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Review

Hydatid fluid as a clinical specimen for the aetiological diagnosis of a suspected hydatid cyst.

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Hydatidosis is mainly diagnosed by radio imaging & anti hydatid antibody detection. The radiological picture of hydatid cyst may mimic other pathologies & serology may be inconclusive. Fluid may at times be aspirated for establishing the etiological diagnosis of a suspected hydatid cyst or may be available postoperatively. The microscopy of the wet mount of aspirated cystic hydatid fluid would be simples test that could be performed to confirm hydatid aetiology of a suspected cyst in a patient treated in hospitals attached with poorly equipped laboratories such as in a district health centre hospital. Detection of specific hydatid antigen in the fluid is an approach alternate to hydatid fluid microscopy. In reference or tertialry level hospitals, the antigen detection in cystic fluid either by enzyme-linked immunosorbent assay (ELISA), counter-current immuno electrophoresis (CIEP) or Co-agglutination (Co-A) could be used as a routine diagnostic test in the laboratories to confirm the hydatid cysts which are otherwise negative by fluid microscopy. Examination of hydatid fluid for diagnosis of CE offers a few advantages. First, microscopy of fluid or Co-A test for detection of antigen is the most rapid 5 min test. Second, examination of cystic fluid is most specific for CE, if the test is positive, no other test is required. Third, the tests are most economical and simple. Both the tests (microscopy and Co-A test) do not require any hi-tech instruments and can be performed with minimum technical expertise available in poorly equipped laboratories. However, extreme caution should be observed in aspiration of hydatid fluid due to risk of anaphylaxis & dissemination.

Keywords : cystic echinococcosis, diagnostic aspiration, hydatid fluid, serodiagnosis.

INTRODUCTION

Cystic echinococcosis (CE) or hydatid disease in humans is a zoonotic infection caused by the larval stage *(hydatid cyst)* of the dog tapeworm, *Echinococcus granulosus*. The condition is endemic in sheep-rearing areas of the Australia, South America, the Mediterranean basin and the former USSR. The disease has also been reported from different parts of India, including non-sheep rearing parts of the country. The annual incidence of CE in different parts of the world has been reported to vary from <1 to 220 per 100,000 persons in various endemic areas. The reported mortality rate due to the disease varies between 2 to 4% (WHO working group, 1996). In India, CE in humans is being increasingly recognized and reported from most parts of the country. This is mainly due to increased detection of the cases as a result of improved imaging tecniques and immunodiagnostic methods. CE in humans has been reported from most parts of India (Dwivedi *et al*, 2002; Rajagopal and Bishwas, 2002; Konar *et al*, 2001; Sodhi *et al*, 2000; Sekar *et al*, 1988;Upadhaya *et al*, 1974; Gupta *et al*, 1966; Purohit *et al*, 1999; Sinha and Sharma, 2001; Thomas *et al*, 1996; Wagholikar and Sikora, 2001; Sandhu *et al*, 2001; Bhat *et al*, 2002; Pushparaj *et al*, 2001; Paul *et al*, 2002; Khursheed *et al*, 2001; Choudhuri *et al*, 1989; Kakrani *et al*, 2000; Ratan *et al*, 1994; Sathyanarayanan *et al*, 1996; Beg and Mansoor, 2002; Usha *et al*, 1994; Pillai and Vasudevan, 1996; Yadav *et al;* 1999; Das and Choudhury, 2002; Parija *et al*, 1983, 1987; Vamsy *et al*, 1991; Parija, 2004*)* CE in humans is acquired by coming in contact with infected dogs harbouring adult *E.granulosus* in their intestine. The eggs of the parasite are the source of infection especially those present on

perianal hair, muzzle and paws. The infection is transmitted orally by ingestion of these eggs from the hands, food or water.

The presence of unilocular hydatid cysts in a tissue or organ is the main pathology of the CE in humans. While, in a majority of cases the condition remains asymptomatic throughout the life, cysts may produce clinical symptoms, often when they attain sufficient size to cause disturbances by exerting mechanical pressure in the surrounding tissues. The growth rate of cysts is highly variable. On an average the cysts grow at a rate of approximately 1cm - 1.5 cm per year and may depend on strain differences. During the natural course of infection, the fate of the hydatid cysts is variable. Some cysts may grow to a certain size and then continue to remain without producing any pathological change for many years. Other cysts may rupture spontaneously or collapse and disappear completely .Hydatid cyst is primarily responsible for the pathogenesis of the disease. CE in human is mostly asymptomatic through out the life in a majority of cases. The hydatid cysts in lung, liver or other organs may be detected accidentally during x-ray examination, body scanning, surgery, or for other clinical reasons. In symptomatic cases, the clinical manifestations are highly variable and non-specific and depend on the organ infected with the cyst, number of the cysts, size of the cysts and their sites within the involved organ, interaction between the expanding cysts and adjacent organ, and complications caused by rupture of cysts. Secondary echinococcosis or disseminated hydatid disease, and notable feature that occur due to the rupture of the primary cyst or by spillage during surgery have also been reported. Moreover, incubation period varies from few months to several years. Since clinical symptoms of the disease are nonspecific and variable, the clinical diagnosis of the condition may be confirmed by imaging techniques (Parija, 2004) and anti hydatid antibody detection. The radiological picture of hydatid cyst may mimic other pathologies & serology for antibody detection may be inconclusive

Hydatid fluid as a clinical specimen

The serum, urine and fluid aspirated from a cyst are used for the diagnosis of CE. Serum is the most frequently used clinical specimen for the demonstration of either antibodies or antigens. A variety of immunoassays (Parija, 1998a) to diagnose CE have been used for the detection of serum hydatid antibodies or antigens with variable results. Most recently, use of urine as a clinical specimen alternative to the serum has been reported (Parija *et al*, 1997; Parija, 1998b) for detection of excreted urinary hydatid antigen. In addition to these specimens, hydatid fluid aspirated from cysts is now becoming increasingly used for establishing the aetiological diagnosis of a suspected hydatid cyst, thereby establishing the specific diagnosis of CE (Craig *et al*, 1986; Parija, 2004; Ravinder and Parija, 1997).

Hydatid fluid is aspirated from hydatid cysts, the majority of which are commonly found in the lungs and liver, where they are surrounded by a pericyst formed by the host tissues. The pericyst encompasses endocyst, which encompasses an outer laminated acellular layer is lined by a thin cellular germinal membrane .The germinal membrane gives rise to brood capsules within which protoscolices develop. The cavity of the cyst is filled with a clear, colour less fluid, containing a large number of brood capsules and free protoscolices.

The hydatid fluid is slightly acidic (pH 6.7), has a low specific gravity (1 - 1.010) and is rich in electrolytes such as sodium chloride, sodium sulphate and salts of succinic acid. A large number of brood capsules and free protoscolices, forming ' hydatid sand ' are found in the fluid The hydatid fluid is antigenic and highly toxic, if ruptured within the human body, can cause anaphylaxis or secondary echinococcosis (Parija, 2004). The fluid is collected by puncture of a cyst and then aspirating the fluid from it.

During the treatment of hydatid cysts by PAIR (puncture-aspiration-injection-reaspiration), the fluid is collected by puncture of the cyst under the supervision of ultrasound or CT. Treatment of hydatid cysts particularly those present in the liver by PAIR has been found to be a promising alternate to surgery (WHO working Group, 1996). The method is carried out by puncture of the cyst under ultrasound guidance, aspiration of cystic fluid, injection of protoscolicidal agents such as 95% alcohol or 20% saturated salt

solution for at least 15 min and then re-aspiration of cystic fluid. This method has been recommended for treatment of hydatid cysts especially for inoperable cysts and for cases with a high surgical risk.

Diagnostic aspiration of cystic fluid is usually carried out in: (1) cysts suspected to be hydatid; (2) cysts clinically thought to be a condition other than CE; and (3) cysts that are removed during surgery from an organ such as the liver. The diagnostic aspiration is carried out to confirm the hydatid aetiology of a suspected cyst. It distinguishes hydatid cyst from those of congenital hepatic cysts, congenital dilatation of the hepatic bile ducts, and other cystic lesions found in the liver.

Microscopy of hydatid fluid

Demonstration of daughter cysts, scolices, hooklets or the fragments of laminated membrane in the centrifuged deposit of hydatid fluid by light microscopy establishes the hydatid aetiology of a suspected cyst (Parija, 2004). Cystic fluid is examined for scolices and hooklets either by making a direct wet mount or preparing a wet mount with a temporary staining agent. The hydatid fluid is centrifuged and the deposit examined in the direct wet mount for scolices and hooklets, which are identified by their typical morphological features. However, since these scolices and hooklets appear transparent and colourless, with poor visibility of internal structures, these are frequently missed in the examination of direct wet mounts. To enhance their visibility, temporary staining agents such as methylene blue or lacto-phenol cotton blue (LPCB) have been used .The LPCB, widely used in a mycology laboratory for wet mount preparation of clinical specimens for fungi, fungal elements, and also used in stool microscopy for intestinal helminthic ova and protozoal cysts (Parija and Prabhakar, 1995) can be used in the wet mount preparation of hydatid fluid (Parija and Ravinder , 1997) . The LPCB wet mount of hydatid fluid is prepared by mixing a drop of LPCB solution with a drop of centrifuged deposit of the hydatid fluid on a microscopic glass slide and covering with a coverslip. The wet mount is examined microscopically initially by using a low power (10 x objectives) and then a high power (40 x objectives). In the LPCB wet mount, the scolices and hooklets are intact and stain deep blue, and can easily be detected and identified.

Other permanent stains have been developed for demonstrating scolices or hooklets (Clave *et al* ,1999) including acid-fast stain (Brundefet, 1973) , Wheatly trichrome (Wheatley ,1951) and Ryan trichrome blue stain (Ryan *et al* , 1993) . The acid-fast stained smear examined under transmitted light shows irregularly stained hooklets, and when examined with a filter set for fluorescence stain shows bright orange-yellow hook lets. Unstained methanol-fixed hooklets appear blue under UV (F-1) and green under violet (F-2) and blue-violet (F-3) light. Hook lets stain light pink in Wheatly trichrome stain, while they stain deep red when stained with Ryan trichrome blue stain (Clavel *et al* , 1999).

The microscopy of cystic fluid has the advantages of being rapid and specific, and is the gold standard in establishing the diagnosis of CE. The procedure, nevertheless, has a few inherent disadvantages. In some situations, protoscolices may not be present as seen in the acephalo cyst or even if present may not be aspirated in the cystic fluid. This means that the procedure sometimes fails to confirm hydatid aetiology of many cysts. Therefore, microscopy alone may not be always sufficient to confirm the aetiological diagnosis of the cyst (Ravinder and Parija, 1997). In such cases, the demonstration of hydatid antigen in the cystic fluid is suggested to be of more value in identifying the cyst as hydatid.

Antigen detection in hydatid fluid

Detection of specific hydatid antigen in the fluid is an approach alternate to hydatid fluid microscopy. Detection of fluid antigen is suggested to be of value in the diagnosis of cases of CE where: (1) aspirated fluid is negative for scolices and hooklets by microscopy. (2) histology is not appropriate such as in acephalo cysts ; and (3) hydatid serology is negative (Ravinder and Parija , 1997) .Enzyme-linked immunosorbent assay (ELISA) (Craig *et al*, 1986) , Co-agglutination (Co-A) (Ravinder and Parija , 1997) and counter-current immunoelectrophoresis (CIEP) (Parija *et al*, 1997), using hyper immune antisera raised against hydatid fluid antigen in rabbits have been employed to

detect hydatid antigen in the cystic fluid. Co-A (Ravinder and Parija, 1997) employs *Staphylococcus aureus* (Cowan's strain I) bearing protein. A sensitised with the purified hyperimmune hydatid antisera as the main reagent. The test was carried out with cystic fluid samples collected from 14 hydatid cysts operated by surgery .The Co-A was found to be 100% sensitive. The test detected hydatid antigen in all 14 samples collected from the cases of surgically confirmed hydatid cysts. The test was more sensitive than that of fluid microscopy, which was positive for only 6-out of 14 fluids, tested. The Co-A could be employed as a simple, rapid and a bedside diagnostic procedure to confirm hydatid aetiology of a cyst (Ravinder and Parija, 1997).

The detection of hydatid antigen in the cystic fluid, as an alternate to microscopy, offers a few additional advantages. First the method is much more sensitive than the conventional cystic fluid microscopy. Second, the hydatid aetiology of the cyst can be confirmed by examination of fluid samples if fluid samples collected not only from clinically suspected cysts but also by examination of the fluid aspirated from doubtful as well as unsuspected cases.

Hydatid fluid is an invaluable specimen for confirming aetiological diagnosis of a hydatid cyst either by microscopy or detection of antigen in the fluid and thereby diagnosing the cases of CE. Examination of hydatid fluid for diagnosis of CE offers a few advantages. First, microscopy of fluid or Co-A test for detection of antigen is the most rapid 5 min test. Second, examination of cystic fluid is most specific for CE, if the test is positive, no other test is required. Third, the tests are most economical and simple. Both the tests (microscopy and Co-A test) do not require any hi-tech instruments and can be performed with minimum technical expertise available in poorly equipped laboratories.

Aspiration of cystic fluid, however, still remains controversial mainly due to its safety. Many still consider direct aspiration of fluid from a case of hydatid cyst to be dangerous and should be avoided due to its inherent risk of possible leakage leading to anaphylaxis or dissemination of scolices in the circulation resulting in secondary echinococcosis at different sites in the body. However, if diagnostic aspiration is performed, more care should be taken so that hydatid cyst is not ruptured during aspiration. Aspirating the fluid by percutaneous drainage (Bastid *et al*, 1994) or aspiration by fine needle aspiration cytology under sonography (Das, 1995) are the suggested procedures having the least risk of leakage, thereby, making aspiration of cystic fluid a relatively safe procedure.

In conclusion, the aspirated fluid may at times be submitted for establishing the etiological diagnosis of a suspected hydatid cyst and after surgical removal of cyst. Simple microscopic exam by wet mount or antigen detection in the fluid may confirm the diagnosis of hydatidosis.

REFERENCES

Bastid C, Azar C, Doyer M. et al. 1994. Percutaneous treatment of hydatid cysts under ultra sonographic guidance. Dig.Dis.Scie. 39:1576-80.

Beg MH, Mansoor T. 2002 Pulmonary hydatidosis - a surgical experience. J Indian Med Assoc.100(1):22-3.

Bhat S, Kamble RT, Sundaram P, Joshi JM. 2002 Transdiaphragmatic extension of hepatic hydatid cyst. Indian J chest Dis Allied Sci.44 (3):191-4.

Brundelet P. 1973. Acid-fast staining of hooklets of *Taenia echinococcus*. Lancet, 1,678.

Choudhuri G, Prasad R, Tantry BV, Sharma MP, Tandon RK. 1989 Poor response to long term albendazole therapy of hydatid liver cysts. Scand J Infect Dis. 1989.21 (3):323-5.

Clavel A, Varea M, Doiz O, Lopez L, Quilez J, Castillo FJ, Rubio,C; Gomez-Lus, R. 1999. Visualisation of hydatid elements: comparison of several techniques. J .Clin.Microbiol. 37,1561-1563.

Craig PS, Bailey W, Nelson GS. 1986. A specific test for identification of cyst fluid samples from suspected human hydatid fluid. Trans. R. Soc. Trop. Med. Hyg; 80:256-7.

Das DK. 1995. Ultrasound guided fine-needle aspiration cytology: diagnosis of hydatid disease of abdomen and thorax. Diag. Cytopathol; 12: 173-176.

Das DK, Choudhury U. 2002 Hydatid disease: an unusual breast lump. J Indian Med Assoc. 100 (5) :327-8.

Dwivedi M, Misra SP, Dwivedi S, Kumar S and Misra V. 2002. Percutaneous treatment of hepatic hydatid cysts using hypertonic saline. J.Assoc. Physicians. India. 50 (5);647-50.

Gupta S, Nanda SN, Sobti P. 1966 Hydatid disease. J Assoc Physicians India. 14 (9):553-6.

Kakrani AL, Chowdhary VR, Bapat VM. 2000 Disseminated pulmonary hydatid disease presenting as multiple cannon ball shadows in human immunodeficiency virus infection. J Assoc Physicians lndia. 48 (12); 1208-9.

Khursheed AS, Abrar AWJariq HN, Shams B. 2001 Open method versus captionnage in management of hepatic hydatidosis in children. Pediatr Surg lnt.17 (5-6):382-5.

Konar K, Ghosh S, Konar S, Bhattacharya S, Sarkar S. 2001 Bilateral ovarian hydatid disease-An unusual case. Indian J Pathol Microbiol. 44(4):495-96.

Parija SC. 1998a. A review of some simple immunoassays in the serodiagnosis of cystic hydatid disease. Acta Tropica; 70; 17-24.

Parija SC. 1998b. Urinary antigen detection for diagnosis of parasitic infections. Parasitol Today; 14: 5-6.

Parija SC. 2004.Text Book of Medical Parasitology. Protozoology and Helminthology. Text and Color Atlas. Madras AIPD, 2nd Ed.

Parija SC, Prabhakar P K. 1995. Evaluation of lacto-phenol cotton blue for wet mount preparation of feces. J .Clin, Microbiolf; 33:1019-1021.

Parija SC, Ravinder PT. 1997. Lacto-phenol cotton blue stain for wet mount preparation of hydatid fluid. Trop. Doct:, 17:108.

Parija, SC, Sheela Devi C. 1999 .Current concepts in the diagnosis of cystic echinococcosis in humans and livestock and intestinal echinococcosis in canine hosts. J. Vet. Parasite. 13 93:102.

Parija SC, Sasikala A, Rao RS. 1987 Serological survey of hydatid disease in Pondicherry, India. *Trans R Soc Trop Med Hyg.* 81: 802-803.

Parija SC, Ravinder PT, Shariff M. 1996. Detection of hydatid antigen in the fluid samples from hydatid cysts by co-agglutination. Trans, R, Soc, Trop, Med, Hyg, ; 90: 255-6.

Parija SC, Ravinder PT, Subba Rao KVSK. 1997. Detection of hydatid antigen in urine by counter-current immuno-electrophoresis. J, Clin, Microbiol; 35: 1571-74.

Parija SC, Rao RS, Badrinath S, 1983 Sengupta DN. Hydatid disease in Pondicherry J. Trop Med Hyg. 86:113-115.

Paul MJ, Shyamkumar NK, Sadhu D.2002 Soft-tissue case 47. Pelvie retroperitoneal hydatid cyst secondary to rupture of a liver hydrated cyst. Can J Surg.45(5);368-391.

Pillai AM, Vasudevan S. 1996 Hydatid cyst of the orbit producing proptosis-a case report. J Indian Med Assoc, 94(3): 116-8.

Purohit M, Srivastava CP, Yadav KS. 1999 Primary mediastinal hydatid cyst. Indian J chest Dis Allied Sci. 41 (1):57-60.

Pushparaj K, Sundararajan M, 2001 Madeswaran K, Ambalavanan S. Primary spinal intradural hydatid cyst-a short report. Neuror) India .49(2):203-4.

