahorea,* in different proglottides region is similar to that of *B. archeilognathi* (Riggs *et al.,* 1987), in the distribution pattern, compared with the hosts. As it is well known that selenium plays a vital role in the sequestration of free radicals and in the cellular antioxidant defense system, the concentration of selenium in the parasite may be affording a protective function to the parasite against such metabolites in the host body. It is also of interest to note that higher the selenium content showing higher values in the growing and mature regions as compared to the gravid regions seem to indicate its indirect role in immunogenecity which is bound to decline during ageing processes.

In the present study the zinc was found in all the different developmental regions of *A.lahorea.* The data also reveal that its concentration is more in the immature and mature regions when compared to the gravid regions. Such high concentration of zinc inside the regions of the parasite may be attributed to the presence of the metabolites like protein, ammo acids and unsaturated fatty acids which are suggested to enhance the zinc absorption (Sandstrom and Lonnerdal, 1989). The lower level of zinc in the gravid regions may be attributed to ageing process as has been suggested by Sandstrom and Lonnerdal (1989). Similar results were reported by Chowdhury and Singh (1989) in *Taenia hydatigena* and in other cestodes (Nadakal and Nair, 1982 and Chowdhury and Singh, 1993). Considering the above references and also the present results it may be apt to suggest that zinc represents an important element involved in altering the genetic potential of cells or tissues and culminating in growth and differentiation. A similar hypothesis has already been advanced regarding the zinc's function in mammalian system (Chester, 1974). Manifold functions that are attributed to zinc in the biochemistry of life include cellular respiration, protein synthesis, synthesis of RNA and DNA, utilization of nitrogen and sulphur. Zinc also plays a significant role in cell division and growth, spermatogenesis and ova formation, integrity of membranes, wound healing, efficiency of immune systems, sensory responses etc. (Fujioka and Liberman, 1964 and Chowdhury and Singh, 1989).

The results of the present study on different electrolytes in *A.lahorea,* in relation to the differentiation of segments imply that each and every individual electrolyte shows significant variation between the immature, mature and gravid proglottid segments. The differential requirement of trace elements is also made obvious. Hence the electrolytes/ trace elements are as important as any other metabolite for the growth and development remains tenable.

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Effect of Dextran and Polyvinylpyrrolidone on the activity of enzymes released extracellulary by *Trypanosoma brucei*

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Trypanosoma brucei **releases the enzymes acid phosphatase and peptidase extracellularly into phosphate/saline/glucose (PSG) pH 8.0 buffer, in which the organism is incubated in *vitro* at 4°c for 5-30 minutes with lysosomotropic compounds dextran and polyvinylpyrrolidone (PVP). The effect of these compounds was evaluated on the activity of the two enzymes. Both compounds promoted a time-dependent release of acid phosphatase but inhibited the release of the peptidase. While dextran had no lethal effect on the survival of the trypanosomes after 30 minutes of incubation, PVP killed most of the parasites after 15 minutes of exposure.**

Key words : Acid Phosphatase, Dextran, Enzyme Release, Peptidase, Proteases, PVP, *Trypanosoma brucei*

INTRODUCTION

Trypanosomes are flagellated protozoan parasites of medical and veterinary importance. In man and domestic animals they exhibit varying degree of pathogenicity. Chemotherapy is still regarded as the most promising approach to the treatment and control of African trypanosomiasis. Endocytosis-Exocytosis is a cellular process that occurs widely in eukaryotic cells. It involves sequential formation and fusion of membrane-bound vesicles, during which time, the macromolecules involved are sequestered or compartmentalized in vesicles. This is an important feature that facilitates direct transfer of substances between the outer and inner cell environment (Silverstain *et al.,* 1989' Blumenthal 1987; Albert *et al*., 1989).