Rajagopal KV, Bishwas R. 2002 Hydatid cyst of the liver presenting as an inferior vena cava obstruction. J Cliin Ultrasound.30 (2): 114-6.

Rattan KN, Gathwala G, Sharma LK, Sodhi D. Abroi P. 1994 Pulmonary hydatid cyst an unusual presentation. Indian Pediatr.31 (1):77-9.

Ravinder P.T, Parija SC. 1997. Counter-current immunoelectrophoresis test for detection of hydatid antigen in the fluid samples from hydatid cysts: a preliminary report. Acta. Tropica; 66 : 169-73.

Ryan NJ, Sutherland G, Coughlan, Globan M, Doultree J, Marshall J, Bain RW, Pediagen J, Dwyer Brian. 1993. A new trichrome-blue stain for detection of microsporidial species in urine, stool and nasopharyngeal specimens. J.Clin.Microbiol; 31, 3264-9.

Sandhu P, Saggar K, Sodhi KS. 2000 Neurological Picture. Multiple hydatid cysts of the brain after surgery. J Neurol Neurosurg Psychiatry.68 (1):97.

Sathyanarayanan MV, Shenoy MG, Pai VM, 1996 Haque PW, Hedge P, Rajan R, Pahuja A. Metabolic acidosis induced by cetrimide-chlorhexidine solution in hydatid cyst surgery. Indian J Gastroenterol. 15(3): 104.

Sekar M, Appaji Rao VN, Ramadass P, Raghavan N. Serological diagnosis of hydatidosis in man. Indian J Parasite). 1988.12 (2):335-338.

Sinha R, Sharma N. 2001. Abdominal hydatids: a minimally invasive approach.JSLS.5 (3):237-40.

Sodhi KS, Saxena A, Sood B, Suri S. 2002 Soft-tissue case 46. Splenic hydatidosis. Can J Surg. 23 (1):35-7.

Thomas A, Bhat S, Jacob AJ. 1996. Echinococcal disease of the kidney-a case report.J Indian Med Assoc.94(2):68.

Upadhyaya GH, Rai P, Shah PK. 1996. Clinical study of hydatid disease in Jam Nagar.J Indian Med Assol. 63 (7):213-6.

Usha Tiwari PV, Khanna A, Garg M. 1994. Hydatid cyst of ovary-a case report. Indian J Pathol Microbio). 37 (3):349-51.

Vamsy M, Parija SC, Sibal RN. 1991. Abdominal hydatidosis in Pondicherry, India, outheast Asian J Trop Med Public Health. 22suppl:365-70.

Wagholikar GD, Sikora SS. Surgical management of liver hydatid. Trop Gastroenterol.2001.22 (3): 159-62.

Wheatley WB. 1951. A rapid staining procedure for intestinal amoebae and flagellates. Am. J.CIin.Pathol; 21,990-1.

WHO Informal working group on echinococcosis 1996. Guidelines for treatment of cystic and alveolar echinococcosis in humans. Bull, WHO; 76:231-42.

WHO Informal working group on echinococcosis 1996. Guidelines for treatment of cystic and alveolar echinococcosis in humans. Bull WHO ; 76:231-242.

Yadav BS,Garg N,Ray D. 1997. Hydatid cyst of heart presenting as ventricular tachycardia. J Assoc Physicians India. 47 (3):342-3.

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Identification and characterization of *Echinococcus granulosus* by PCR-RFLP technique in Tabriz district

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Hydatidosis is a zoonotic disease that occurs in intermediate hosts by larval stage of *Echinococcus granulosus* a cestode. Studies demonstrated that in the endemic area *E. granulosus* show an intraspecies variation which affects epidemiology, pathology, control and prevention of hydatidosis. With molecular methods, nine distinct genotypes (G_1 - G_9) have been identified within *E. granulosus* till date. Seventy five isolates including 20 cattle, 40 sheep and 15 human isolates were collected from Tabriz industrial slaughterhouse and hospitals. DNA extraction was performed, using SDS and proteinase K with Cetyl trimethyl ammoniun bromide (CTAB) and PCR for ITS1 fragment was done. PCR products of all isolates were restricted with *Rsa* I and *Hpa* II. The PCR-RFLP products were electrophoresed and stained. Study of PCR amplified bands showed that human, cattle and sheep isolates were similar in size (approximately 1 kb). PCR-RFLP patterns of ITS1 fragment of all isolates with restriction enzymes were identical as follows, *Rsa* I bands under UV showed two bands approximately 600bp and 300bp. *Hpa* II bands were as follows: two approximately 600bp and 200bp which were recognizable under UV and small fragments which were not recognizable. Based on PCR-RFLP patterns of ITS1 fragment produced with endonucleases enzyme digestion in animal and human isolates, it can be concluded that a single strain of *Echinococcus granulosus* (sheep strain) is dominant genotype in this region.

Keywords: *Echinococcus granulosus*, Iran, PCR, Strain Variation

INTRODUCTION

Hydatidosis is a significant economic and public health problem in Iran. High prevalence rates have been reported in various species of domestic animals, including sheep, cattle, camels and goats, and at least 2 distinct cycles of transmission are thought to occur (Fasihi harandi et al 2002). Incidence rate of cystic echinococcosis in Iran is 1.2 per 100 000 inhabitants. Maximum infection rate is in Khorasan province (North-east part of Iran) with 44.5 per 100 000 inhabitants and minimum is in Hormozgan province (South part of Iran) with 0.1 per 100 000 inhabitants (Noorjah 1987). Infection rate in the west part of Iran (including study area) is 5.55% (Zarif Fard *et al* 1999). In definitive hosts the highest prevalence was detected in dogs from the rural areas of Isfahan (city in central part of Iran) (63.3%) and the lowest, in dogs from those of Sistan (city in South-east of Iran) (3.3%) (Eslami and Hosseini 1998). Human cases of cystic hydatidosis are also regularly reported from medical centers in different parts of the country (Mobedi et al 1971; Bastami and Dehdashti 1995; Fasihi harandi et al 2002) However, the sources of infection to humans, and particularly the role of intermediate host reservoirs, remain to be determined (Fasihi Harandi et al 2002) Studies have demonstrated that in the endemic area, *Echinococcus granulosus* shows an intraspecies variation which affects epidemiology, pathology, control and prevention of hydatidosis (Thompson and Lymbery 1996). Many different techniques were used to determine this variation especially PCR-RFLP (Thompson and Lymbery 1996). With molecular tools, nine distinct genotypes (G_1 - G_9) were identified within *Echinococcus granulosus* to date (Thompson and McManus 2002). In this study, we have tried to determine the genotype of larval stage *of Echinococcus granulosus* tape worm in patients, sheep and cattle in Tabriz, the north-west city of Iran using PCR-RFLP method.

* Corresponding Author

MATERIAL AND METHODS

A total of 60 hydatid cysts (20 cattle and 40 sheep) from naturally infected intermediate hosts (sheep and cattle) were collected from Tabriz industrial slaughterhouse. Human isolates (15 cases) were obtained from Tabriz Imam Khomeynei hospital. To define the fertility of the cysts, wall fragments and hydatid fluid were observed under an optical microscope to determine the presence of protoscolices. Parasite materials (protoscolices in the case of fertile cysts and germinal layer in the case of nonfertile cysts) were washed three times in sterile phosphate-buffer-saline, pH 7.2, and then preserved in 70% ethanol at -20°C .After three washings with sterile distilled water, the specimens were crushed in liquid nitrogen by three times freeze and then digested in 200µg/ml proteinase K and 0.5% sodium dodecyl sulphate (SDS) for 2 days at 50°C. Then DNA was extracted using phenol- chloroform extraction method with some modification and using CTAB. Tissues of intermediate hosts were used as negative control. The PCR to amplify ITS1 fragment was performed by using BD1 (5′-GTCGTAACAAGGTTTCCGTA-3′)and 4S(5′-TCTAGATGCGTTCGAA(G/A)TGTCGATG-3′) [(Fasihi harandi et al 2002; Rosenzvit *et al* 1999; Ahmadi *et al* 1999)TIB MOLBIOL Germany] primers (forward and reverse, respectively] Reactions were carried out in a final volume of 50 µl containing dNTP 0.2 mM dNTP, 2 Mm, $MgCl_2$ 1µM primers (each), Taq 1.5 DNA polymerase Units in final concentration. Each reaction was subjected to an initial denaturation at 95°C for 60 sec., 55°C for 60 sec. (annealing), 72°C for 90 sec. (extension) and a final extensionat 72°C for 7 min. Amplificated ITS1 fragments with expected length (1kb) were purified by using High Pure PCR Product Purification Kit (Roche). The purified PCR products were digested by two restriction endonucleases [Rsa I and Hpa II (Roche)]. The size of the restricted products were assessed by electrophoresis in 1% (w/v) Tris-acetate/EDTA (TAE) agarose gel, stained with 0.5 µg/ml ethidium bromide. The size of fragments was determined by using known size markers, λDNA *EcoRI/ HindIII* digest (Fermantas). The DNA Marker contained 13 discrete fragments (in base pairs): 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831,564, 125.

RESULTS

Two protocols were used for DNA isolation. In the first protocol (protocol 1) phenol/chloroform extractions were made and DNA was precipitated with ethanol (Sambrook and Russell 2001). With first method we could not obtain suitable DNA for the polymerase chain reaction analysis from germinal layers. The presence of PCR inhibitors such as polysaccharides can account for this. We employed CTAB to remove them from DNA preparations. DNAs isolated by this protocol(protocol2) allowed reliable PCR results (Fig.1). Extracted DNAs were used for amplification of ITS1 region by PCR s in material and methods. After electrophoresis, amplified products were studied

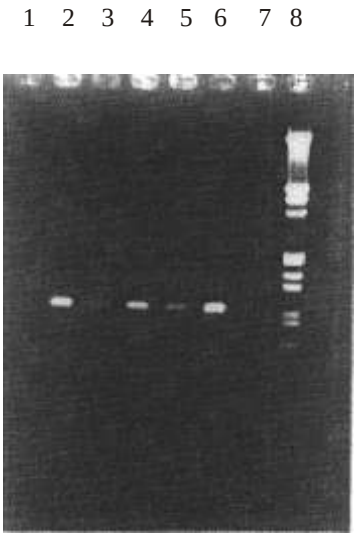

Fig. 1: Comparison between DNA extraction with and without CTAB in PCR results

1. Negative control
2. PCR band using human germinal layer DNA extracted with CTAB
3. PCR band using human germinal layer DNA extracted without CTAB
4. PCR band using cattle germinal layer DNA extracted with CTAB
5. PCR band using cattle germinal layer DNA extracted without CTAB
6. PCR band using sheep germinal layer DNA extracted with CTAB
7. PCR band using sheep germinal layer DNA extracted without CTAB
8. Size marker Lambda DNA/ *Eco*RI+ *Hind* III

under UV. Transilluminator study of amplified bands showed that human, cattle and sheep isolates are similar in size each band of approximately 1Kb. (Fig. 1). After purification of PCR products by High Pure PCR Product Purification kit and digestion with endonucleases (Rsa I and Hpa II), electrophoresis was done. Study of Rsa I digested product under UV showed two bands of approximately 600 bp and 300bp where as Hpa II digested products showed two bands of approximately 600 bp and 200bp which were recognizble under UV and small fragments which were not recognizable(Fig. 2)

Discussion

Cystic hydatid disease, caused by larval stage of *Echinococcus granulosus,* is medically and economically one of the most important of the zoonoses (Togerson 2003; Thompson and McManus 2001). Considerable genetic and phenotypic variation has been demonstrated in *Echinococcus,* with the recognition of a series of what appear to be host-adapted genotypes or strains (Thompson and McManus 2001), which are likely to represent distinct species (Thompson *et al* 1995). Therefore, there is a need to characterize the etiological agents in different endemic areas using molecular epidemiological techniques, in order to determine transmission patterns, particularly where there is the possibility of interaction between cycles (Thompson *et al* 1998). The isolation and purification of the nucleic acid template is an important first step toward achieving high amplification efficiency and specificity in PCR. We used both protoscolices and germinal layer for DNA extraction whereas other studies use only protoscolices (Scott et al 1997; Zhang *et al* 1998 a & b; Ahmadi *et al* 1999; Rosenzvit *et al* 1999; Fasihi harandi *et al* 2002). This was very important for molecular study of non-fertile cysts. We used initially a general method for DNA extraction using SDS and proteinase K (Sambrook and Russell 2001), but we could not obtain DNA suitable for the polymerase chain reaction analysis from germinal layers using conventional techniques, and further purification steps were needed. The presence of PCR inhibitors in these DNA samples can account for this. It is possible that these inhibitors came from the laminar layer, which was not separated from the germinal layer. Polysaccharide-containing laminar layers, which were tightly adhered to the germinal layers, were not removed from the preparations used for DNA extraction. Since polysaccharides are described as potent DNA modifying enzyme inhibitors, we employed CTAB to remove them from DNA preparations. This cationic detergent has been employed successfully to remove polysaccharides and other enzyme inhibitors found in biological specimens (Yap and Thompson 1987). PCR-RFLP patterns of human, cattle and sheep isolates in our study has shown similar characteristics and it seems that, there is same genotype of *E.granulosus* that cause cystic hydatidosis in this region. In other studies (Zhang *et al* 1998(a); Ahmadi *et al* 1999; Fasihi harandi *et al* 2002;) similar results were obtained with some difference. In

1 2 3 4 5 6 7 8 9 10

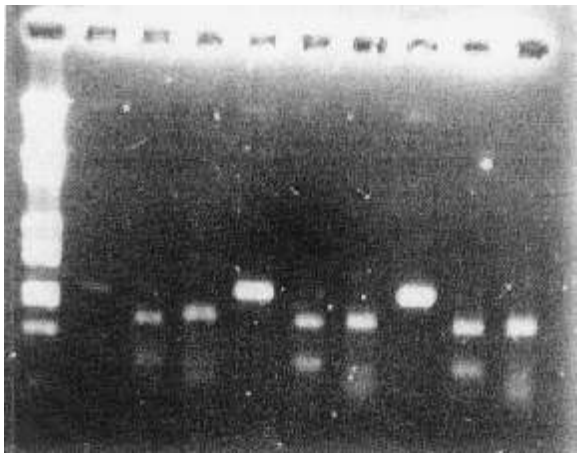

Fig. 2. ITS1 PCR and RFLP patterns of *E. granulosus* isolates in agarose gel 1 %

1. Size marker λDNA/EcoRI, *Hind* III
2. ITS1-PCR from human origin
3. ITS1 from human origin digested with *Rsa* I
4. ITS 1 from human origin digested with *Hpa* II
5. ITS1-PCR from cattle origin
6. ITS1 from cattle origin digested with *Rsa* I
7. ITS 1 from cattle origin digested with *Hpa* II
8. ITS1-PCR from sheep origin
9. ITS1 from sheep origin digested with *Rsa* I
10. ITS 1 from sheep origin digested with *Hpa* II

the present study because of the reason of rare camel and goat slaughtering and low usage of them for eating we can not obtain these samples. Two main strains of *E.granulosus* (sheep and camel strains) are found in Iran and at least 2 distinct cycles of transmission are thought to occur (Zhang *et al* $1998_{(a)}$; Ahmadi *et al* 1999); Fasihi harandi *et al* 2002;). The results of the present study reveal that sheep strain of *Echinococcus granulosus* is most important strain in this region and most of the infections in this province are due to this strain and the presence of one fertile cyst in one cattle may indicate that cattle can act as a reservoir of sheep strain but this finding requires further evaluation. Thus for disease control programs we must pay attention to this and focus our control programs in sheep and cattle.

REFERENCES

Ahmadi N, Dalimi A, Sadeghizadeh M, 1999. Molecular study of human, sheep and camel hydatid cysts isolates in Iran using PCR method. Modarres, 2(2): 53-56.

Bastani B and Dehdashti F, 1995. Hepatic hydatid disease in Iran, with review of the literature. Mount Sinai Journal of Medicine, 62: 62-69.

Eslami A and Hosseini SH, 1998. *Echinococcus granulosus* infection of farm dogs in Iran. Parasitology Research, 84: 205-207.

Fasihi Harandi M, Hobbs RP, Adams PJ, Mobedi I., Morgan-Ryan UM, Thompson RCA 2002. Molecular and morphological characterization *of Echinococcus granulosus* of human and animal origin in Iran. Parasitology, 125: 367-373.

Mobedi I, Arfaa F, Farahmandian I. 1971. Studies on echinococcosis in Iran. Acta Medica Iranica, 14:221-229.

Noorjah N, 1987. Hydatidosis-Echinococcosis in Iran, Evaluating Economic Losses due to the Disease. Ph.D thesis (in Persian), Tehran University of Medical Sciences, 1987.

Rosenzvit MC, Zhang L, Kamenetzky L, Canova SG, Guarnera EA, McManus DP, 1999. Genetic variation an epidemiology *of Echinococcus granulosus* in Argentina. Parasitology, 118: 523-530.

Sambrook J and Russell DW, 2001. Molecular cloning- A laboratory manual, Volume 2, Third edition. Cold Spring Harbor Laboratory Press, New York, pp 405-520.

Scott JC, Stefaniak J, Pawlowski ZS, McManus DP 1997. Molecular genetic analysis of *Echinococcus granulosus* human hydatid cases from Poland: identification anew (G9) genotype. Parasitology, 114: 37-43.

Thompson RCA, Lymbery AJ, Constantine CC, 1995. Variation in Echinococcus: towards a taxonomic revision of the genus. Advance in Parasitology, 35: 145-176.

Thompson RCA and Lymbery AJ 1996. Genetic variability in parasites and host-parasite interactions. Parasitology, 112: 517-522.

Thompson RCA, Constantine CC, Morgan UM, 1998. Overview and significance of molecular methods: what role for molecular epidemiology? Parasitology, 117: 161-175.

Thompson RCA and McManus DP, 2001. Aetiology: parasites and life cycles. In: Eckert, J: Manual on Echinococcosis in Humans and Animals a Public Health Problem of Global Concern, World Organization for Animal Health (OIE), Paris, pp 1-19.

Thompson RCA and McManus DP, 2002. Towards a taxonomic revision of the genus Echinococcus. Trends in Parasitology, 18(10): 452-457, 2002.

Torgerson PR, 2003. Economic effects of echinococcosis. Acta Tropica, 85: 113-118, 2003.

Yap KW and Thompson RCA, 1987. CTAB precipitation of Cestode DNA. Parasitology Today, 3:220-222.

Zarif Fard MR, Abshar N, Akhavizadegan MA, Motamedi GR 1999. Seroepidemiological survey of human hydatidosis in western part of Iran. Archive Razi Institue, 50: 71-75.

Zhang L, Eslami A, Hosseini SH, McManus DP 1998(a). Indication of the presence of two distinct strains of *Echinococcus granulosus* in Iran by mitochondrial DNA markers. American Journal of Tropical Medicine and Hygeine, 59(1): 171-174.

Zhang L, Chai JJ, Jiao W, Osman Y, McManus DP 1998(b). Mitochondrial genomic markers confirm the presence of the camel strain (G6 genotype) of *Echinococcus granulosus* in north-western China. Parasitology, 116: 29-33.

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Genetic Variation among filarial species as detected by random amplified polymorphic DNA (RAPD)

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Random Amplified Polymorphic DNA (RAPD) analysis technique is a powerful tool for detecting diversity among morphologically indistinguishable population of organisms. Identification of genetic variation among filarial species may be useful in correlating clinical spectrum of the disease with parasite heterogencity. In a preliminary effort in this direction genomic DNA isolated from various life stages viz. adult, microfilariae and infective larvae of three filarial species *Brugia, malayi, Setaria cervi* and *Acanthocheilonema viteae* were subjected to PCR amplification employing commercially available preimers. Amplified products were size fractionated on 2% agarose gel and visualized under UV illumination. Different species of filarial parasites gave different fingerprinting pattern with same primer while various life cycle stages of the same species, as expected, yielded similar pattern. Thus RAPD appears to be a good tool to study the genetic polymorphism in filarial parasites.

Keywords: Acanthocheilonema viteae, Brugia malayi, RAPD, Setaria cervi

INTRODUCTION

Lymphatic filariasis is a major public health problem particularly in tropical and subtropical countries affecting nearly 120 million people worldwide (Michael, Bundy and Greenfell, 1996) The disease exhibits wide spectrum of variation in clinical manifestations ranging from microfilariaemic to chronic symptomatic cases. In India, majority of elephantiasis cases are found in southern coastal states. In northern states like UttarPradesh, Madhya Pradesh and Bihar, in spite of the occurrence of mosquitoes, the vector, in huge number, people are found mostly normal or asymptomatic microfilariaemic. Individual immunological status of a person has ben considered as the major cause of such a diverse spectrum of the disease. Beside this variation in parasite population has also been now taken into account as an important factor. Hence characterization of various strains of the parasite and their regional distribution warrants thorough investigation.

Defining distinction between different species, *Wuchereria bancrofti or Brugia malayi,* for example on the basis of morphological characteristics alone is not possible. Attempts were therefore made in some laboratories for defining intra and inter species variation on the basis of morphological and isoenzymic patern (Backett and Mcdonal, 1972; Cutillas *et al*1995). However these parameters also appeared to have only limited application. Fingerprinting of genomic DNA has recently emerged as a powerful tool for the analysis of such differences from small organisms to large animals including human beings. Random amplified polymorphic DNA (RAPD) analysis provides a valuable tool for determining intraspecies and interspecies variation at genetic level (Williams *et al* 1990; Welsh and McLelland, 1990). It was of considerable interest therefor to study RAPD pattern for some filarial species with a panel of primers.

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MATERIAL AND METHODS

Adult Parasites

Setaria cervi were collected from the peritoneal folds of freshly slaughtered Indian water buffalo (*Bubalus bubalis* Linn) from a local slaughterhouse . *Acanthocheilonema viteae* infection was maintained in *Mastomys coucha* and the worms were collected from subcutaneous pockets of the anaesthetized animals carrying 75 to 120 days old infection. *Brugia malayi*, on the other hand, were collected from peritoneal cavity of birds (*Meriones unguiculatus*) after 120-180 days of inoculation with infective larvae (L3).

Larval Stages: Infective larvae of A.*Vitea* were recovered from ticks (*Ornithodoros moubata*) infected by feeding on microfilaraemic mastomys at least 30 days earlier. L3 of *B. malayi* on the other hand was obtained from gently crushed mosquitoes (*Aedes aegypti* on 9 ±1 day of infective feeding on a donor mastomys. Larvae were collected by Baermann apparatus and washed free of vector tissues. The infection was transmitted to vertebrate host by inoculating 10 live L3 *of A. viteae* and *B. malayi* subcutancously in the neck region of mastomys. Microfilariae (mf) of both species were collected in PBS by teasing the heart and lungs of heavily microfilaraemic mastomys harbouring respective infections. Phosphate buffered saline, pH 7.2 (PBS) containing mf was passed through a fine mesh to remove tissue pieces and the filtrate was then passed through 5.0 *µm* membrane filters. The filter membrane was suspended in lukewarm PBS for 10-15 minutes and mf were pelleted by centrifugation at 90 g x 5 min.

Isolation of DNA

The protocol of Sambrook, Fritsch and Maniatis (1989) was followed with minor modifications to isolate DNA. In brief, fresh or frozen (after thawing) worms were taken and minced to small pieces with scissors and 10%homogenate (w/v) was prepared in homogenizing buffer (Tris, 0.3 M, pH 8.0;NaCl 0.1M; Sucrose 0.2M; EDTA 0.01M) SDS was added to a final concentration of 1% and proteinase K to IOU/ml. The mixture was vortexed briefly and incubated at 37°C for overnight. One-tenth volume of 8M-sodium acetate was added and thoroughly mixed. The mixture was reincubated at 4°C for 2 hr and subjected to two extractions with 1:1 mixture of phenol and chloroform followed by one extraction with chloroform alone. DNA from the aqueous phase was precipitated by the addition of 2.5 volumes of chilled absolute ethanol. The precipitated DNA was recovered by centrifugation at 1,500 x g for 10 min. The DNA was washed once with 70% ethanol and resuspended in minimum volume of TE buffer (Tris 0.3M, pH 8.0, EDTA 0.01 M) To this solution was added the solution of DNase free RNase to a final concentration of 20µg/ml. The mixture was incubated for 30 min at 37°C and extracted with phenolchloroform as described above. Purity of the sample and quantitation of DNA was done spectrophotometrically by taking absorbance at 260 and 280 nm.

RAPD Analysis

Amplification of DNA and detection of amplified products by agarose gel electrophoresis was done as per instructions provided by M/S Amarsham Pharmacia Biotech (U.K.) with RAPD analysis kit. Briefly 25 µl reaction volume was set up in a 200µl thin wall PCR tube containing a single RAPD bead, 20 ng template. DNA, 25 pmoles of a single RAPD primer and sterile triple distilled water. The contents of the tube were gently vortexed and centrifuged for 30 sec. The tubes were placed in a PTC - 100 thermocycler (MJ Research, Inc., USA) using following cycle profile: 1 cycle at 95° C for 5 min followed by 45 cycles at 95° C for 1 min, 36° C for 1 min, 72°C for 2 min. The fingerprinting pattern of the randomly amplified DNA was analyzed by agarose (2%) gel electrophoresis and visualised in Transilluminator (UVP Gel Documentation System). The six primers used for RAPD analysis designated as primer 1 to 6 were having the following sequence

Primer 1	(5'-d [GGTGCGGGAA]-3')
Primer 2	(5'-d [GTTTCGCTCC]-3')
Primer 3	(5'-d[GTAGACCCGT]-3')
Primer 4	(5'-d[AAGAGCCCGTJ-3')
Primer 5	(5'-d[AACGCGCAAC]-3')
Primer 6	(5'-d[CCCGTCAGCA]-3')

RESULTS

High quality i.e. pure and undegraded DNA is the prime requirement for the production of better quality of RAPD pattern. Hence purity of DNA from various preparations was ensured by determining the ratio of optical densities at 260 and 280 nm which always ranged betwen 1.8 to 2.0, indicating towards the purity of the isolated DNA (data not given). Also the DNAs used in the present study were undegraded since a single band was observed in ethidium bromide banding pattern (Fig. 1, lanes 2 and 7: Fig 2 lanes 13,14 and 15) Initially the PCR was set up with 10 and 20ng of *S.cervi* genomic DNA as template; the bands with 10 ng DNA were very faint and even some were invisible (Fig 1, lane 3), but those obtained with 20ng DNA were very clear and visible (Fig 1, lane 4). Hence in all the subsequent experiments 20ng DNA was used as the template. The Validity of PCR test was further affirmed by amplifying two control *Escherichia coli* DNA BL21 (DE3) and (CLa) (Fig.1, Lane 5, 6), Provided by the supplier with RAPD kit, which produced banding pattern exactly similar to that

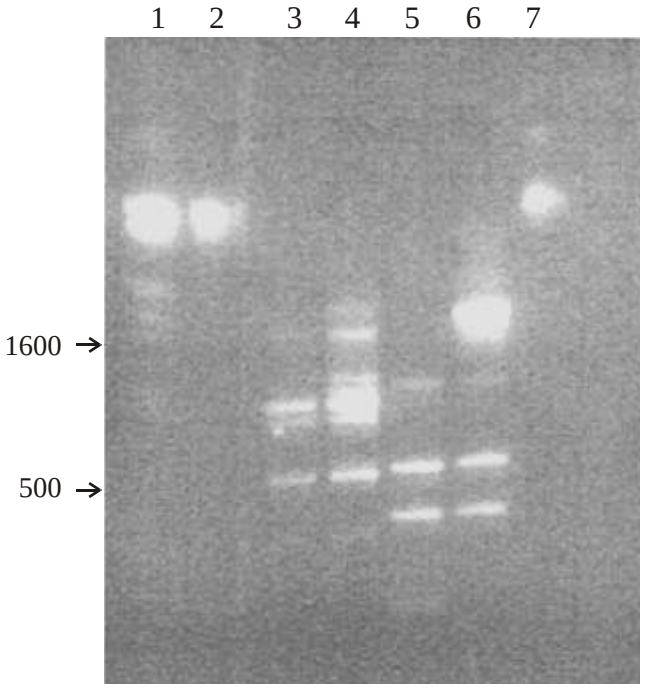

Fig. 1: Lane 1: Marker (λDNA / Eco*RI* + Hind III double digest), Lane 2& 7 : genomic DNA of *S.Cervi*, Lanes 3 to 6 : RAPD pattern with primer 2 and DNA of *S. cervi* (10ng); (20ng); *E. coli* BL 21 (DE3) 10ng & *E.coli* BL21 (Cla) 10ng respectively.

reported by the supplier. Fig. 2 shows fingerprints produced by DNA of adults *S.Cervi* with primers 1 to 6 (lanes 1-7) as well as by *B.malayi* and *A. viteae* with

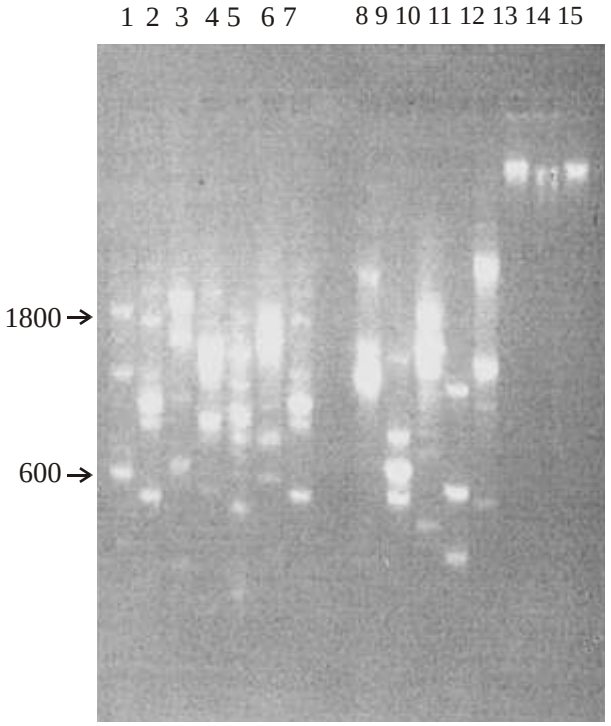

Fig. 2: RAPD pattern with filarial genomic DNA. Lanes 1 to 6: *S.cervi* (batch I) with Primers 1 to 6 respectively. Lane 7 : *S.cervi* (batch II) with primer 2, Lane 8: *B malayi* with Primer 2, Lane 9: *A. viteae* with Primer 2, Lanes 10 to 12 : Control *E. Coli* BL 21 (DE3) DNA with primers 1 to 3 respectively. Lanes 13 to 15: DNA of *S.cervi, B. malayi and A.viteae* respectively.

primer 2 (lanes 8 to 9), respectively. It can be noticed that all the primers with *S.cervi* DNA generated PCR products between 300 to 1800 bp, but the prominent products differed from primer to primer used. Thus primer 1 yielded the product of 600 bp as the most prominent band while other primers produced prominent band of 800, 1800, 1050, 750 and 1200 bp size respectively. When the fingerprints of S. cervi, B. malayi and A. viteal (lanes 7,8 & 9) were compared with each other, they yielded recognizable distinct pattern. Fingerprinting of different life stages viz. adult, microfilariae and L3 of *B. malayi* and *A. viteae* with primers 1 to 6 gave interesting pattern(Figs 3 & 4). Different life stages of the same parasite with same primer yielded identical pattern (Lanes 3-8) whereas same life stage of different species yielded altogether

different pattern (Lanes 1,4 and 2,5). For example with primer 6, adults, mf, and L3 of *B. malayi* (Lanes 11, 12 & 13) yielded similar pattern but mf of adults of *A. viteae* (Lanes 15 & 16) had a pattern quite different to that shown by any stage of *B.malayi*.

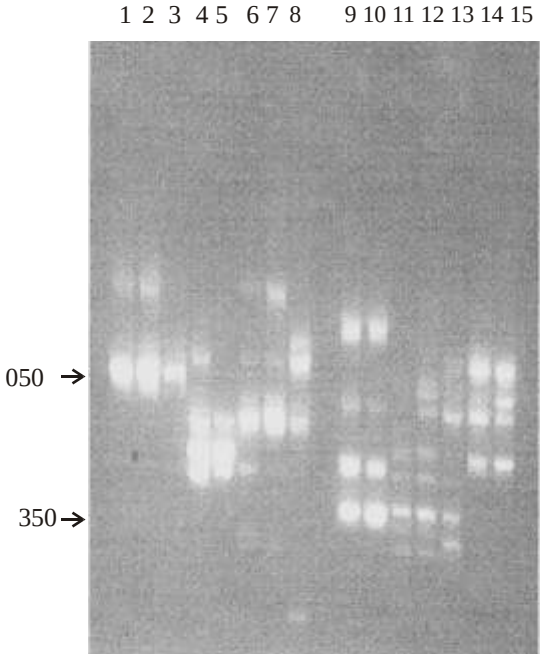

Fig. 3: RAPD Pattern with genomic DNA from various life cycle stages of filarial parasites and primers 2, 4 & 6 respectively. Lanes 1, 6 & 11 : Adult *B. Malayi*, Lanes : 2, 7 & 12 : *B. malayi* microfilariae Lanes 3, 8 & 13 : *B.malayi* infective larvae (L3), Lanes 4, 9 & 14: Adult *A. viteae* . Lanes 5, 10, & 15 : *A viteae* microfilariae

Discussion

In this preliminary report it has been shown that the banding pattern of the filarial DNA after PCR amplification followed by separation on agarose gel is complex yet distinct and characteristic to the parasite species (Fig.) The difference in the banding spectrum depends on the composition of primer taken as well as the parasite used. Different species viz. *A. Viteae B. malayi* and *S.cervi* exhibited distinct pattern but also shared *some* bands, indicating the presence of common sequences also. A few bands for each species were more prominent than others. For instance, with primer 2, *S.cervi* had a prominent band of 850 bp while *B.malayi* and *A.viteae* had those of 1000 and 600 bp respectively (Fig 2, lanes 7,8,9). This may be due to either better sequence homology between some portion of the genomic DNA and the primer used or due to repetitiveness of a particular sequence in the parasite genome. It is important to mention that many repeats of 322 bp sequence (equivalent to 10% of total genome) have been reported in DNA of both *B. malayi* and *B. pahangi* (Williams, DeSimon and McReynolds 1988; McReynolds, DeSimon and Williams 1986) Nevertheless one can not deny for the importance of homology to PCR. It is because if the prominent band appears due to repetitive sequence only, it should be present in all the lanes irrespective of the primer used. However, since the position of prominent band varies with the primer used, it strongly turns the possibility in favour of good similarity in the sequence of primer and that of a certain portion of the genomic DNA.

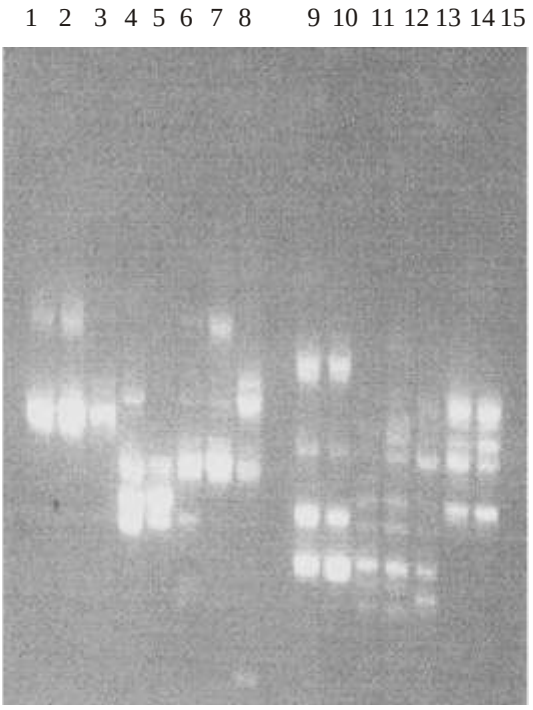

Fig. 4: RAPD Pattern with genomic DNA from various life cycle stages of filarial parasites and primers 1, 3 & 5 respectively. Lanes 1, 6 & 11 : Adult *B. malayi Lanes 2, 7 & 12: B. malayi* microfilariae, Lanes 3, 8 & 13 : *B. malayi* infective larvae, Lanes 4, 9 & 14 : Adult *A. viteae.* Lanes 5, 10 & 15: *A. viteae* microfilariae.

One of the most prominent drawbacks of RAPD analysis is that often the pattern are not very reproducible because even the slightest alteration in the PCR conditions or components may alter the

banding pattern (McKeand 1998; Thompson, Constantine and Morgan, 1998) It is interesting to note that reproducibility is not a problem in the present study since the genomic DNA of *S.Cervi* of the same batch when amplified on different dates (Fig. 1, lane 4; Fig. 2, lane 2) or the DNA from different batches of *S. cervi* amplified same day (Fig.2, lanes 2 and 7) yielded exactly identical banding pattern. RAPD analysis has been successfully applied to many parasites viz. *Trypanosomes* (Majiwa *et al.* 1993; Steindel *et al* 1993) *Giardia* (Morgan *et al.*, 1993); *Cryptosoridium* (Morgan *et al;* 1995) and *Leishmania* (Andresen *et al.* 1996; Motazedian *et al.*, 1996) Thus the present study along with earlier studies (mentioned above) by others suggests that RAPD analysis can be very well used for the genetic characterization of species/strains of filaraial parasites. This may provide an excellent tool to correlate the disease spectrum with the genetic polymorphism of the parasite. Furthermore since all the stages of the same parasite exhibit identical banding, spectra, DNA from any stage may be utilized for identification and characterization of filarial species and their isolates.