The processes of endocytosis-exocytosis occur through the flagellar pocket in many species of trypanosomes (Opperdoes *et al.,* 1987; Langreth and Balber 1975) for the secretion of proteases and nutrient uptake (Knowles *et al.,* 1987; Nwagwu *et al.,* 1988; Huet *et al.,* 1992, Webster and Marsh 1986; Okenu and Opara, 1996). The released protease(s) have been implicated in the pathology of the disease such as platelet aggregation, blood coagulation and thrombocytopaenia (Nwagwu *et al.,* 1989; Davis *et al.,* 1974). Considering the importance of endocytosis-exocytosis in trypanosomes, inhibiting or inducing these processes would be a step in the right direction towards the development of effective chemo-therapeutic agents and/or vaccines against trypanosomiasis. We had, previously shown that the released proteases include proteolytic bands of Mr 200, 106, 93, 63, 48, 40 and 25 KDa among which are cysteine and trypsin-like serine proteases as well as acid phosphatase (Okenu and Opara 1996; Ekpo *et al.,* 1996 and Steiger *et al*, 1980). We have also demonstrated that lysosomotropic drugs such as chloroquine, retinal, swainsonine, dextran and polyvinylpyrrolidone (PVP) inhibit or activate the release of these enzymes (Opara *et al.,* 1994; Opara and Okenu 1996). In this report we have evaluated the effect of the uptake of dextran and PVP on the survival of trypanosomes and the effect of these compounds on the activity of two of the enzymes released by T. *brucei* namely acid phosphatase and peptidase.

MATERIALS AND METHODS

Fluorogenic peptide substrates with a carboxyl-terminal 7-amino-4-trifluoromethyl Coumarin (AFC) were purchased from Enzyme Systems Product Livermore, Ca, (USA) while 4-methylumbelliferyl

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phosphate (4-MUP), 4-methylumbelliferone, Dextran and PVP were purchased from Sigma Chemicals Co., St. Louis (USA). The Kontron's Spectro-Fluorometer SFM was used to determine relative fluorescence. All other reagents used were of the highest quality, commercially available.

EXPERIMENTAL DESIGN

Trypanosoma brucei (stock Gboko CT 70) was used for this study. Six Wister rats weighing between 200-300g were inoculated intraperitoneally with 10^6 trypanosomes per rat. After 72 hours or peak parasitaemia, blood was withdrawn from the animals by cardiac puncture and trypanosomes were isolated as described previously (Lanham and Godfrey, 1970).

Trypanosomes were incubated at a concentration of 1 x 10^8 organisms/ml phosphate-saline-glucose (PSG) buffer, PH 8.0 at 4° for 30 mins, an optimal in *vitro* condition which maintains the integrity of the organisms (Okenu and Opara, 1996). They were pelleted immediately after incubation at 3,000g for 10 min. and survival i.e the number of actively motile organisms was determined by counting with a haemocytometer. The supernatant fraction obtained after pelleting the trypanosomes served as the source of Trypanosome Affected Supernatant (TAS), and was concentrated using Amicon's Centricon - 10 micro concentrator and dialysed in 0.1m Tris-HCI, pH 6.8. The protein content of TAS was determined by the method of Bradford (1976) with Bovine Serum Albumin as standard. The reaction mixture comprised of 800 µl of TAS and 200 µl of Bio-Rad dye concentrate. Absorbance was determined at 595 nM.

INCUBATION OF TRYPANOSOMES WITH DEXTRAN AND PVP

Trypanosomes (1 x $l0^8$ml) were incubated in PSG, pH 8.0 with a solution of dextran or PVP at 4°C for 35 min. the concentration of dextran and PVP was kept at 1%. The control consisted of an equivalent concentration of trypanosomes incubated in PSG, pH 8.0 at 4°C for 35 min, without dextran and PVP.

SURVIVAL OF TRYPANOSOMES

An aliquot of the incubation mixture was transferred to a haemocytometer chamber and observed under the microscope to ascertain whether the trypanosomes were actively motile, sluggish or dead. This was done before and after incubation with and without the compounds. The number of live and actively motile trypanosomes was determined as described previously by Okenu and Opara (1996).