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REFERENCES

Andresen, K, Ibrahim, ME, Theander, TG, Kharazmi, A.1996. Random amplified polymorphic DNA for differentiation of *Leishmania donovani* isolates from Sudan. Transaction of Royal Society of Tropical Medicine and Hygiene. 90: 204-205

Becket, EB, Medonal, WW. 1972. The morphology of the rectal protrusion of *B.pahangi* and subperiodic *B.malayi* larvae as a method of differentiating betwen the two species. Annals of Tropical Medicine and Parasitology. 66: 135- 141.

Cutillas C, Espina C, Spakulova M, Arias P. 1995. Differential diagnosis of lung nematode parasites from livestock by electrophoresis techniques. International Journal of Parasitology. 25: 215-220.

Majiwa, PAO, Maina M, Waitumbi JN, Mihok S, Zweygarth E. 1993. *Trypanosoma* (Nannomonas) *congolense*: molecular Characterization of a new genotype from Tsavo, Kenya. Parasitology. 106: 151-162.

Mckeand JB. 1998. Molecular diagnosis of parasitic nematodes. Parasiotology. 117: S87-S96

McReynolds LA, Desimone SM, Williams SA. 1986. Cloning and comparison of repeacted DNA sequences from the human filarial parasite *Brugia malayi* and the animal parasite *Brugia pahangi*. Proceding of National Academy of Sciences, USA. 83: 797-801

Michael E., Bundy D AP, Grenfell B. 1996. Reassessing the global prevalence and distribution of lymphatic filariasis Parasitology. 112: 409-428

Morgan UM, Constantine CC, Greene WK, Thompson RCA. 1993. RAPD (Random amplified Polymorphic DNA) analysis of Giardia DNA and correlation with isoenzyme data, Transaction of the Royal Society of Tropical Medicine and Hygiene . 87 702-705.

Morgan UM, Constantine CC,O'donghue P, Meloni BP, O'brien PA, Thompson RCA. 1995. Molecular characterization of crytosporidium isolates from human and other animals using RAPD (Random Amplified Polymorphic DNA) analysis. American Journal of Tropical Medicine & Hygiene. 52: 559-564

Motazeidian H, Noyes H, Maingon R. 1996. *Leishmania* and *Sauroleishmania*: The use of random amplified polymorphic DNA for the identification of parasites from vertebrates and invertebrates. Expermintal Parasitology. 83: 150-154

Sambrook J., Fritsch EF, Maniatis T. 1989. Molecular cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press NewYork

Steindel M, Neto ED, DE Menezes, CLP Romanha, A Simpson, AJG. 1993 Random amplified Polymorphic DNA analysis of *Trypanosoma cruz'istrains* Molecular and Biochemical Parasitology. 60: 71-80,

Thompson RCA, Constantine CC, Morgan UM, 1998. Overview and significance of molecular methods; What role for molecular epidemiology? *Parasitology* 117: S 161-S175

Welsh J, Mclelland M, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acid Research. 18: 7213-7218.

Williams JG K, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research. 18; 6531-6535

Williams SA, Desimone SM, McReynolds A. 1988. Species Specific oligonucleotide probes for the identification of human filarial parasites. Molecular and Biochemical Parasitilogy. 28 : 163-170

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Spatial variation in the infection of the mudsnail *Cerithidea cingulata* by larval trematodes in the southern shore of Kuwait Bay

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The component community of larval trematodes infecting the marine prosobranch mudsnail *Cerithidea cingulata* was examined at five different sites along the southern shore of Kuwait Bay. In total 14 trematode species were encountered representing the families: Cyathocotylidae (2), Hemiuridae (1), Echinostomatidae (2), Haplosplanchnidae (1), Heterophyidae (3), Microphallidae (1), Philophthalmidae (2) and Plagiorchiidae (2). The hemiurid (cystophorus) and haplosplanchnid species use fish as definitive host while the other 12 species use waterfowls. Ten of the encountered species occurred in snails collected at Khwaisat (824 snails examined), 9 at Sulaibikhat (442 snails examined), 9 at Doha (581 snails examined), 6 at Shuwaikh (300 snails examined) and 5 at Kuwait City (1262 snails examined). The overall prevalence of infections was spatially variable among sampling sites: 48.0% at Sulaibikhat, 12.9% at Doha, 8.0% at Shuwaikh, 7.0% at Khwaisat and 1.9% at Kuwait City. The prevalence of infection for each species was generally below 3.3%, except for cyathocotylid-II and the microphallid, reaching 36.4% at Sulaibikhat and 7.2% at Doha, respectively. Trematode life-cycle patterns, distribution and diversity of waterfowl populations, coastline topography, tidal cycle and current movements are probably major contributors to spatial variation in the component community of trematodes in C. *cingulata* in Kuwait Bay.

Keywords: *Cerithidea cingulata*, Gastropoda, Kuwait, Spatial variation, Trematoda.

The structure and organization of the component community of larval trematodes parasitizing invertebrate intermediate hosts are highly complex resulting from long and intense relationship between host and parasite populations. The dynamics of this relationship are under constant manipulation by complex sets of biotic and abiotic factors controlling trematode transmission cycles and determining strategies (Esch *et al*., 1990). In marine and freshwater systems, local ecological factors and processes, host and parasite population characteristics and parasite recruitment are among the major factors generating variations in larval trematode community structure in snail intermediate hosts (Sousa, 1990).

Studies on trematodes infecting snails in Kuwait Bay have revealed rich and diverse taxa exhibiting temporal trends in transmission patterns (Abdul-Salam *et al.*, 1994; Abdul-Salam *et al.*, 1997; Abdul-Salam and Sreelatha, 1998, 1999; Al-Kandari *et al.*, 2000). The present study is the first attempt to investigate spatial variation in the component community of trematodes in a snail intermediate host in the Bay. It is focused on infections in the mudsnail *Cerithidea cingulata* (Gmelin, 1791), the most prevalent mollusc in the intertidal zone of the Bay.

The five snail-sampling sites are located at the southern shore of Kuwait Bay (Figure 1). The Bay is characterized by extensive intertidal mudflats formed by the settling of silt drifted by the rivers Tigris and Euphrates through the Shat al-Arab waterway. Environmental conditions are similar in the snail-sampling sites. Tides are diurnal, varying from 3.5 to 4.0 m. At high tide, the tidal flat constitutes an important nursery ground for larvae and juveniles of many invertebrate and fish taxa, and at low tide, the

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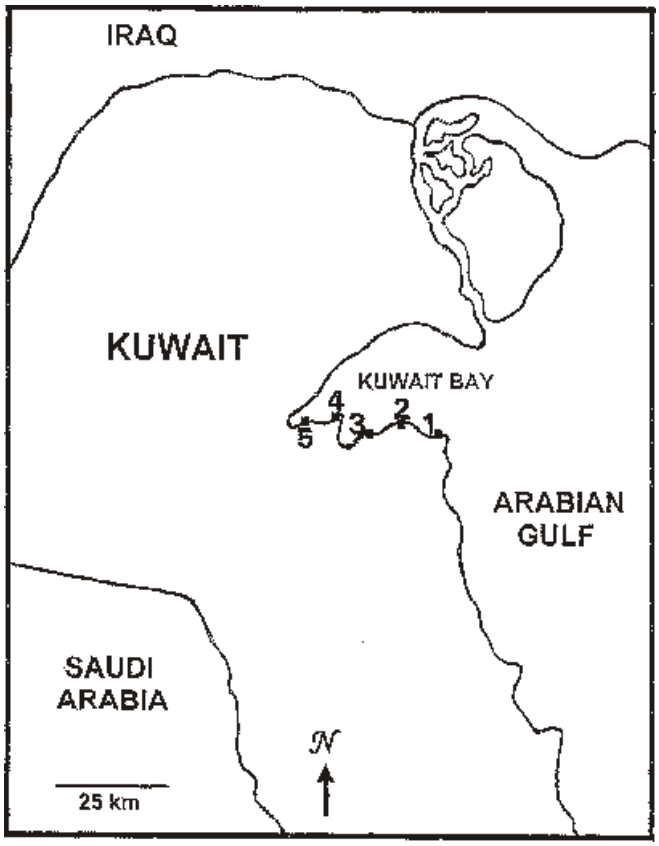

Figure 1. Map of Kuwait Bay showing snail-sampling sites. 1, Kuwait City; 2, Shuwaikh; 3, Sulaibikhat; 4, Doha; 5, Khwaisat.

mudflats serve as major resting and feeding grounds for large populations of resident and passage migrant waterfowls. Shoreline of the sampling sites is typical of the muddy tidal flats of the Bay. The Doha, Khwaisat and Sulaibikhat sites are relatively undisturbed, while Kuwait City and Shuwaikh sites are heavily urbanized and most of the tidal area is reclaimed for commercial, industrial or recreational purposes. *Cerithidea cingulata* is the dominant gastropod in the intertidal mudflats of the Bay occurring in dense aggregates at the lower shore. Snails representing all shell length were collected from the intertidal zone at different times between 1997 and 2002.In the laboratory the shell was gently crushed and the soft tissues were removed and examined under a stereomicroscope for the presence of larval trematode infections, which are identified according to Cable (1956) and Schell (1985).

A total of 14 different trematode species were encountered in C. *cingulata* from the five sampling-sites. The number of snails examined and prevalence of trematode species encountered at the sampling-sites are shown in Table I, and the life-cycle patterns for the encountered families (Schell, 1985) are summarized in Table II. The cystophorus and haplosplanchnid species use fish as definitive host while the other 12 species use waterfowls. The trematode component of the parasite community comprised 10 species at Khwaisat, 9 species at each Doha and Sulaibikhat, 6 species at Shuwaikh and 5 species at Kuwait City. The species, cyathocotylid-II, the haplosplanchid, heterophyid-I and philophthalmid-I, were commonly found at all sampling sites, while cyathocotylid-I, echinostome-I, heterophyid-II, plagiorchiid-I and plagiorchiid-II were rarely encountered. The overall prevalence of the infections is spatially variable among the sampling sites, ranging from 1.9% at Kuwait City to 48.0% at Sulaibikhat. Prevalence of cyathocotylid-II (36.4%) at Sulaibikhat and the microphallid (7.2%) at Doha was highest compared to the other three sites.

Ecological processes such as population structure of the definitive and intermediate hosts and habitat characteristics contribute to structuring parasitic trematode communities in marine and freshwater snails (Rohde, 1981; Curtis and Hurd, 1983; Fernandez and Esch, 1991; Sousa, 1993). Results of the present study show that composition and prevalence of trematodes infecting the mudsnail C. *cingulata* are spatially variable among different habitats along the southern shore of Kuwait Bay. A high species composition (9-10 species) was recorded at Doha, Khwaisat and Sulaibikhat, all of which represent undisturbed areas located in the vicinity of Al-Jahra Birds Sanctuary. The Sanctuary provides a favorable resting ground for diverse taxa of resident, winter visitor and migrant waterfowls. On the other hand, the overall prevalence of the infections was highest at Sulaibikhat (48.0%) and Doha (12.9%), areas with vast intertidal mudflats serving as major resting and feeding ground for abundant populations of waterfowls. The species composition was lowest (5-6 species) at Kuwait City and Shuwaikh, areas with high human population density and activity. Coastal habitat disturbances influence trematode component community by upsetting the ecology of intermediate and definitive hosts populations and the biology of

Table I : Prevalence (%) of larval trematodes infecting the mudsnail *Cerithidea cingulata* at five sampling sites in the southern shore of Kuwait Bay, between 1997 and 2002. CtI, Cyathocotylid-I; CtII, Cyathocotylid-II; Cp, Cystophorus; EcI, Echinostome-I; EcII, Echinostome-II; Hp, Haplosplanchnid; HtI, Heterophyid-I; HtII, Heterophyid-II; HtIII, Heterophyid-III; Mc, Microphallid-I; PhI, Philophthalmid-I; PhII, Philophthalmid-II; PgI, Plagiorchid-I; PgII, Plagiorchiid-II.

Site	Snail Examined	Prevalence (%) of trematode species													
		CtI	CtII	Cp	EcI	EcII	Hp	HtI	HtII	HtIII	Mc	PhI	PhII	PgI	PhII	Total
Doha	581		1.03	0.52		0.52	0.68	2.24		0.17	7.23	0.34		0.17		12.90
Khwaisat	824		0.73	0.24			1.6	0.73	0.73	0.49	1.82	0.36	0.12		0.24	7.04
Kuwait City	1262		0.24	0.24			0.16	1.11				0.16				1.90
Shuwaikh	300		0.66	0.66			3.00	2.00				1.33	0.33			8.00
Sulaibikhat	442	2.2	36.40		0.90	0.23	2.04	1.58			3.39	0.23	0.91			47.96
Total	3409	0.2	5.22	0.29	0.12	0.12	1.09	1.35	0.18	0.15	2.11	0.35	0.18	0.03	0.06	11.52

parasite life-cycles (Lafferty, 1997). The deposition of anthropogenic sediment in coastal areas around Kuwait City and Shuwaikh Harbor is bound to have profound effects on the diversity and abundance of potential hosts, i.e., waterfowls, fish, crustaceans and molluscs. Bustnes and Galaktionov (1999) showed that deposited sediments alter environmental factors such as temperature, salinity, pH and oxygen tension that may influence trematode community structure by affecting hatching, longevity and activity of the infective stages, cercariae and miracidia.

Bird trematodes constitute the dominant component of the larval trematode community of *C. cingulata* in Kuwait Bay, as 86% of the encountered species are parasites of waterfowls. Other studies on trematode fauna of snails in Kuwait Bay have also revealed high proportions of species that use waterfowl as definitive hosts (Abdul-Salam and Al-Khedery, 1992; Al-Kandari *et al.*, 2000). This phenomenon is not surprising knowing that the Bay is located on the crossroads of two major bird migration routes between continents; the first route extends from eastern Europe to the Indian subcontinent, and the second, from the Caspian Sea to Central Africa. In addition to the passage migrants, many waterfowls travel from northern latitudes to spend the entire winter in the mild climate of Kuwait Bay. Studies have shown that the distribution of the final hosts, in particular waterfowls, is one of the major contributors to spatial richness and distribution of trematode infections among molluscs in marine and freshwater habitats (Esch and Fernandez, 1994; Kube *et al.*, 2002). Sousa (1990) investigated spatial patterns of trematode infections in C. *californica* in the western coastline of the United States and concluded that: (1) patterns of trematode species diversity in the snail were independent of interspecific antagonism among trematode species, density of snail hosts, or variation in host size, and (2) spatial and temporal variation in the abundance of

Table II : Life-cycle patterns of families of trematodes infecting the mudsnail *Cerithidea cingulata* at the southern shores of Kuwait Bay (after Schell, 1985).

Trematode family	Trematode Species	Second intermediate host	Definitive host
Cyathocotylidae Poche, 1962	Cyathocotylid-I Cyathocotylid-II	Fish	Birds
Hemiuridae Luhe, 1901	Cystophorus	Copepods	Fish
Echinostomatidae Looss, 1902	Echinostomatid-I Echinostomatid-II	Annelids, fish or molluscs	Birds
Haplosplanchnidae Poche, 1926	Haplosplanchnid	Not required*	Fish
Heterophyidae Odhner, 1914	Heterophyid-I Heterophyid-II Heterophyid-III	Fish	Birds
Microphallidae Travassos, 1920	Microphallid	Crustaceans	Birds
Philophthalmidae Travassos, 1918	Philophthalmid-I Philophthalmid-II	Not required*	Birds
Plagiorchiidae Luhe, 1901	Plagiorchiid-II Plagiorchiid-II	Molluscs or insects	Birds

* Cercariae encyst as metacercariae on vegetation or other substrates.

infective stages in the parasite life-cycle is the primary determinant of trematode component community structure. Accordingly, the detected overall high trematode composition and prevalence in *C. cingulata* is probably contributed by rich avifauna providing a constant influx of trematode eggs into the Bay. Studies have shown that trematode eggs deposited by waterfowls in a certain area are often drifted by current before they are eaten by snails or miracidia hatch (Probst *et al.*, 2000; Smith, 2001). Therefore, it is possible that the final spatial distribution of trematodes in *C. cingulata* in the Bay is also influenced by distribution patterns of trematode eggs by different stochastic mixing processes, i.e., semi-diurnal tidal current movement and anticlockwise circulation of the water mass in the Arabian Gulf.

The present study reveals for the first time spatial heterogeneity of trematode parasite communities in a snail in the southern coastline of Kuwait Bay. Variations in trematode life-cycle pattern, waterfowls distribution, diversity and abundance, coastline topography, tidal cycle and currents movement are probably major contributors to the spatial heterogeneity. Understanding the processes of structuring assemblages of trematode parasites in Kuwait Bay system requires in depth studies of prevailing environmental factors, and the biology and ecology of the encountered species.

REFERENCES

Abdul-Salam J and Al-Khedery B. 1992. The occurrence of larval Digenea in some snails in Kuwait Bay. Hydrobiologia 248: 161-165.

Abdul-Salam J and Sreelatha BS 1998. A list of larval digenetic trematodes parasitizing some marine invertebrates of Kuwait Bay. Kuwait Journal of Science and Engineering 25: 409-34.

Abdul-Salam J and Sreelatha, BS. 1999. Component community structure of larval trematodes in the snail *Cerithium scabridum* from Kuwait Bay. Current Science 77:1416-1417.

Abdul-Salam J Sreelatha BS. and Al-Kandari, W. 1997. Temporal variations in the infection of population of *Clypeomorus bifasciata* (Gastropoda: Prosobranchia) bya digenean microphallid larva in Kuwait Bay. Journal ofHelminthology 71: 1-7.

Abdul-Salam J Sreelatha BS and Askhanani H 1994. Seasonal prevalence of trematode cercariae in *Clypeomorus bifasciata* (Gastropoda: Prosobranchia) in Kuwait Bay. Folia Parasitologica 41: 247-252.

Al-Kandari WY, Abdul-Salam J and Meakins, R. 2000. Temporal variations in the infection of a population of *Cerithidea cingulata* by larval trematodes in Kuwait Bay. Journal ofHelminthology 74: 17-22.

Cable RM 1956. Scientific survey of Puerto Rico and the Virgin Islands. Marine cercariae of Puerto Rico. New York Academy of Sciences 14: 491-577.

Curtis LA and Hurd LE 1983. Age, sex and parasites: spatial heterogeneity in a sandflat population *ofllyanassa obsoleta*. Ecology 64: 819-829.

Esch GW and Fernandez JC 1994. Snail-trematode interactions and parasite community dynamics in aquatic systems: a review. American Midland Naturalist 131:209-237.

Esch GW, Shostak AW, Marcogliese DJ and Goater TM 1990. Patterns and processes in helminth parasite communities: an overview. In Parasite Communities: Patterns and Processes. Esch, G. Bush, A. and Aho, J. (Eds.). Chapman and Hall, London,pp.1-20.

Fernandez JC and Esch GW 1991. Guild structure of the larval trematodes in the snail *Helisoma anceps*. Journal ofParasitology 77: 937-944.

Kube J Kube S and Dierschke V. 2002. Spatial and temporal variations in the trematode component community of the mudsnail *Hydrobia ventrosa* in relation to the occurrence of waterfowl as definitive hosts. Journal ofParasitology 88: 1075-1086.

Lafferty KD 1997. Environmental parasitology: what can parasites tell us about human impacts on the environment? Parasitology Today 13:251-255.

Probst S Kube J and Bick A 2000. Effects of winter severity on life history patterns and population dynamics of *Hydrobia ventrosa* (Gastropoda: Prosobranchia. Archiv fur Hydrobiologie 148: 383-396.

Rohde H 1981. Population dynamics of two snail species, *Planaxis sulcatus* and *Cerithium moniliferum*, and their trematode species at Heron Island, Great Barrier Reef. Oecologia 49: 344-352.

Schell SC 1985. Handbook of Trematodes of North America, North of Mexico. University Press of Idaho, Moscow, Idaho, p. 263. Smith, N.F. 2001. Spatial heterogeneity in recruitment of larval trematodes to snail intermediate hosts. Oecologia 127: 115-122.

Sousa W 1990. Spatial scale and the processes structuring a guild of larval trematode parasites. In Parasite Communities: Patterns and Processes. Esch, G. Bush, A. and Aho, J. (Eds.). Chapman and Hall, London, pp. 41-68.

Sousa W 1993. Interspecific antagonism and species coexistence in a diversity guild of larval trematode parasites. Ecological Monographs 63 : 103-128.

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The penetration of *Plasmodium* into red blood cell is a protective mechanism of malaria parasite against high levels of accummulated nitric oxide in blood circulation

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In addition to numerous cytokines, nitric oxide (NO) is thought to be an important mediator and key signaling molecule of malaria pathology. It is suggested that NO is critical for intracellular parasites such as *Plasmodia.* The current study has investigated the involvement of down/upstream molecules including reactive nitrogen intermediate (RNI) and inducible nitric oxide synthase (iNOS) in both *P. berghei* parasitised (PRBC) and uninfected red blood cells (URBC). The actual intra- and extra- cellular pattern of RNI and iNOS is suggested to be related to *Plasmodium* activities. The RNI accumulation was measured by Griess micro assay (GMA) and the iNOS expression was detected by Western blot (WB) technique. The results showed that the RNI level inside the PRBC and URBC were low at baseline detection level by GMA whereas the RNI outside RBC (Plasma) showed significantly higher concentration *($p<0.05$)*. Moreover, the iNOS protein was expressed only in blood of malarial animals, but not in control group, which indicates a contribution of host's white blood cells (WBC) in production of iNOS in response to malaria infection. The data of this study revealed that *Plasmodium* may be sensitive to toxic effects of high NO levels or its down/upstream molecules (RNI, iNOS) in blood circulation, therefore its penetration into RBC could be a protective mechanism for parasite, which in red blood cells is constantly surrounded by hemoglobin (Hb), a very potent scavenger for NO.

Key words : Haemoglobin, iNOS, Nitric Oxide, *Plasmodium*, RBC, RNI.

INTRODUCTION

Potential effector mechanisms of immunity against malaria include antibodies, macrophages, cytotoxic T-cells, cytokines and a variety of other soluble mediators (Hommel, 1996). NO is produced by many cells and released into the circulation or trapped by other cells. Mononuclear and several other types of mammalian cells produce NO after stimulation with cytokines, bacterial endotoxin or antigens of infectious agents (Oliveira *et al*, 1998). It is a chemical messenger mediating events in a large number of biological systems, including the brain, cardiovascular, endocrine, immune, pulmonary and renal systems (Rockett *et al*, 1992). NO and RNI may react in several ways to cause cell death by diffusing into cells as well as the formation of nitrosothiol groups, leading to inactivation of enzymes or changes in protein functions (Ellis *et al*, 1998) Figure 1).

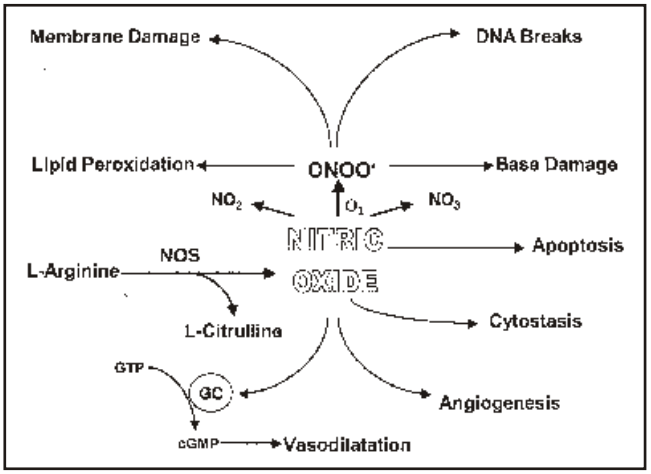

Figure 1 : Biochemical pathway of nitric oxide, **NOS -** nitric oxide synthase, **ONOO -** peroxynitrite, **GC -** guanylate cyclase, **GTP -** guanine triphosphate, **cGMP -** cyclic guanosine monophosphate.

A role for antiparasitic effects of NO and RNI have been demonstrated *in vivo* and *in vitro* against a number of parasites including : *Plasmodium spp., Leishmania spp., Toxoplasma gondii, Schistosoma spp.* and *Trypanosoma brucei* (Clark *et al,* 1996; Motard *et al,* 1993; Rockett *et al,* 1991; Roitt *et al,* 1998) NO may be a key mediator in the immune responses against malaria (Bogdan *et al,* 2000) or a critical signaling molecule of malaria pathology (Taylor *et al,* 1997). In malaria infected patients, NO is generated by macrophages and appears to be responsible for the malaria tolerance (Clark *et al,* 1996), inhibition of malaria parasites activity (Rockett *et al,* 1992) or may even be involved in the killing of infected hepatocytes (Hommel, 1996). However, there are some contradictory reports about the role of NO and related molecules in malaria. Some researchers propose that NO has a protective role in malaria (Scheller *et al,* 1997; Chiwakata *et al,* 2000; Amante and Good, 1997), whereas others conclude it is of little protective importance and may even contribute to the development of severe malaria (Jones *et al,* 1996; Clark *et al,* 1992; Rocket *et al,* 1994). The possible antiparasitic or pathological roles of NO in murine models of malaria have been reviewed, but it remains an unanswered question as to whether the actions of NO are protective or damaging or both during malaria (Mohan and Stevenson, 1998). In view of the ubiquitous occurrence of NO, its short biological life and the multiple tissues affected by *Plasmodia,* it is not surprising that the overall value of NO in the host response to malaria is a matter for debate (White, 1998; Rockett *et al,* 1994).

The half-life of NO in biological samples is reported to be short about 5 s or less (Ellis *et al,* 1998). NO in the presence of oxygen is unstable and it rapidly decomposes to yield NO_2 (Jourd'heuil *et al,* 1997). Hb catalyses the conversion of NO_2 to NO_3, therefore, most of the decomposed NO appearing in plasma is in the form of NO_3 (Granger and Kubes, 1983). The GMA used in this study, measures both free nitrite and the larger nitrate pool, these values are commonly used as a measure of RNI (Nahrevanian and Dascombe, 2001). The GMA detects stable NO metabolities that accumulate NO produced both locally and from the sites far away (White, 1998).

The levels of NO and its up/downstream metabolities are generally measured in plasma, the surrounding medium for RBC, however, understanding the amount of NO production inside the RBC seems to be important for parasite survival. The blood circulation may also play a key role to transfer NO and RNI from target organs to other parts, in order to eliminate these toxic materials by exposing to scavenger sites (Nahrevanian and Dascombe, 2001). One of the mechanisms which may explain NO scavenging in RBC, is reaction of nitrite with Hb to form nitrosothiol-Hb and met-Hb (Rockett *et al,* 1991), which may provide a less toxic condition for *plasmodia* survival.

The objective of this study was to measure RNI levels inside the PRBC and comparison with that in plasma. The RNI measurement inside both PRBC and URBC, in addition iNOS detection in both malaria and control groups may represent the actual levels of NO production, which could be critical for intracellular *Plasmodia.* In this study RNI production was assayed in plasma and RBC lysates of both malaria and control groups. In addition, the expression of iNOS protein in blood homogenates is investigated by WB. The detection of RNI and iNOS as down/upstream NO metabolities may clarify the real pattern of NO production in both sides of membrane of RBC during this murine malaria infection.

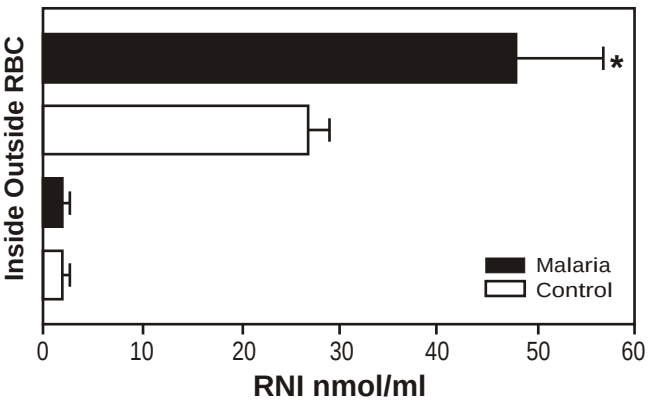

Figure 2 : The concentration of RNI inside and outside RBC, Values of RNI from RBC haemolysed suspensions and plasma samples are expressed as mean ±SEM, $n=5$. Malarial animals were inoculated with *P. berghei* and control mice received and equivalent volume of diluted URBC. Statistical analysis were determined by Student's *t*-test with a significant difference of $*P<0.05$ using Graph Pad Prism.

MATERIALS AND METHODS

Animals

Animals used in this study were male MF1 mice supplied by the Biological Services Unit, University of Manchester. Animals were housed in plastic cages at room temperature (19-22°C), on a 12h light (08:00-20:00) and 12h dark (20:00-08:00) cycle, with unlimited access to food (CRM feeding pellets, SDS) and tap water. Experiments were licensed under the Animals Act (UK Scientific Procedures) 1986. In compliance with the conditions of this license, infected animals were humanely killed at the onset of the terminal phase of malaria.

Experiments and groups

Ten mice were used in this study, divided into two groups i.e. malaria and control groups. Plasma samples and RBC hemolysed suspensions were collected for RNI and iNOS measurement using GMA and WB respectively.

Malaria parasite

P. berghei N/13/1A/4/203 originally obtained from the School of Tropical Medicine, University of Liverpool, U.K. was used in this study. Parasite was maintained by blood passage in mice when active parasites were required; otherwise it was stored at -70°C in Alsever's solution (2.33% glucose, 0.52% NaCl and 1% sodium citrate in deionized water) and glycerol (9:1 parts by volume).

Inoculation of malaria parasite

Mice were inoculated (0.2 ml, i.v.) into a tail vein with blood from a donor *P. berghei* infected mouse diluted with 0.85% saline to contain $2x10^7$ PRBC. Control animals received an equivalent volume (0.2 ml/mouse, i.v.) of 85% saline diluted URBC.

Parasitaemia

In malarial animals parasitaemia was determined using blood smears stained with Leishman's stain (Sigma Chemical Co., USA) prepared in methanol at 2mg/ml. PRBC were counted in five different fields, each of approximately 200 cells. Results are expresed as the mean percentage (%) of erythrocytes containing Leishman positive bodies.

Preparation of plasma

Mice were terminally anaesthetised by inhalation of diethyl ether (BDH, England) and blood taken by cardiac puncture into a 1 ml syringe containing 50 i.u. heparin (Monoparin, CP Pharmaceuticals Ltd., Wreham, UK). Plasma was prepared by centrifuging blood at 3000 rpm (MSE Centaur 2, UK) for 10 min and stored at -70°C until assayed. After blood collection, animal were humanely sacrificed by cervical dislocation.

Lysis of RBC by hypotonic shock

Blood was taken by cardiac puncture under terminal general anaethesia and RBC pellet prepared by centrifugation. This method was adapted from Rockett *et al*. (1994) with modification of using direct lysis in hypotonic deionised water (d.H_2O). The method is based on osmosis and the effect of hypotonic solutions on cells, which leads to water flow through cell membranes. The protocol applied was as follows. Blood samples were centrifuged at 2500 rpm (Centriguge, MSE, Centaur 2, Co., UK) for 10 min, then plasma and buffy coat removed as supernatant. Pellets were washed with PBS and centrifuged 10 min at 2500 rpm (3 times). d. H_2O was added in equal volume to sedimented RBC; contents were mixed vortexed thoroughly for 30s, incubated for complete haemolysis at room temperature (19-22°C) for 30 min and finally centrifuged 15 min at 3000 rpm. Supernatants were stored at -20°C until subjected to GMA. The amounts of RNI at the end of calculations were multiplied by two, because of the 1:1 dilution of RBC in d. H_2O used during cell lysis.

Griess Micro Assay (GMA)

The GMA was adapted from the methods of Rockett *et al*. (1994) and Amante and Good (1997) to assay nitrite. RNI was measured in samples using the Griess reaction after converting nitrates to nitrites with nitrate reductase treatment. 60 µl samples were treated with 10 µl nitrate reductase (NAD[P] H Aspergillus species, Sigma Chemical Co. UK) and 30µ1 NADPH β-nicotinamide adenine dinucleotide phosphate (Sigma Diagnostics, St Louis, USA). 200µl Griess reagent

(5% phosphoric acid, 1% sulfanilic acid and 0.1% N (l-naphthyl-l)-ethylendiamine dihydrochloride (NED), all from Sigma Chemical Co. UK, dissolved in 100 ml d.H_2O) was then added and proteins subsequently precipitated by 200 µ1 trichloroacetic acid 10%, (BDH, England). Tube contents were vortex mixed and centrifuged at 15000 rpm (Model 1-13 Microcentrifuge, Sigma, UK). Duplicate 200µl of supernatants were transferred to a 96-well flat-bottomed microplate (Costar, USA) and absorbance read at 520 nm using a microplate reader (Dynatech, MRX, USA).

Western Blotting (WB) for detectionof iNOS

The protocol for WB was adapted from Park *et al.* (2000) and tests were done using a antibody according to the instructions of the manufacturer of the kit (Transduction Laboratory, Lexington, USA). Blood samples were taken from mice under terminal anaesthesia and transferred immediately onto dry ice. Then 500 µl of boiling lysis buffer [Sodium dodecyl sulfate (SDS, 1g), sodium orthovanadate (0.184g) and tris buffer (1.21 g), pH=7.5, gradually added to d.H_2O to have a final volume of 100 ml] was added to 100 µl blood, homogenised (for 10-60 s), boiled for 2 min at 95°C and centrifuged at 13,000 rpm (Model 1-13 Microcentrifuge, Sigma Co., UK) for 5 min. An aliquot of the samples was diluted 10-fold for protein content determination. SDS polyacrylamide gel 8% was prepared and samples were boiled at 95°C for 5 min prior running in gel. 10µl sample (10µg protein) was loaded per lane along with marker using a micro syringe (Hamilton Bonaduz AG, Switzerland) and electrophoresed in a mini-tank initially for 10 min at 90 V then for 60 min at 120 V. Nitrocellulose membrane 0.45 µm (Hybond ECL, Amersham Pharmacia Biotech, UK), blot and buffered-papers were placed respectively in semi-dry transfer-blot (Bio-Rad Co., UK) After 60 min at 18 V, proteins were transferred to nitrocellulose then stained with a 10% Ponceau S Red (Sigma-Aldrich, Poole, UK) for 30s. Gels were also stained with Coomassie Blue (Sigma-Aldrich, Poole, UK) for 30 min. Blots were blocked with a 5% blocking solution [5 g Marvel dried skimmed milk (Premier Brands Ltd., Co., UK) made up in 100 ml TBS/Tween (33.3 ml of NaCl, 5 M was added to 10 ml Tris-HCl, 1 M and 0.5 ml stock Tween-20 and made up volume to 1L with d.H_2O)] for 2 h at room temperature (19-22°C) or overnight at 4°C to prevent non-specific binding. Primary rabbit anti-iNOS polyclonal Ab (Transduction Laboratory, Lexington, USA) was diluted (1:10,000) in a 5% blocking solution, added to the membranes, incubated overnight at 4°C and washed 3 times for 10 min with TBS/Tween with agitation. Membranes were then incubated with secondary goat anti-rabbit IgG: horseradish peroxidase (HRP, Transduction Laboratory, Lexington, USA) for 1 h at room temperature with agitation. Both antibodies were diluted according to manufacturer recommendations. Membranes were finally washed 3 times for 10 min each with TBS/Tween and incubated with enhanced chemiluminescent (ECL) reagent (New England Biolabs Co., UK), Chemiluminescent Substrate LumiGLO® for 1 min, excess solution was removed, wrapped in plastic and placed into a light tight developing cassette. They were then exposed to X-ray Hyper film ECL™ (Amersham Pharmacia Biotech, UK) in a dark room for 10-60 s. The ECL reagent reacts with the HRP-conjugate to emit light, which is detected by placing film over the membrane. Film was processed by film processor (Kodak, X-OMAT M35, CO., UK) and membranes were stained with Higgins Black India ink (diluted 1:1,000 in wash buffer) to confirm the same bands with that in X-ray film.

Statistical analysis

Values for RNI concentration are presented as the mean $\pm$ standard error of mean (SEM) for groups of n=5 mice. The significance of difference ($P<0.05$) was determined by Student's *t*-test using GraphPad Prism Software (GraphPad, San Diego, California, USA).

RESULTS

The RNI concentrations inside the RBC were low (1.9 $\pm$ 0.3 nmol/ml), which is the baseline level of RNI measured by the GMA in this experiment. Although, no difference was observed in the inside RNI levels between URBC and PRBC hemolysates, however, it showed a significantly higher levels outside RBC (plasma) of both malaria and control groups (URBC : inside 1.9 $\pm$ 0.3, outside 26.8 ± 2 .1: PRBC inside 1.9 ±

0.3 outside 47.7 ± 9.1 nmol/ml, $P<0.05$, n=5) (Figure 2). In addition, the iNOS protein was detectable only in blood of malarial animals, but not in control group, which emphasises the involvement of blood components in iNOS induction during malaria infection (Figure 3).

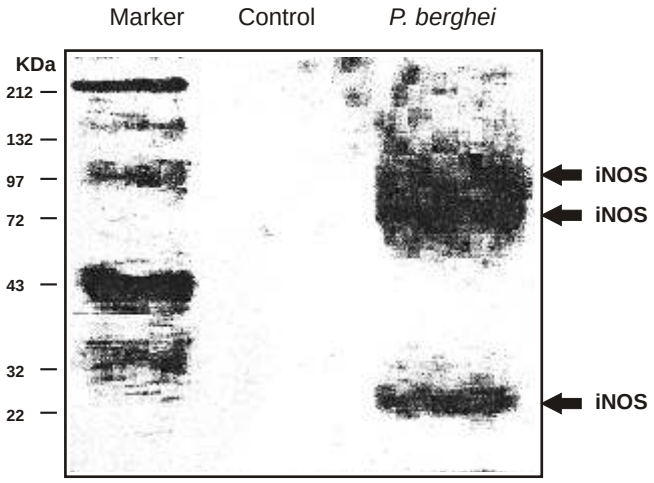

Figure 3 : Western blot of iNOS protein expression in blood of malaria and control group, Lanes : Marker : Mol wt. marker; control URBC; *P. berghei :* PRBC taken from infected animals onday 4 Blood samples were homogenised with lysis buffer and proteins were determined with Bradford assay using albumin standard. Samples were then boiled for 5 min at 95ºC in sample buffer, then 10 µl of each protein was separated by SDS-PAGE 8%, transferred electrophoretically to a nitrocellulose membrane 0.45 µm, probed with a rabbit polyclonal anti-iNOS Ab followed by a goat anti-rabbit IgG conjugated with HRP directed to LumiGLO® Chemiluminescent Substrate and finally detected in a hyper film by a Kodak film processor.