DETERMINATION OF ENZYME ACTIVITY IN TAS AFTER INCUBATION WITH DEXTRAN AND PVP

i) Peptidase

The activity of the released peptidase was determined as described by Steiger *et al.* (1980) using valine-lysine-arginine-7-amino-4-trifluoro-methyl coumarin (VKKK-AFRC) as the substrate. The reaction mixture contained 200µl of TAS, 1790 µl of 100 nM Tris-HCI, pH 7.0 and 10 µl of substrate, which started the reaction.

The mixture was incubated for 2 hours at 28°C. The control contained only 10 µl of substrate and Tris-HCI buffer, TAS was omitted. The absorbance at 368 nM excitation and 494 emission was determined spectrofluoro-metrically. The control reaction mixtures were prepared to allow for non-enzymatic hydrolysis of the substance.

ii) Acid Phosphatase

The activity of acid phosphatase in TAS was determined as described by Steiger *et al.* (1980) using 4-methylumbelliferyl phosphate (4-MUP) as the substrate. The reaction mixture contained 500 µl of 4-MUP, 500 µl of 0.05M sodium acetate- acetic acid buffer, pH 5.0 and 1000 µl of concentrated and dialysed TAS added to start the reaction. The mixture was incubated at 37°C for 10 min., at the end of which 3ml glycine-NaOH buffer, pH 10.4 was added to stop the reaction.

The control mixture contained 500 µl of substrate and 500 µl of buffer but no TAS. Serial dilutions of 4-MUP from 0.002-µmole-0.2µmole per ml in acetate buffer with 3ml glycerine-NaOH were prepared and used as standard for measuring the amount of the product in the reaction. The relative fluorescence at 364 nM excitation and 448 emission of the assay mixture was determined spectrofluorometrically.

RESULTS

The survival of the trypanosomes, the activities of peptidase and acid-phophatase are presented in Table 1 and figure 1. Table 1 shows that 1% (w/v) dextran had no effect on the trypanosomes after 30 min. of incubation while PVP killed some trypanosomes within 15 min. and suppressed motility of the survivors. Percentage survival of 95% at 15 min. and 46% at 30 min was observed. Fig. 1 and Table 1 shows that dextran inhibited the activity of peptidase from 0.90 at 5 min to 0.30 after 30 min of incubation while PVP blocked peptidase activity almost completely from 0.16 at 5 min to 0.10 after 30 min of incubation. Dextran and PVP promoted the activity of acid phosphatase from 1.05 at 5 min to 2.61 and 2.30 at 30 min of incubation respectively.

DISCUSSION

The compounds used in this study, dextran and PVP are non-digestible polysaccharides, which are localized in the lysosomes of trypanosomes when taken up (Deams *et al.*, 1961; De Duve *et al.*, 1974; Fairlamb and Bowmen 1980).

The results showed that 1% (w/v) dextran had no effect on trypanosomes after incubation for 30 min, but PVP at a concentration of 10% suppressed activity and actually killed some trypanosomes after 15 min of incubation. This could be due to an overloading effect on the lysosomes resulting from a rapid uptake of the polysaccharide without a compensatory rapid clearance rate in the form of exocytosis. We had documented a similar result elsewhere (Opara and Okenu, 1996).

Under the influence of dextran there was a time-dependent inhibition of peptidase release, but with PVP there was a complete inhibition independent of the time of incubation. It is possible that these compounds either inhibited the actual release of peptidase by trypanosomes into the medium or they inhibited the activity of the enzymes already released in TAS. There was an increase in protein content of TAS when the compounds were incubated with TAS suggesting that some of these protein released could be