DISCUSSION

Although, the protective immune response against malaria parasite is multifactoral and the final effecter molecules that mediate parasite death are not known, in the current study, NO and its down/upstream molecules have been implicated (Dascombe and Nahrevanian, 2003). The increase in RNI accumulation and iNOS expression during *P. berghei* malaria suggests that NO and its metabolites have a role during this murine malaria infection (Nahrevanian and Dascombe, 2001). NO is a freely diffusible molecule through the RBC membrane (Balmer et al, 2000), however, the GMA detected low level inside the RBC suspensions. The levels of RNI in plasma (outside RBC) was at its high effective concentration (27 times), which could be one of the reasons for parasite to penetrate into RBC membrane to avoid high toxic level of NO and other possible cytokines.

The probable mechanism of low RNI level inside RBC may be that haemoglobin (Hb) binds to NO and downstream metabolites e.g. RNI, but not to upstream factors e.g. iNOS (Nahrevanian and Dascombe, 2001), which emphasis that Hb scavenges NO and prevents nitrite formation. On the other hand, NO is known to react rapidly with oxy-Hb to give nitrate and met-Hb. Even if NO is cytotoxic for *Plasmodia,* some would argue it is an unlikely effecter molecule *in vivo* against erythrocytic parasites, which are constantly surrounded by a potent NO scavenger such as Hb (Jones et al, 1996). The scavenging of NO by Hb in blood vessels should represent a significant sink for this molecule (Lancaster, 1997), which could explain why it is present in such small amounts inside red blood cells (Granger and Hibbs, 1996). Hb within the RBC may prevent NO from reaching the *Plasmodia* (Jones et al, 1996) and thus inhibits its toxic effects. Therefore, scavenging of NO by Hb, decreases its cytotoxic effects against intracellular *plasmodium* and explains its penetration into RBC which is a protective intracellular medium (Taylor-Robinson, 1998). Figure 4).

Furthermore, Hb may serve a dual role, as a scavenger and also as a donor for NO. It is thought an equilibrium between binding of NO to Hb (NO scavenging) and).

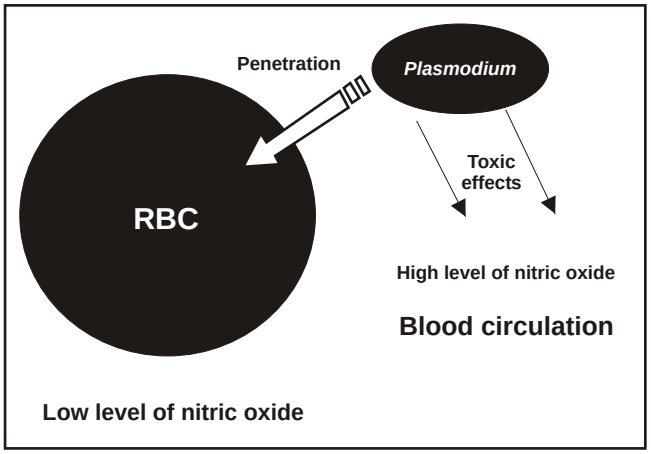

Figure 4 : Proposed pattern of protection in *Plasmodium* against toxicity of high level of accumulated NO in blood circulation

unbinding (NO releasing) is reached at a given, variable oxygen tension (Taylor-Robinson, 1998). Morever, the sensitivity of malaria parasites to NO increases with the decreasing oxygen tension. This is because, at lower oxygen tension, Hb releases NO rather than binding the molecule, making it available to exert antiparasitic effect (Taylor-Robinson and Looker, 1998). It is suggested that a negative feedback by high level of NO can reduce the amount of NO production as well (Taylor *et al*, 1997). This feedback starts when NO concentration is saturated in the circulation *in vivo;* it can stop or may reverse the process of L-arginine oxidation to L-citrulline. This novel mechanism was identified whereby NO down-regulated iNOS gene expression, possibly to limit overproduction during the septic response (Stevenson *et al*, 1995). In addition, an inhibitory mechanism by haemozoin leads to reduction of RNI overproduction (Prada *et al*, 1996). NO could react with intra-parasitic molecules and participate in the assembly of free haeme into haemozoin or formation of S-nitrosothiols (Ghigo *et al*, 1995).

The iNOS activity was very high in the lysate of PRBC in current study, while not measurable in that of URBC, suggesting it's role in parasite-host interaciton. The iNOS protein was detected in 3 bands in malaria blood lysates including 22,72 and 97 KDa fragment sizes. This may be because NOS isozymes are fairly complex haeme proteins containing two identical, independent subunits (Mayer, 1998); alternative splicing of sequences in the NOS gene may be responsible for expression of different-sized NOS proteins (Park *et al*, 2000). The migration of purified NOS as multiple bands on SDS-PAGE has been reported previously by Ghigo et al. (1995) and Lyons *et al*. (1992). While NO itself may not be inhibitory to parasite development, its downstream products may have some anti-plasmodial activity (Jones *et al*, 1996). Taken together, this study and the date provided by others indicates that the expression of iNOS in murine malaria is detectable using WB technique; however its detection is depending on sensitivity of enzyme, its proteolysis during protein preparation, its rapid reaction in NO process and its short half-life as an enzyme.

The actual levels of RNI may indicate the involvement of NO during infection with *P. berghei,* however its contribution in malaria might be pathological or preventive. The pathological consequences of lethal malaria, including hepato-splenomegaly, anaemia, weight loss, hypothermia and a reduction of locomotor activity, may be related at least in a part to over-production of NO and RNI (Nahrevanian and Dascombe, 2002). The data of this study revealed that the *Plasmodium* may be senstive to toxic effects of high levels of NO in blood circulation, therefore its penetration into RBC could be protective mechanism of this intracellular protozoa. Perhaps, NO comes from several cellular sources, which can contribute towards the protective immune responses against *Plasmodia.* Further investigation in defining these sources will be important for the understanding of cell-mediated defence mechanism(s) in malaria.

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REFERENCES

Amante FH and Good, MF. 1997. Prolonged Thl-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. Parasite Immunol 19:111-126.

Balmer P, Phillips HM, Maestre AE, McMonagle FA and Phillips RS. 2000. The effect of nitric oxide on the growth of *Plasmodium falciparum, P. chabaudi* and *P. berghei* in vitro. Parasite Immunol 22: 97-106.

Bogdan C, Rollinghoff M and Diefenbach A. 2000. The role of nitric oxide in innate immunity. Immunological Review 173: 17-26.

Chiwakata CB, Hemmer CJ and Dietrich M. 2000. High levels of inducible nitric oxide synthase mRNA are associated with increased monocytes count in blood and have a beneficial role in *Plasmodium falciparum* malaria. Infecion and Immunity 68: 394-399.

Clark IA, Rockett KA and Cowden WB. 1992. Possible central role of nitric oxide in conditions clinically similar to cerebral malaria. Lancet 340 : 894-896.

Clark IA, al-Yaman FM, Cowden WB and Rockett KA. 1996. Does malarial tolerance, through nitric oxide, explain the low incidence of autoimmune disease in tropical Africa? Lancet 348:1492-1494.

Dascombe MJ and Nahrevanian H. 2003. Pharmacological assessment of the role of nitric oxide in mice infected with lethal and nonlethal species of malaria. Parasite Immunology, 25(3): 149-159.

Ellis G, Adatia I, Yazdanpanah, M and Makela, SK. 1998. Nitrite and nitrate analyses: a clinical biochemistry perspective. Clinical Biochemistry 31: 195-220.

Ghigo D, Todde R, Ginsburg H, Costamagna C, Gautret P, Bussolino F, Ulliers D, Giribaldi G, Deharo E and Gabrielli G. 1995. Erythrocyte stages *of Plasmodium falciparum* exhibit a high nitric oxide synthase (NOS) activity and release an NOS-inducing soluble factor. Journal of Experimental Medicine 182: 677-688.

Granger DL and Kubes P. 1983. Nitric oxide as anti-inflammatory agent. In: Packer, L. (Ed.), *Nitric oxide, Part B;* physiological and pathological processes, 269th Edition, Academic Press, pp.435-441.

Granger DL and Hibbs JB. 1996. High-output nitric oxide: weapon against infection? Trends in Microbiology 4 : 46-47.

Hommel M. 1996. Immunology of malaria. In: World Health Organization (Ed.), WHO, Health co-operation papers, Quaderni di Cooperazion Sanitaria, pp. 53-70.

Jones IW, Thomsen LL, Knowles R, Gutteridge WE, Butcher GA and Sinden RE. 1996. Nitric oxide synthase activity in malaria-infected mice. Parasite Immunol 18: 535-538.

Jourd'heuil D, Kang D and Grisham MB. 1997. Intreactions between superoxide and nitric oxide: Implications in DNA damage and mutagenesis. Frontier in Biosciences 2: 189-196.

Lancaster JRJ. 1997. A tutorial on the diffusibility and reactivity of free nitric oxide. Nitric Oxide 1: 18-30.

Lyons CR, Orloff GJ and Cunningham JM. 1992. Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. Journal of Biological Chemistry 267: 6370-6374.

Mayer B. 1998. Structure and function of Nitric Oxide Synthase. Nitric oxide: Biology and Chemistry 2: 390-391.

Mohan K and Stevenson MM. 1998. Acquired immunity to asexual blood stages In: Sherman, I.W. (Ed.), Malaria: Parasite Biology, Pathogenesis and Protection, Washington DC: ASM Press, pp. 467-493.

Motard A, Landau I, Nussler A, Grau G, Baccam D, Mazier D and Targett GA. 1993. The role of reactive nitrogen intermediates in modulation of gametocyte infectivity of rodent malaria parasites. Parasite Immunol 15 : 21-26.

Nahrevanian H and Dascombe MJ. 2001. Nitric oxide and reactive nitrogen intermediates in lethal and nonlethal starins of murine malaria. Parasite Immunol 23: 491-501.

Nahrevanian H and Dascombe MJ. 2002. Expression of inducible nitric oxide synthase (iNOS) mRNA in target organs of lethal and non-lethal strains of murine malaria. Parasite Immunol 24: 471-478.

Oliveira DM, Silva-Teixeira DN, Carmo SA and Goes AM. 1998. Role of nitric oxide on human schistosomiasis *mansoni:* upregulation of *in vitro* granuloma formation by N omega-nitro-L-arginine methyl ester. Nitric Oxide: Biology and Chemistry 2: 57-65.

Park CS, Krishna G, Ahn MS, Kang JH, Chung WG, Kim DJ, Hwang HK, Lee JN, Paik SG and Cha YN. 2000. Differential and constitutive expression of neuronal, inducible and endothelial nitric oxide synthase mRNAs and proteins in pathologically normal human tissues. Nitric Oxide 4: 459-471.

Prada J, Malinowski J, Muller S, Bienzle U and Kremsner PG. 1996. Effects of *Plasmodium vinckei* hemozoin on the production of oxygen radicals and nitrogen oxides in murine macrophages. American Journal of Tropical Medicine and Hygiene 54: 620-624.

Rockett KA, Awburn MM, Cowden WB and Clark LA. 1991. Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. Infection and Immunity 59 : 3280-3283.

Rockett KA, Cowden WB, Awburn MM and Clark IA. 1992. Arginine utilization and induction of nitric oxide synthase: cytokine-induced release of nitric oxide *in vivo* and its implications for the pathogenesis of cerebral malaria. In: Moncada, S. Martella, M.A. Hibbs, Jr.J.B. and Higgs, E.A. (Eds.) The biology of nitric oxide: 2 Enzymol Biochem and Immunol, Portland Press, London, UK pp. 135-137.

Rockett KA, Awburn MM, Rockett EJ, Cowden WB and Clark IA. 1994. Possible role of nitric oxide in malarial immunosuppression. Parasite Immunol 16: 243-249.

Roitt IM, Brostoff J and Male D. 1998. Immunology. 5th edition Mosby Publication, London, pp.132-314.

Scheller LF, Green SJ and Azad AF. 1997. Inhibition of nitric oxide interrupts the accumulation of CD8+ T cells surrounding *Plasmodium berghei-infected* hepatocytes. Infection and Immunity 65: 3882-3888.

Stevenson MM, Tam MF, Wolf SF and Sher A. 1995. IL-12-induced protection against blood-stage *Plasmodium chabaudi AS* requires IFN-gamma and TNF-alpha and occurs *via* a nitric oxide- dependent mechanism. Journal of lmmunol 155: 2545-2556.

Taylor BS, Kim YM, Wang Q, Shapiro RA, Billiar TR and Geller DA. 1997. Nitric oxide down-regulates hepatocyte-inducible nitric oxide synthase gene expression. Archive in Surgery 132: 1177-1183.

Taylor-Robinson AW. 1998. Nitric oxide can be released as well as scavenged by hemoglobin: relevance to its antimalarial activity. Parasite Immunol 20: 49-50.

Taylor-Robinson AW and Looker M. 1998. Sensitivity of malaria parasites to nitric oxide at low oxygen tensions. Lancet 30(351): 1630.

White NJ. 1998. Malaria Pathophysiology. In: Sherman IW (Ed.) Malaria, Parasite Biology, Pathogenesis and Protection, Washington DC, ASM Press, pp. 371-385.

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Leishmania donovani : Effect of pH on the infectivity of axenic amastigotes

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The promastigotes were transformed into amastigotes by giving acid and heat shock in modified NNN medium. Two different media were used for transformation i.e., NNN medium with basic MEM (pH-7.2) and NNN medium with acidic MEM (pH-5.5) as overlay. The promastigotes were cultured in these two media for 72 hrs at 22 ± 1°C and then incubated at 37 ± 1°C in 5% CO_2 for transformation into amastigotes. Within 96 hrs the promastigotes transformed into amastigotes as assessed by light microscopy. The infectivity of amastigotes was checked in vitro in mouse peritoneal macrophages and in vivo in Balb/c mice. A significant increase in percentage of infected macrophages was observed in cultures infected with amastigotes grown in acidic medium as compared to those grown in basic medium and promastigotes.Similarly, a significant increase in weights of spleen and liver and parasite load was observed in mice infected with amastigotes grown in acidic medium as compared to those grown in basic medium and promastigotes.

Key words : Amastigotes, *Leishmania donovani*, NNN medium, Promastigotes.

INTRODUCTION:

Leishmanial parasites have a simple digenetic life cycle, proliferating as extracellular flagellated promastigotes in the alimentary tract of the insect vector and as obligate intracellular amastigotes in the phagolysosomal vacuoles of mammalian macrophages. Promastigotes are flagellated, motile and vary from rounded and stumpy forms to elongated, highly motile metacyclic promastigotes. They range in size from 15-26 µm and grow at ambient temperatures ranging from 22°C to 26°C. On the other hand, the amastigotes are oval, intracellular and measure 2-3 µm in diameter. They have a relatively large, eccentrically located nucleus, and a bar shaped mitochondrial structure, the kinetoplast. Amastigotes are adapted to mammalian body temperature and the acid environment of the macrophage phagolysosome where reside(Chang et al 1985). The transformation of promastigotes to amastigotes occurs during phagocytosis by host macrophages with exposure in the parasites to an acid environment and increased temperature. The drastic morphological changes that the parasite undergoes during its life cycle are believed to be induced by the differences in the temperature, pH and chemical composition of the environment encountered in the host and in the vector. Comparative studies indicate that the morphology, physiology and antigenic composition of amastigote stage is distinct from that of promastigote stage (Coombs *et al.* 1988; Eperon and McMohan Pratt, 1989b).

The promastigotes can be easily cultured in vitro in several biphasic and monophasic media (Hendricks *et al.* 1978).The first in vitro cultivation of *Leishmania* was accomplished using a modified version of biphasic NNN medium (Nicolle, 1908) which was developed originally for the cultivation of trypanosomes (Novy and McNeal, 1905). Since then, several modifications in these media have led to the isolation and maintenance of different species (Ray, 1932; Rao *et al.* 1984). Supplementing liquid phase of NNN medium with minimum essential medium is suitable for in vitro cultivation of *L . donovani* promastigotes. However the long-term cultivation of axenic amastigotes is still a challenge. Several factors are essential for stabilization and maintenance of such cultures and it appears that each species requires

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special conditions relating to temperature, pH and nutrients.The short term in vitro cultivation of amastigotes has been achieved by acid shock and heat shock treatment (Mukadda *et al.* 1985; Doyle *et al.* 1991). The first successful cultivation of *Leishmania donovani* axenic amastigotes was achieved by Doyle *et al.* 1991 in 100% Foetal Calf Serum. Later a standard growth medium consisting of RPMI-1640, supplemented with 10% FCS and with a pH of 5.5 to 6.0 was developed (Antioine *et al.* 1990; Joshi *et al.* 1993). Amastigote like forms of *L. donovani* have also been cultured in NNN medium at a temperature of 34 ± 1°C and changes in several membrane parameters studied.(Gupta *et al.* 1996). But no studies are available regarding the use of two stresses i.e pH and temperature for transformation of promastigotes into amastigotes in vitro especially in NNN media .(Gupta *et al* 1996).

So, the present study was undertaken to transform promastigotes into amastigotes and to establish the long term culture of axenic amastigotes of *Leishmania donovani* in modified NNN medium (acidic and basic) and to check their infectivity in vivo in Balb/c mice and in vitro in mouse peritoneal macrophages.

MATERIAL AND METHODS:

Parasite: Indian strain of *Leishmania donovani*, viz; MHOM/IN/80/Dd8 was obtained from London School of Tropical Medicine and Hygiene, London. The promastigotes of this strain were maintained in the modified NNN medium by serial subcultures (Rao *etal.* 1984). Two different media were used for cultivation of parasites:

1. Culture of amastigotes in Modified NNN medium (basic):Log phase promastigotes maintained in the BOD incubator at 22 $\pm$ 1°C were used for transformation to amastigotes. The pH of the MEM which was used as overlay was adjusted to 7.2. The cultures were incubated at a temperature of 37°C with a relative humidity of 92-95% and 5% CO_2 in a CO_2 incubator.

2. Culture of amastigotes in Modified NNN medium (acidic):Log phase promastigotes maintained in the BOD incubator at 22 $\pm$1°C were used for transformation to amastigotes. . The pH of the MEM which was used as overlay was adjusted to 5.5. The cultures were incubated at a temperature of 37°C with relative humidity of 92-95% and 5% CO_2 in a CO_2 incubator.

In vitro infection of peritoneal macrophages:

The peritoneal macrophages were harvested from normal animals and infected with promastigotes and axenic amastigotes grown in acidic and basic medium. Peritoneal exudate was extracted according to method of Stuart (1978). The exudate was centrifuged at 1200 rpm for 10 minutes at 4°C. The cells collected were washed three times with PBS-EDTA and finally cells (10^6/ ml) were suspended in RPMI-1640 containing 10% FCS. Then the cells were dispended on sterilized cover slips kept inside disposable petriplates and incubated for 24 hrs at 37°C in 5% CO_2 humid atmosphere. The non-adherent cells were removed by washing and macrophage cultures were incubated for 24 hrs before infecting with promastigotes or amastigotes.

The amastigotes (grown in acidic and basic medium) and promastigotes in the late log phase were centrifuged and washed thrice with PBS, and finally suspended in RPMI-1640, 10% FCS in the conc. of 1 x 10^7 cells/ml. The parasites were added in the ratio 5:1 (promastigotes or amastigotes: macrophage) on the macrophage cultures and incubated again at 37°C in 5% CO_2 humid atmosphere. The cover slips were taken out at various intervals (24, 48, 72, 96 and 120 hrs) and washed with fresh medium. The slides were fixed, stained with Giemsa and observed under light microscope.

In vivo infection of Balb/c mice :

Promastigotes and amastigotes in the stationary phase of growth (5-7 days old culture) were harvested from acidic and basic NNN medium and finally suspended in PBS at a conc. of 10^8 parasites per ml. A 0.1 ml of suspension was injected intracardially into each mice. Five mice each infected with promastigotes or amastigotes (grown in acidic and basic medium) were sacrificed on different post infection days. Liver and spleen were weighed. A cut section of liver was rinsed with MEM and repeated imprints were made on clean glass slides from the cut end. The smears were allowed to air dry and fixed in methanol and stained in Giemsa.

The slides were examined under light microscope. A total of 200 cells were counted and the number of amastigotes in these cells were counted. The parasite load was assessed in terms of Leishman Donovan Units (LDU) and calculated as follows (Bradley and Kirkley, 1977).

$$LDU = \frac{\text{No. of amastigotes}}{\text{No. of liver cell nuclei}} \times \text{weight of organ(mg)}$$

RESULTS:

In vitro infection of peritoneal macrophages:

The peritoneal macrophages infected with either promastigotes or amastigotes (grown in acidic and basic medium) were observed after 24 hrs of infection, 90 ±0.5% of the cells were found to be infected with promastigotes as compared to 98 ± 2.5% of cells found to be infected with amastigotes grown in acidic medium(Fig.l). The percentage of infected macrophages increased further after 48, 72, 96 and 120 hrs both with promastigotes as well as amastigotes (grown in both, acidic and basic medium). The number of infected macrophages and number of parasites per macrophages were found to be more when infected with acidic amastigotes than the basic amastigotes and promastigotes (Fig. 2).

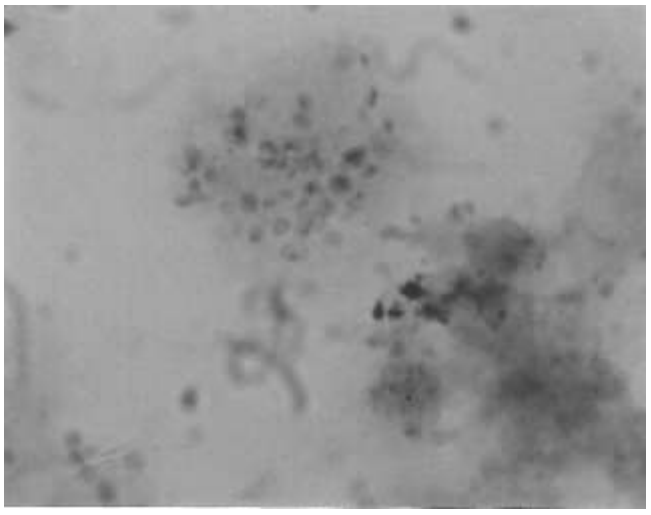

Figure 1 : Peritoneal macrophages infected with acidic amastigotes of *Leishmania donovani* showing amastigotes inside the cells (1000 X).

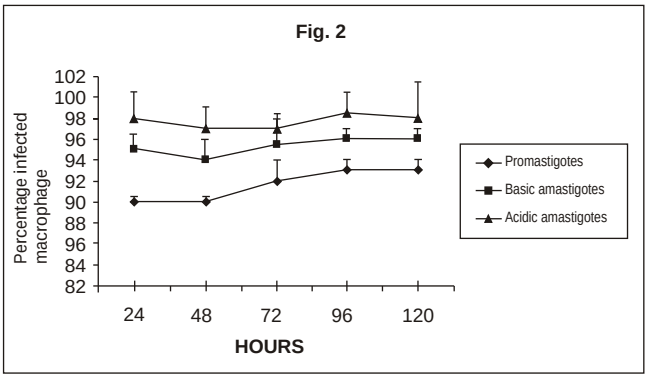

Figure 2 : Percentage infection of macrophages with promastigotes, basic amastigotes and acidic amastigotes of *Leishmania donovani.*

In vivo infection of Balb/c mice:

Axenic amastigotes (grown in acidic and basic medium) and promastigotes, when inoculated intracardially into the Balb/c mice resulted in the increase in weight of spleen, liver and parasite load. A significant increase in the spleen weight was observed in infected animals as compared to normal controls($p<0.001$,Fig 3).Similarly the mean weights of liver also increased significantly in all the infected group of animals($p<0.001$,Fig 4). The parasite load as measured by LDU increased significantly from 7 p.i.d onwards and kept on increasing till 35 p.i.d. in all infected animals but the increase was significantly more in animals infected with acidic amastigotes as compared to basic amastigotes and promastigotes (Fig.5).

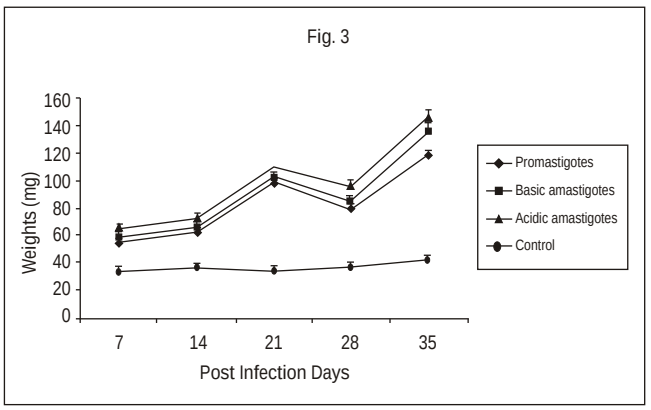

Figure 3 : Weights of spleen (mg) of Balb/c mice infected with promastigotes, basic amastigotes and acidic amastigotes of *Leishmania donovani.*

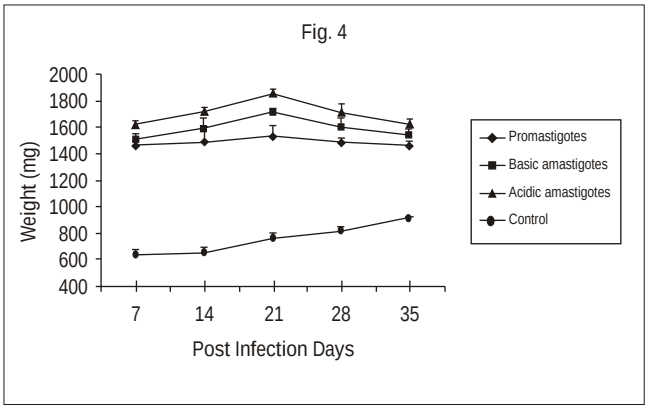

Figure 4 : Weights of liver (mg) of Balb/c mice infected with promastigotes, basic amastigotes and acidic amastigotes of *Leishmania donovani.*

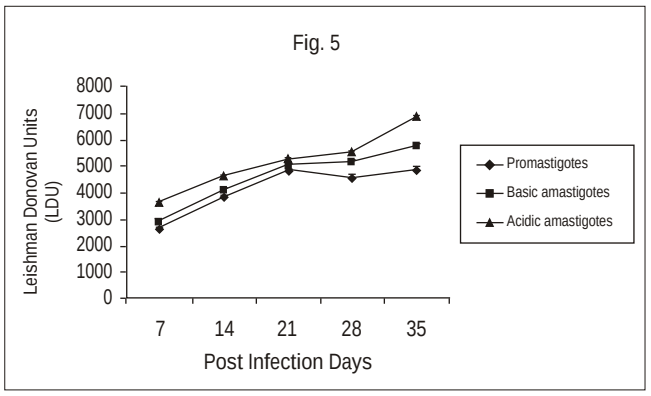

Figure 5 : Leishman Donovan Units in Balb/c mice infected with promastigotes, basic amastigotes and acidic amastigotes of *Leishmania donovani.*

DISCUSSION:

Protozoan of genus *Leishmania* are obligate intracellular parasites that cycle between the midgut of sand flies and phagolysosome of mammalian macrophages and therefore are exposed to extreme environmental changes. These environmental changes trigger a developmental programme in the parasites. Thus following heat shock, promastigotes differentiate to amastigotes. Promastigotes also respond to acidification of their environment by changing the expression of a number of genes. However, the combination of both low pH and high temperature induces the transformation of the promastigote to the amastigote (Zilberstein and Shapira, 1994).

In vitro cultivation of promastigotes is accomplished in various biphasic and monophasic liquid media. But the cultivation of amastigotes has been hampered greatly as they were cultured in macrophage cell lines, which provided limited amounts of purified organisms (Looker *et al.* 1986). A number of investigators have used elevated temperatures to induce the conversion of promastigotes to amastigote like forms, but these forms proved incapable of subsequent propagation at the higher temperature and reverted to promastigotes if the temperature was reduced (Smejkal, 1988).

The first successful attempt at long term propagation of amastigote like forms of *L. pifanoi* was done by Pan in 1984 by elevation of temperature from 26°C to 37°C. Later, amastigote like forms of *L. panamensis* and L. *donovani* were also reported (Eperon and McMahon Pratt, 1989a). Exposure of Leishmanial promastigotes of L. *mexicana*, *L. braziliensis* and L. *panamensis* species to a temperature of 33°C-37°C in vitro caused their transformation to amastigote like forms (Pan, 1984, Stinson *et al.* 1989). Successful cultivation of amastigotes of *L. donovani* axenically was accomplished by several workers. (Doyle *et al.* 1991; Joshi *et al.* 1993; Chattopadhyay, 1996) in different media such as 100% foetal calf serum and RPMI-1640 plus 10% FCS at a pH of 5.5 to 6.0. Axenically amastigotes have also been cultured successfully in NNN medium by elevation of the temperature to 35°C-37°C. (Gupta *et al.* 1996) and lowering the pH of the RPMI-1640 media (Chattopadhyay, 1996).

In the present study the axenic amastigotes were cultured in C02 incubator in modified NNN medium by increasing the temperature and lowering the pH. This is the first report of culturing of amastigotes in NNN medium by increasing the temperature to 37° C and lowering the pH of the MEM to 5.5, which is used as overlay.

Infectivity of axenic amastigotes was checked in vitro in mouse peritoneal macrophages. Parasite could be seen after 24 hrs of infection inside the macrophages in the amastigote form. This is the usual time taken for the establishment of infection (Berman and Lee, 1984). Both promastigotes and amastigotes entered the macrophages and transformed into amastigotes, but the percentage of the cells infected with acidic amastigotes was more as compared to basic amastigotes and promastigotes. More than 90% of the macrophages were found to be infected within 24 hrs. But the number

of parasites per macrophage was more when the macrophages were infected with acidic amastigotes as compared to basic amastigotes and promastigotes.

When the parasites were injected intracardially into Balb/c mice, an increase in the weights of spleen, liver and parasite load was observed in all the three groups of animals, but the mice infected with acidic amastigotes showed maximum increase in all the three parameters. The increased infectivity of axenic amastigotes had been shown earlier for L. *braziliensis panamensis, L. donovani* and *L. major* (Smejkal *et al.* 1988; Al Bashir *et al.* 1992). But no studies were available as regards the comparison of infectivity of axenic amastigotes obtained from basic and acidic NNN medium. This study shows that the axenic amastigotes grown in acidic medium are more infective and that external pH, rather than the temperature may act as the main trigger for expression of newer proteins which may provide protection against the hostile environment of the phagolysosome (Handman and Greenblatt, 1977, Gottlieb and Dwyer, 1981) and may play a role in increased survival rate of amastigotes (Mauel, 1984). It has earlier been reported that changing only the temperature is not sufficient to generate a long term amastigote like culture and it is the combination of an elevation in temperature and a decrease in pH which facilitates the establishment of stable axenic amastigote like cultures (Bates *et al.* 1992, Joshi *et al.* 1993).

Use of amastigote as an antigen is of great importance in the diagnosis of leishmaniasis as amastigote is the stage which exists in human host and cause disease. In the present study we cultured the axenic amastigotes in modified NNN medium in basic and acidic conditions. The infectivity of the axenic amastigotes grown in acidic medium was found to be more as compared to other parasites. It is thereby suggested that these axenic amastigotes grown in acidic NNN medium could be used for development of a serodiagnostic test as the culture medium is economical as compared to 100% FCS and RPMI-1640+FCS used by earlier workers(Doyle et al. 1991, Joshi etal. 1993) and simple to prepare in research laboratories where there is paucity of funds.

REFERENCES:

Al Bashir NT, Rassam MB and AL Rawi BM. 1992. Axenic cultivation of amastigotes of *Leishmania donovani* and *Leishmania major* and their infectivity. Annals of Tropical Medicine and Parasitology. 86 (5); 487-502.

Antoine JC, Prina E, Jouanne C and Bongrand P. 1990. Parasitophorous Vacuoles of *Leishmania amazonensis* - Infected macrophages maintain an acidic pH. Infection and Immunity. 58: 779-787.

Bates PA, Robertson CD, Tetley L and Coombs GH. 1992. Axenic cultivation and characterization of *Leishmania mexicana* amastigote like forms. Parasitology. *105:* 193-202.

Berman JD and Lee LS. 1984. Activity of antileishmanial agents against amastigotes in human monocyte derived macrophages and in mouse peritoneal macrophages. Journal of Parasitology. 70: 220-225.

Bradley DJ and Kirkley J. 1977. Regulation of Leishmania population within host I. The variable course of *Leishmania donovani* infection in mice. Clinical and Experimental Immunology. 30; 119-129.

Chang KP and Hendricks LD. 1985. Laboratory cultivation and maintenance of Leishmania. In "leishmaniasis" (KP Chang and R Bray eds.) pp. 213-244. Elsevier Science Publisher BV Amsterdam.

Chattopadhyay R, Kaur S, Ganguly NK and Mahajan RC. 1996. Antigenic differences between axenic amastigotes and promastigotes of *Leishmania donovani.* Indian Journal of Medical Research. *104:* 349-354.

Coombs GH, Robertson CD and Mottram JC. 1988. Cysteine proteinases of leishmaniasis. In "Biochemical Protozoology" (G.H. Coombs and M.J. North eds.) pp 208-220. Taylor and Francis, London.

Doyle PS, Engle JC, Pimenta PFP, Pinto deSilva P and Dwyer DM. 1991. *Leishmania donovani:* Long term culture of axenic amastigotes at 37 °C. Experimental Parasitology. 73 : 326-334.

Eperon S and McMahon-Pratt D. 1989a. Extracellular cultivation and morphological characterization of amastigote-like forms of *Leishmania panamensis* and *Leishmania braziliensis.* Journal of Protozoology. 36 : 502-510.

Eperon S and McMahon-Pratt D. 1989b. Extracellular amastigote-like forms of *Leishmanai panamensis* and *Leishmania braziliensis* II. Stage and specific monoclonal antibodies. Journal of Protozoology. 36 : 510-518.

Gottlieb M and Dwyer DM. 1981. Protozoan parasite of humans : Surface membrane with externally disposed acid phosphatase. Science. *212 :* 939-940.

Gupta N, Goyal N, Kumar R, Aggarwal AK, Prahlad KS and Rastogi AK. 1996. Membrane characterization of amastigote - like forms of *Leishmania donovani.* Tropical Medicine International Health *1 (4):* 495-502.

Handman E and Green blatt CL. 1977. Promotion of leishmanial infections in non-permissive host-macrophages by conditioned medium. Zeit schrift fur Parasiten Kunde 53: 143-147.

Hendricks LD, Wood DE and Hajduk ME. 1978. Haemoflagellates : commercially available liquid media for rapid cultivation. Parasitology. 76 : 309-316.

Joshi M, Dwyer DM and Nakhasi HL. 1993. Cloning and characterization of differently expressed genes from *in vitro* grown "amastigotes" of *Leishmania donovani.* Molecular and Biochemical Parasitology. 58 : 345-354.

Looker DL, Martinez S, Horton JM and Marr JJ. 1986. Growth of *Leishmania donovani* amastigotes in the continuous human macrophage cell line U937 : Studies of drug efficiency and metabolism. Journal of Infectious Disease. *154* : 323-327.

Mauel J. 1984. Mechanisms of survival of protozoan parasites in mononuclear phagocytes. Parasitology. 88 : 579-592

Mukadda AJ, Meade JC, Glaser TA and Bonventre P. 1985. Enhanced metabolism of *Leishmania donovani* amastigotes at acidic pH : an adaptation of intracellular growth. Science. 229: 1099-1101.

Nicolle C. 1908. Culture du parasite du bouton d' orient, CR. Academic Science, Paris. (146); 842.

Novy FG and McNeal WJ. 1905. Journal of Infectious Disease. 2 : 256. Cited by Chang, K.P. and Hendricks, L.D. 1985. Laboratory cultivation and maintenance Leishmania. In "Leishmaniasis" (Chang, K.P. and Bray, R.S. eds.) pp 213-244. Elsevier Science Publishers, BV Amsterdam.

Pan AA. 1984. *Leishmania mexicana :* Serial cultivation of intracellular stages in a cell free medium. Experimental Parasitology. 58 : 72-80.

Rao RR, Mahajan RC and Ganguly NK. 1984. Modified media for in vitro cultivation of Leishmania promastigotes. A comparative study Bulletin PGI. *18:* 125-128.

Ray JC. 1932. Cultivation of various Leishmania parasites on solid medium. Indian Journal of Medical Research. 20 : 355.

Smejkal RM, Wolff R and Olenick JG. 1988. *Leishmania braziliensis panamensis :*Increased Infectivity Resulting from heat shock. Experimental Parasitology. *65 :* 1-9.

Stinson S, Sommer JR and Blum JJ. 1989. Morphology of *Leishmania braziliensis :* Changes during reversible heat-induced transformation from promastigote to an ellipsoidal form. Journal of Parasitology. 75 : 431-440.

Stuart AE, Hebeshaw JA and Davidson AE. 1978. Phagocytes in vitro. In 'Handbook of Experimental Immunology' (D M Weir) Vol. 2, ch. 31, Blackwell Oxford.

Zilberstein D and Shapira M. 1994. The role of pH and temperature and in the development of Leishmania parasites. Annals Review on Microbiology. *48 '*. 499-570.