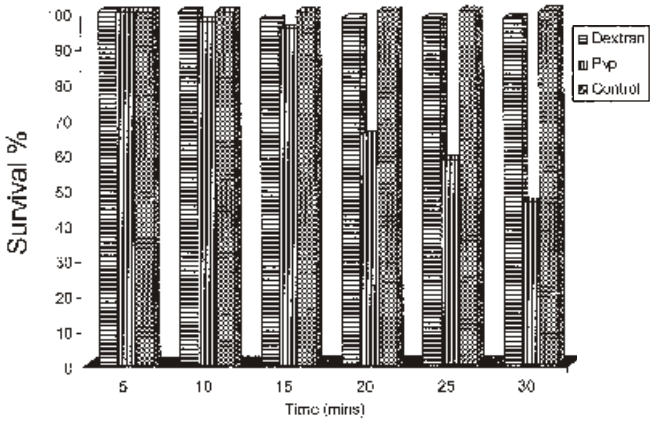

Fig.1:Effect of dextran and PVP on the survival (%) of Tryanosomes

Table I : Effect of Dextran and PVP on Peptidase and Acid Phosphatase Activity of Trypanosome Affected Supernatant

	Peptidase Acivity			Acid Phosphatase Activity		
Time (Min)	Dextran	PVP	Control	Dextran	PVP	Control
5	0.90±0.01	0.16±0.00	1.20+0.01	1.05+0.01	1.05±0.02	1.05±0.00
10	0.81±0.00	0.16±0.00	1.15±0.02	1.30±0.01	1.49±0.01	1.49±0.01
15	0.63±0.03	0.15±0.01	1.25±0.00	1.50±0.02	2.00±0.2	1.49±0.01
20	0.50±0.01	0.11±0.02	1.70±0.01	1.80±0.03	2.04±0.02	1.60±0.02
25	0.45±0.00	0.11±0.01	2.10±0.02	2.00±0.01	2.20±0.00	1.80±0.02
30	0.30±0.02	0.10±0.01	2.61±0.01	2.61±0.02	2.30±0.01	2.18±0.03

The results represent the mean and SD of peptidase and acid phsophatase released by Trypanosomes in three separate experiments performed in duplicates.

proteases. This observation is in accord with the documented report of Evans and Ellis (1983), Nwagwu *et al.* (1988, 1989) and Huet *et al.* (1992) that T. *brucei* release protase extracellularly. Gordon *et al.* (1974) observed that endocytosis of latex, an indigestible particle, by macrophages stimulated increased release of plasminogen, a neutral protease. It may therefore be speculated that the uptake of dextran and PVC may either stimulate the release of protease with other protein or inhibit the activity of the enzymes after they had been released into TAS. Axline and Cohn (1970) had earlier shown that ingestion of a non-digestible particle PolyvinylToluene inhibited lysosomal enzyme activity whereas activity rose to a peak when a digestible material, such as sheep erythrocyte was endocytosed by macrophage. The possibility of inhibition of actual protease(s) released in this study is not excluded.

Dextran and PVP stimulated acid phosphatase activity. A time-dependent increase in activity was observed with dextran.

Survivability study showed that trypanosomes incubated with dextran were still active after 30 min and it is possible that active exocytosis might have been occurring as the compound was internalized. This may be responsible for the high enzymes activity of the TAS incubated with dextran. Certainly, in PVP the loss of viability of the trypanosomes observed after 15 min hindered enzyme release and a drop in the activity.

The difference in the effect of dextran and PVP on the activity of the two enzymes is striking. While the activity of peptidase was inhibited, acid phosphatase activity was promoted. This difference may be due to difference in the rate of exocytosis of the respective enzymes. The location of acid phosphatase on the membrane of the flagellar pocket (Steiger *et at.* 1980) which is the site of endocytosis-exocytosis (Opperdoes *et al.* 1987) may possible facilitate it being exocytosed much more readily in response to an induced and sustained endocytosis of non-digestible materials like dextran and PVP.

Since proteases released by T. *brucci* have been implicated in platelet aggregation, blood coagulation and thrombocytopaenia, inhibition of protease release could therefore be useful in studying these complications. Also, the effect of PVP on survival of trypanosomes provides intriguing information as to the possibility of using it or its analogue as a potent inhibitor. It would be worthwhile that a complete understanding of these processes and the application of current information would strengthen the efforts towards developing new chemotherapeutic agents against trypanosomiasis.

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Short Communication

On a new species of the genus *Hexacanalis* Perrenoud (1931) from the spiral valve of *Dicerobatis erregoodoo*

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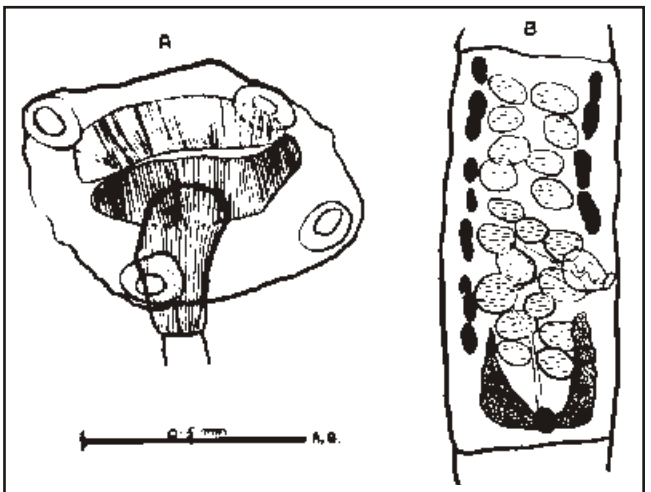
The present communication deals with a new species of the genus *Hexacanalis* Perrenoud (1931) *vzi. Hexacanalis govindi* n.sp. collected from spiral valve of *Dicerobatis eregoodoo.* The species differ from all other known species of the genus in having scolex oval, compressed on all sides, the testes are of medium size, oval in shape, pre-ovarian 20 in numbers, cirrus pouch medium, short, oval, cirrus is coiled, ovary 'U' shaped, compact, vagina is thin tube, the viteilaria are follicular in single row.

KEY WORDS : Cestode, *Hexacanalis*, Marine fish, *Trygon*

The genus *Hexacanalis* Perrenowd (1931) was recorded by Southwell (1911) as *cephalobothrium* with its type species *C. abruptum* from *Pteroplates micura* and *Dasybatus kuhli* at Ceylon (Trivandum coast) and he also reported another species *C.variabilis* from *Pristis cuspidatus* and *Dasybatus kuhli* at Ceylon. Perrenowd (1913) synomysied these species and created a new genus *Hexacanalis* with its type species *H. abruptus* and the name *H. variabilis* was proposed for *C. variabilis*. He recorded the genus from the same locality (*Ceylon*). Shinde and Deshmukh (1979) reported two new species i.e. *H. zugei* and *H. Yamaguti* from *Trygon zugei* and *Dicerobatis eregoodoo* from Ratnagiri and Veraval (West coast of India) respectively. Shinde et al (1982) added two new species i.e. *H. trygoni* and *H. ratnagirensis* from *Trygon zugei* Later on Murlidhar (1986) added one new species i.e. *H. indraji* from *Trygon sephen* at Kakinada (A.P.) East coast of India.



Hexacanalis govindi n.sp.
A - Scolex B - Mature Segment

Fifteen specimens of the cestode parasites were obtained from the spiral valve of *Dicerobatis eregoodoo,* at Veraval, Gujarat (West coast of India).

The worms were collected washed and fixed in 4% formalin, stained with Harris haematoxylene, dehydrated cleared in xylene, mounted in Canada balsum. Drawings were made with the aid of Camera lucida, identification was carried out with the help of systema Helminthum Vol-II (Yamaguti, 1960) all measurements are in millimeters.

***Hexacanalis govindi* n.sp.**

Measurements in mm		
Organs	**Length**	**Width**
Scolex	0.514	0.610
Sucker	0.270	0.145
Neck	0.329	0.164 to 0.106
Mature segment	0.853	0.388
Testes	0.106 to 0.062	0.062 to 0.485
Cirrus pouch	0.121	0.081
Cirrus	0.265	0.014
Vas deferens	0.101	0.009
Genital pore	0.043	0.019
Ovary	0.270 to 0.200	0.072 to 0.0029
Vagina	0.450	0.0009
Ootype	0.052	0.052
Vitellana	0.101 to 0.032	0.033 to 0.019

Diagonosis and Relationships

1. The worm under discussion differs from *H. abrupts* which is having the scolex 1.3 X 1.2, neck absent, testes 62 to 73 in number, 40 to 45 on aporal side and 22 to 28 on poral side, ovary dumb-bell shaped, without acini, rsceptaculum seminis present and vitellaria follicular with constant globular acini.

2. The present cestode differ from *H. zugei* which is having the testes 42 to 47 in number almost in two fields, in 3 to 4 rows, large, ovary single mass, small, oval compact, genital pores at middle of the segment and vitellaria granular, subcorticular with wide strip.

3. The present worm differs from *H. ratnagiriensis* having scolex 0.5 x 0.91, testes 35 in number, in two lateral fields (18 in poral and 17 in aporal side), ovary single mass, roughly quadrangular, compact, vagina posterior to cirrus pouch and vitellaria granular with wide strips.

In the view of differences, the present form is regarded here to constitute a new species for which the name *Hexacanalis govindi* n.sp. is proposed in honour of Ex. Prof. G.B. Shinde who has contributed a lot in the field of Cestodology.

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Short Communication

Prevalence of *Cysticercus tenuicollis* infection in slaughtered sheep and goats at Kakinada, Andhra Pradesh

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A study was undertaken to know the prevalence of *Cysiticercus tenuicollis* infection in slaughtered sheep and goats at Kakinada, A.P. This was recorded to be 2.36% in sheep and 1.62% in goats with an overall occurrence of 2.34% in both the ruminants. The age wise percentage of occurrence recorded in animals below and above four years of age revealed slightly higher percentage in older animals.

Key words : *Cysticercus tenuicollis*, Goats, Prevalence, Sheep

Cysticercus tenuicollis normally occurs in greater omentum, mesentry and serosal surface of organs of domestic as well as wild ruminants (Soulsby, 1982). Prevalenc of *C.tenuicollis*, has been earlier recorded in sheep and goats in Tamilnadu (Jayathilakan et al., 2000 and Raman and Rajavelu, 2000) and Orissa (Dash et al., 2000) states in India.

In view of protecting sheep and goats from *C.tenuicollis* infection by taking necessary preventive measures a study was undertaken to determine the prevalence of *C.tenuicollis* infection in slaughtered sheep and goats at Kakinada, Andhra Pradesh.

Table I
Species wise prevalence of *C.tenuicollis* infection in sheep and goats

S.No.	Species	No. of animals examined	No. of positives	% of positives
1.	Sheep	20,888	495	2.36
2.	Goats	798	13	1.62
	Total	21,686	508	2.34

X^2 Test Calculated Value (1.84) is less than the table value at 5 percent level (3.84) of significance for 1 degrees of freedom for species wise prevalence of *C.tenuicollis* infection in between sheep and goats.

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The prevalence of *C.tenuicollis* infection was ascertained by examination of cysts found in the omentum, mesentry, peritoneal cavity and liver in the carcasses of sheep and goats slaughtered at municipal house, Kakinada in the regular meat inspection and the identification of cysts of *C.tenuicollis* was done at Animal Health Center, Kakinada on the basis of morphology described by Soulsby (1982) during the year 2001-2001. The prevalence of *C.tenuicollis* infection in relation to species and age was analysed and presented in tables I and II respectively.

Out of 20,888 sheep and 798 goat carcasses examined, 495 and 13 were found positive for *C.tenuicollis* infection in sheep and goats respectively. The overall prevalence of *C.tenuicollis* infection was found as 2.34% in sheep and goats during the study period.

The species wise prevalence was higher in sheep (2.36%) as compared to goats (1.62%). However this was not statistically significant. Jayathilakan et al., (2000) reported *C.tenuicollis* infection in 15.68% and 13.33% of sheep and goats respectively. Soulsby (1982) mentioned that the prevalence of *C.tenuicollis* infection in sheep is high.

The age wise prevalence revealed higher infection in the age group above 4 years (2.42% in sheep and 1.77% in goats) when compared to below 4 years (2.15% in sheep and 1.28% in goats), these, however, are not statistically significant. There is also no significant difference in the prevalence of

Table. II
Age wise prevalence of *C.tenuicollis* infection in sheep and goats

S.No.	Age	No. of animals examined			No. of positives			% of positives		
		Sheep	Goats	Total	Sheep	Goats	Total	Sheep	Goats	Total
1.	Below 4 Years	4,538	234	4,772	98	3	101	2.15	1.28	2.11
2.	Above 4 Years	16,350	564	16,914	397	10	407	2.42	1.77	2.4
	Total	20,888	798	21,686	495	13	508	2.36	1.62	2.34

(1) X^2 test Calculated Value (1.10) is less than the table value at 5 percent level (3.84) of significance for 1 degrees of freedom for age wise prevalence of *C.tenuicollis* infection in sheep

(2) X^2 test Calculated Value (0.03) is less than the table value at 5 percent level (3.84). of significance for 1 degrees of freedom for age wise prevalence of *C.tenuicollis* infection in goats

(3) X^2 test Calculated Value (0.45) is less than the table value at 5 percent level (3.84). of significance for 1 degrees of freedom for prevalence of *C.tenuicollis* infection in between sheep and goats for below 4 Years

(4) X^2 test Calculated Value (0.99) is less than the table value at 5 percent level (3.84). of significance for 1 degrees of freedom for species wise prevalence of *C.tenuicollis* infection in between sheep and goats for above 4 Years

C.tenuicollis infection between two animal species in each age group of below 4Years and above 4Year Lapage (1956) mentioned that *C.tenuicollis* infection is often found in older sheep. This might be due to decrease in resistance in older animals and increase in the chances of exposure to infection with the advancement of age.

Further studies are to be undertaken to determine the extent of damage done i.e., pathogenecity in the organs in which these cysts are present/attached particularly the liver of these small ruminants. A study to be made in human cases as well, simultaneously, to evaluate the public health importance of this metacestode, if any, in this region of A.P.

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Short Communication

Prevalence of Fascioliasis in Goats in Kanpur

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The prevalence of fascioliasis in goats due to *Fasciola gigantica* in Kanpur city of Utter Pardesh was studied between February 2000 to January 2003. On detailed examination of 13259 livers and same number faecal samples from slaughtered goats during the period of 3 years revealed that a total of 487 (3.67%) livers were found infected due to Fasciola gigantica. Year wise infections were recorded, of which 4.99 percent was in the year 2000-2001, 3.10 percent in the year 2001-2002 and 3.72 percent was in the year 2002-2003. The highest prevalence was noted during rainy seasons in the present study.

KEY WORD : *Fasciola gigantica.* Goats, Prevalence, Season.

Goats are mainly used as a source of meat, milk and wool. The losses of such precious animals are the economic losses of the nation. According to Arora (1967) in Bareilly, the losses were calculated to be Rs.4.178 million due to rejection of livers. Boray, (1985) estimated the economic losses due to fascioliasis to be about U.S.$ 2000 Million per annum world over. Punchauri *et al.*, (1988) reported 22.31 percent loss of goat livers due to fascioliasis. Liver disease in Indian goats is of prime concerned not only for the health of people of Kanpur, but also for that of the nation. Keeping in view the role of fascioliasis infection in liver disease, the present study was conducted to determine the prevalence in goat livers in the City of Kanpur.

The postmortem examination of 13259 goats between the age of one and two years was carried to assess the prevalence of fascioliasis in these animals. Liver and faecal samples of slaughtered animals were collected randomly and examined for the presence of the flukes and their eggs.

Parasitological techniques : Flukes recovered from each of infected liver were counted and morphologically identified as *FascioJa gigantica.* The faecal samples were examined by direct smear, sedimentation technique and Zinc sulphate floatation technique for the presence of fluke eggs as per the standard procedure described by Sastry (2000). The trematode eggs were identified on the basis of morphological details described for *Fasciola gigantica* eggs by Yamaguti, (1975). Counting of eggs was done by Mc. Master eggs counting technique, Kelly (1974).

The result indicated that the prevalence of fascioliasis caused by *Fasciola gigantica* in goats in Kanpur between Feb. 2000 to Jan. 2003 was 3.67 percent. Th; peak incidence of infestation in our study was observed during the rainy seasons (6.19%) in 2001, (5.69%) in 2002, (6.25%) in 2003. Moderates percentage of infection was observed in summer (4.44%) in 2001, (4.18%) in 2002, (4.25%) in 2003, and low percentage during winter (3.89%) in 2001, (2.40%) in 2002, (2.58%) in 2003 (Table).

The high percentage of infection during rainy seasons might be attributed to contamination of pasture with metacercaria and increased grazing time.

Prevalence of fascioiiasis in goats and their seasonal variations of each year have been shown in Table. The seasonal prevalence of fascioiiasis in the three consecutive years was observed as 3.67 percent, which is in close proximity with that observed by Singh, R. et al., (1993) reported 6.40 percent in Ranchi, Bihar, India during a 2 year period. In the present study incidence was more prevalent 4.99 percent in a year 2000-2001 than 2001-2002 and 2002-2003. The highest season wise prevalence was observed during the rainy season (August to October). This is in agreement with the reports of Singh B.P. (2001). This season encourages the propagation of snail population, which serves as an intermediate host for

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Table

Prevalence of Fascioliasis in Goats between Feb.2000 to Jan. 2003

SEASONS	YEAR									SEASONAL
	2000-2001			2001-2002			2002-2003			
	NGE	NGI	%	NGE	NGI	%	NGE	NGI	%	Percentage
SPRING (Feb. to April)	270	9	3.33	755	23	3.04	2010	65	3.23	3.19
SUMMER (May to July)	225	10	4.44	382	16	4.18	1010	43	4.25	4.26
RAINY (Aug. to Oct.)	1340	83	6.19	1107	63	5.69	320	20	6.25	5.99
WINTER (Nov. to Jan.)	950	37	3.89	4580	110	2.40	310	8	2.58	2.65
TOTAL	2785	139	4.99	6824	212	3.10	3650	136	3.72	3.67

1. NGE - Number of goats examined 2785+6824+3650 = 13259
2. NGI - Number of goats infected 139+212+136 = 487 (3.67%)

cercaria larvae. The lowest prevalence was observed during winter season. It seems probable there! ore that the infective stages (cercaria, metacercaria,infection) die at lowest temperature. While in Kashmir, the prevalence of fascioiiasis is found to be fairly high about 54 percent during winter and lowest (33 percent) during summer (Singh,B.P.2001). This difference indicates the adaptability according to the climatic conditions existing in different geographical areas.

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CONGRATULATIONS !

Dr. S.K. GUPTA, Associate Professor, Deptt. of Vety. Parasitology, CCS HAU, Hisar has been awarded the Best Teacher Award, for the year 2001-2002 by CCS Haryana Agricultural University, Hisar in recognition of his significant contribution in undergraduate and postgraduate teaching in the field. This award was given to him by His Excellency Dr. A.P.J. Abdul Kalam, President of India on 19.4.2003 during the 22nd Convocation of the University.

BOOK REVIEW

The book 'Remembering Dr. S.C. Dutt' edited by Prof. M.C. Agarwal, Head, Deptt. of Parasitology, College of Verinary Science and Animal Husbandary, Jawaharlal Nehru Krishi Vishwa Vidayalaya, Jabalpur, India is a compilation of biographical information about Late Dr. S.C. Dutt, the eminent parasitologist. The book also contains a compilation of Dr. S.C. Dutt Memorial lectures and invited articles on 'Present Problems in Parasitology' The book is an inspiration for young scientists to follow the path of Prof. Satish Chandra Dutt and is a nice tribute by the students to their teacher.

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CONTENTS

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