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Evaluation of double aldehyde stabilized Human O red blood cells in the indirect haemagglutintion test for diagnosis of neurocysticercosis

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In the present study various methods for stabilzation of the Human 'ORBCs' including the double aldehyde stabilization (DAS) method are evaluated for use in the IHA for the diagnosis of neurocysticercosis. Blood samples were collected from 30 cases suspected of neurocysticercosis. Control blood samples were obtained from 15 normal healthy persons. The stabilized RBCs were sensitized with *C. cellulose* complete homogenate antigen for use in the IHA for detection of specific cysticercus antibodies in the serum. A titre of $\geq$ 64 was considered to be diagnostic of cysticercosis. At this diagnostic titre , 27 sera were positive by the IHA using DAS RBCS , where as 21, 19 and 23 sera were positive by the IHA using formaldehyde , pyruvic aldehyde and glutaraldehyde stabilized RBCs respectively . The results of the present study in neurocysticercosis shows that the IHA using sensitized DAS cells were found to be more sensitive than that using formaldehyde, pyruvic aldehyde or glutaraldehyde stabilised RBCs.

Key words:Double aldehyde stabilization, Indirect haemagglutination test, Neurocysticercosis,

Introduction

Neurocysticercosis (NCC) is a common parasitic disease of central nervous system and is an important cause of death and epilepsy in tropical countries like India. The condition is acquired by the ingestion of vegetables and water contaminated with human excreta containing eggs of *Taenia solium.* Serological tests play an important role not only in the diagnosis of cases but also in the serosurveillance of the disease in the community. The indirect haemagglutination (IHA) test, because of its simplicity and economy, is frequently used for the purpose.

The IHA test in NCC is a simple and-rapid test that uses red blood cells (RBCs) coated with cysticercus antigen to detect specific antibodies in the serum . The sensitivity of this test largely depends on the source of RBCs and methods employed to stabilize the RBCs for coating the antigen to the RBC surface. Human blood group O, sheep, or Turkey red blood cells (RBCs) are frequently used as carriers of antigen to detect specific antibodies in serum of the patients (Kumar and Gaur, 1989). These RBCs are stabilized either by treating with a single stabilizing agent such as glutaraldchyde (GA) pyruvic aldehyde(PA) or formaldehyde before they are tanned sensitised with cysticercus antigens. In last many years various studies have shown the usefulness of double aldehyde stabilization of RBCs to be more sensitive procedure than single aldehyde stabilization of RBCs in the IHA test for the diagnoses of cystic echinococcosis, amoebiasis, malaria and lymphatic filariasis (Parija et al, 1985 Parija et al, 1986; Sengupta et al, 1986; Dubey et al, 1989; Parija et al, 1989). In the method of double aldehyde stabilization, the RBCs are stabilized by treating sequentially with pyruvic aldehyde, tannic acid and glutaraldehyde.

In the-present study various methods for stabilzation of the Human O RBCs including the double aldehyde stabilization method are evaluated for use in the IHA for the diagnosis of NCC.

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Materials and Methods

Sera

Blood samples were collected from the following groups:

Group 1 : It included 15 suspected cases of NCC with multiple cystic lesions demonstrated by imaging studies

Group 2 : It included 15 suspected cases of NCC with single cystic lesion demonstrated by imaging studies

Group 3 : It included 15 normal healthy persons.

Serum samples were separated and stored at -20 °C till use.

Cysticercus antigen

The source of the parasite material for cysticercus antigen was pig muscles naturally infested with *Cysticercus cellulosae,* which was obtained during slaughtering in a local slaughter house. *Cysticerci* were dissected free from surrounding tissue into sterile PBS 7.2 containing protease inhibitor PMSF (0;006%) at room temperature, followed by washing many times in the same buffer. Preparation of *C. cellulosae* complete homogenate antigen was done as per the procedure described by Sreenivasamurthy et al. 1999. Briefly, in this procedure, nearly 100 cysts were washed thrice in PBS pH 7.2 to remove extraneous matter. The cysts were homogenized in a glass tissue homogeniser with PBS (pH 7.2) containing PMSF (0.006%). Homogenization was done under cooling condition. The homogenized tissue suspension was then sonicated 8 times at 12kH with 30 seconds cooling interval. Each cycle of sonication was for 1 minute. The sonication was done under cooling condition in an ice bath. The sonicated material was centrifuged at 4°C for 30 minutes at 14,000 rpm. The supernatant was collected as the complete homogenate antigen, aliquoted to 1 ml cryoprotected vials and stored at -20°C.

Human RBCs

Human blood group O RBCs were collected into Alsever's solution. The cells were washed thrice in phosphate-buffered saline (PBS, pH 7.2, 0.15 M) and treated with the stabilizing agent within 48hours.

Stabilization of RBCs

Single aldehyde stabilization

a) *Stabilization with formaldehyde:* The method of Fulthrope *et al,* (1961) as cited by Herbert (1978) was followed. To a suspension of human RBC (10ml of packed cells in 500 ml of PBS pH 7.2), 50ml of formalin (aqueous formaldehyde, 40% W/V) was added slowly from a burette, over a period of 30 minutes The mixture was left overnight at 4° C and a further 50 ml of formalin was then added in one lot. Twenty-four hours later the preserved cells were washed thrice in PBS pH 7.2 and stored at 4°C as a 50% V/V suspension in 1 in 200 dilution of formalin in PBS pH 7.2.

b) *Stabilization with glutaraldehyde: The* RBCs were stabilized with glutaraldehyde as per the method described by Avenneas et al (1969). Briefly, 10ml of 5% V/V aqueous glutaraldehyde was added to 1ml of 10% suspension of human RBC in PBS, and the mixture was stirred by a magnetic stirrer for 4 hours as 30% V/V suspension in PBS.

c) *Stabilization with pyruvic aldehyde: The* RBCs were stabilized by pyruvic aldehyde as per the method described by Parija *el al* (1986). A reaction mixture of the following substances, added in the order given, was prepared and subsequently kept in the cold: 1.7%NaCl, 12 ml; 40% V/V aqueous pyruvic aldehyde, 4 ml; 1% Na_2Co_3 35ml; Sorensen's phosphate buffer (pH 7.2) 7ml. To this 10 ml of a 50% suspension of human RBC was added. The mixture was stirred for 30 min and stored for 24 hours at 4°C.The cells were washed thrice in PBS and stored as a 2.5% V/V suspension at 4°C.

Double aldehyde stabilization

Double aldehyde stabilization (DAS) with pyruvic aldehyde and glutaraldehyde: Double aldehyds stabilisation (DAS) of RBCs was carried out as per the method described by Parija and Ananthakrisnnan (1985). The RBCs were first stabilized with pyruvic aldehyde as mentioned above. To a 2.5% suspension of the pyruvic aldehyde stabilized RBCs, an equal

volume of cold tannic acid (1 in 25000 in PBS pH 7.2) was added and the mixture was kept at 4°C for 30 minutes. The cells were then washed thrice in PBS and made upto a 4% suspension in the same diluent. To this was added an equal volume of 2% V/V aqueous glutaraldeliyde in PBS pH 7.2 over a magnetic stirrer which was run for 2 hours. DAS cells were then washed thrice with PBS pH 7.2 and stored as a 50% V/V suspension in this diluent.

Sensitisation of RBCs

Sensitization of single aldehyde stabilized cells with cysticercus antigen: To a 2.5% suspension of single aldehyde stabilized cells (formaldehyde, pyruvic aldehyde or gluataraldehyde) an equal volume of tannic acid (1 in 25000 in PBS pH 7.2) was added and the mixture was kept al 4°C for 30 minutes. The cells were then washed thrice in PBS and made upto a 4% suspension in tile same diluent. After tanning, these cells were sensitized with the optimum sensitizing dose (OSD) of cysticercus antigen, in the same way as followed for sensitization of the DAS cells (Farshy and Kagan., 1972) as mentioned below.

Sensitization of double aldehyde stabilized (DAS) cells with cysticercus antigen : The DAS RBCs were sensitized with the cysticercus antigen as described by Farshy and Kagan (1972) Briefly, one volume of packed DAS RBCs were suspended in 10 volumes of an OSD of the antigen (described below) prepared in PBS pH 6.4. The RBCs and antigen mixture was incubated at 50°C in water bath for 5 minutes, followed by overnight storage at 4°C , then incubated at 50°C again for 5 minutes. The sensitized cells were washed thrice with PBS pH 7.2 and stored in small volumes as a 10% suspension in PBS pH 7.2 at 4 °C until used.

Determination af optimal sensitizing dose (OSD) of cysticercus antigen: The optimal sensitizing dose (OSD) of cysticercus antigen was carried out by chequer board titration using RBCs sensitized with different concentrations of antigen against serial dilutions of positive control sera. The OSD of the antigen was determined to be 1 in 80 dilution of the stock antigen in PBS-7.2.

IHA procedure

The test was performed as described by Parija and Ananthakrishnan (1985). It was performed in 96-well U bottomed microtitre plates using a 1% suspension of cysticercus antigen sensitized RBCs in the diluent (PBS pH 7.2 containing 0.1% bovine serum albumin). To each well 25 µl of a serial dilution of sera in the diluent followed by 25µl of antigen sensitized RBCs was added; the plates were then briefly agitated by hand and iucubated at room temperature. The results were read by observing the settling patterns of the haemagglntinated RBCs in the plate after incubation for overnight at 4 ° C .Adequate control sera were put with each test.

Results

Agglutinated human O RBCs settled after overnight incubation at 4 ° C and demonstrated a definite pattern of haemagglutination with the cysticercus positive

Table I :- Summary of results of IHA test done using RBCs stabilized by various methods, with sera of patient and control groups.

Study group		Total No of cases	No. of sera showing a positive titre (>64) with various stabilization methods			
			FA	PA	GA	DA
I.	Cases with multiple cystic lesions on CT	15	13	11	12	14
II.	Cases with single cystic lesion on CT	15	8	8	11	13
III	Normal sera	15	1	0	1	2

Table II :- Titres of IHA in patients' sera Using Human O' RBC stablized with various aldehydes

Study group	Serum No.	RBC's stabilized with Formaldehyde	RBC Stabilized with Pyruvic aldehyde	RBCs stabilized with glutaraldehyde	RBC stabilized with double aldehyde
Suspected cases of neurocysticerosis (multiple cystic lesions on CT)	1	1 : 128	1 : 64	1 : 128	1 : 128
	2	1 : 32	1 : 8	1 : 16	1 : 128
	3	1 : 128	1 : 128	1 : 256	1 : 512
	4	1 : 64	1 : 64	1 : 128	1 : 256
	5	1 : 16	1 : 16	1 : 8	1 : 16
	6	1 : 64	1 : 128	1 : 128	1 : 256
	7	1 : 64	1 : 64	1 : 128	1 : 256
	8	1 : 64	1 : 128	1 : 128	1 : 256
	9	1 : 64	1 : 128	1 : 128	1 : 128
	10	1 : 128	1 : 128	1 : 128	1 : 256
	11	1 : 128	1 : 256	1 : 256	1 : 1024
	12	1 : 128	1 : 64	1 : 128	1 : 256
	13	1 : 64	1 : 32	1 : 16	1 : 128
	14	1 : 64	1 : 32	1 : 16	1 : 128
	15	1 : 128	1 : 64	1 : 128	1 : 256
Suspected cases of neurocysticerosis (single cystic lesion on CT)	1	1 : 16	1 : 16	1 : 32	1 : 128
	2	1 : 32	1 : 32	1 : 32	1 : 32
	3	1 : 128	1 : 128	1 : 128	1 : 512
	4	1 : 256	1 : 256	1 : 256	1 : 512
	5	1 : 256	1 : 128	1 : 256	1 : 1024
	6	1 : 64	1 : 64	1 : 128	1 : 256
	7	1 : 32	1 : 32	1 : 64	1 : 128
	8	1 : 64	1 : 32	1 : 128	1 : 128
	9	1 : 64	1 : 128	1 : 128	1 : 128
	10	1 : 64	1 : 64	1 : 64	1 : 128
	11	1 : 32	1 : 32	1 : 32	1 : 64
	12	1 : 16	1 : 8	1 : 16	1 : 32
	13	1 : 32	1 : 64	1 : 128	1 : 128
	14	1 : 32	1 : 16	1 : 32	1 : 64
	15	1 : 128	1 : 128	1 : 64	1 : 128
Normal sera	1	1 : 16	1 : 16	1 : 32	1 : 32
	2	1 : 32	1 : 16	1 : 16	1 : 16
	3	1 : 32	1 : 32	1 : 32	1 : 32
	4	1 : 16	1 : 32	1 : 32	1 : 32
	5	1 : 32	1 : 32	1 : 16	1 : 16
	6	1 : 16	1 : 16	1 : 16	1 : 32
	7	1 : 16	1 : 16	1 : 16	1 : 16
	8	1 : 16	1 : 16	1 : 32	1 : 32
	9	1 : 64	1 : 32	1 : 64	1 : 64
	10	1 : 16	1 : 32	1 : 32	1 : 64
	11	1 : 32	1 : 16	1 : 16	1 : 16
	12	1 : 32	1 : 32	1 : 32	1 : 32
	13	1 : 16	1 : 32	1 : 32	1 : 32
	14	1 : 16	1 : 16	1 : 32	1 : 32
	15	1 : 8	1 : 16	1 : 16	1 : 8

sera . A titre of > 64 was considered to be diagnostic titre of cysticercosis based on the litres obtained with normal control sera (mean titre+2 SD). At this diagnostic titre. out of 30'suspected cases in study groups 1 and 2 , 27 sera were positive by the IHA using DAS-RBCs , where as 21 sera , 19 sera and 23 sera were positive by the IHA using formaldehyde , pyruvic aldehyde and glutaraldehyde stabilized RBCs respectively (Table I).

Table II shows the IHA titres obtained with the four types of stabilized human O RBCs. With most of the sera from cases with cysticercosis. the DAS cells gave titres 2-4 times higher titer than those obtained with single aldehyde stabilized cells. Only two sera in the normal control group showed a titre of 64 with DAS cells.

Discussion

Indirect haemagglutination (IHA) test, because of its simplicity and economy, is being widely used by many workers in the serodiagnosis of various parasitic diseases such as amoebiasis (Parija et al 1987 , Wiwanitkit, 2002) , leishmaniasis (Iqbal *et al.,* 2002), malaria (Ray *et al.,* 1983), toxoplasmosis (Soliman *et al.,* 2001) , echinococcosis (Parija *et al.,* 1985; Rigano *et al.,* 2002), schistosomiasis (Van Gool *et al.,* 2002), and many other parasitic diseases (Ikeda T et al, 1979; Ganguly NK et al, 1981; Gam et al, 1987; Comelissen JB et al. 1992; Maleewong W et al, 1998). Human (Herbert, 1978), sheep (Sawada et al, 1982; Rai et al, 1992) , turkey (Singh *et al,* 1980) , goose (Mann*e*n *et al.,* 1984), bovine and swine (Holmgren, 1973) red blood cells (RBCs) are frequently used in the IHA test.

The sensitivity of IHA is greatly enhanced by prior treatment of RBCs with tannic acid (Boyden, 1951). Cole and Farrell (1955) improvised the technique by introducing the method of stabilization of RBCs. The erythrocytes can be stabilized by various chemicals such as aldehyde (eg.formaldehyde, pyruvic aldehyde and glutaraldehyde), chromic chloride, etc. Besides stabilization, aldehydes also react with free amino groups, sulphydryl groups and to some extent imidazole groups, rendering the surface of the RBC more anionic hence facilitating the attachment of the protein antigens to them (Farshy and Kagan, 1972). Ling(1961), studying the effect of aldehydes on RBCs , reported that in addition to their function as stabilizing agent aldehydes served as excellent protein coupling agents. The use of fixed sensitized erythrocytes improves the practicality of the IHA assay by (i) eliminating the need to sensitize cells each time the test is performed; (ii) reducing waste of the purified antigen; and (iii) reducing test-to-test variation (Barrett, 1985).

The use of DAS cells by various workers in the Field have shown to increase sensitivity of the IHA in the diagnosis of malaria, amoebiasis, cystic echinococcosis, filariasis and various other parasitic diseases .Therefore, in the present study single aldehyde stabilized cells as well as DAS cells sensitized with cysticercus antigen were evaluated in the IHA for diagnosis of NCC. The result of the present study in NCC is in conformity with this (Table II) . The IHA using sensitized DAS cells were found to be more sensitive than that using either formaldehyde , pyruvic aldehyde or glutaraldehyde stabilised RBCs .

Cysticercosis as an important public health problem, hence a simple and inexpensive test such as IHA will be useful not only for diagnosis of cases but also for serosurveillance of the disease in the community.

References

Cornelissen JB, de Leeuw, WA and van der Heijden PJ, 1992. Comparison of an indirect haemagglutination assay and an ELISA for diagnosing Fasciola hepatica in experimentally and naturally infected sheep. Vet Q 14(4). 152-6.

Dubey *ML,* Sharma SK, Ganguly NK and Mahajan RC, 1989. Seroepidemiological study of malaria in a rural population of Chandigarh. Indian Journal of Malariology. 26(4): 187-90.

Farshy DC and Kagan IG 1972. Use of stable sensitized cells in indirect microhaemagglutination test for malaria American Journal Tropical Medicine and Hygiene. 21(6):868-72.

Gam AA, Neva FA and Krotoski WA, 1987. Comparative sensitivity and specificity of ELISA and IHA for serodiagnosis of strongyloidiasis with larval antigens. American Journal Tropical Medicine and Hygiene. 37(1): 157.61.

Ganguly NK, Mahajan RC, Vasudev V, Radha Krishna V, Anand BS and Dilawari JB, 1981. Comparative evaluation of indirect haemagglutination and immunofluorescence tests in serodiagnosis of giardiasis. Indian Journal Medical Research. 73 Suppl: 111-3.

Herbert WJ, 1978. Passive haemagglutination with special reference to the tanned cell technique. In Handbook of experimental immunology, ed Weir, D.M. 3rd ed, chapter 20, Oxford.

Holmgren N 1973. An indirect haemagglutination test for detection of antibodies against Mycoplasma pneumoniae using fomialinized tanned swine erythrocytes. Ada Vet Scand 14:353-5.

Ikeda T, Aoki Y, Tada I, Recinos MM, Ochoa JO and Molina PA 1979. A sero-epidemiological study ofo'ncliocerciasis with the indirect hemagglutination test. Journal of Parasitology. 65(6):855-61.

.Iqbal J, Hira PP, Saroj G, Philip R, Al-Ali F, Madda PJ and Sher A, 2002. Imported visceral leishmaniasis: diagnostic dilemmas and comparative analysis of three assays. Journal of Clinical Microbiology. 40(2):475-9.

Kumar D and Gaur SN 1989. Comparative evaluation of various inununodiagnostic tests for the diagnosis of Taenia solium cysticercosis in pigs, using fractionated antigens. Journal of Helminthology. 63(1):13-7.

Malccwong W, Inlapan PM, Wongkham C, Pajongthanasaris MP, Morakote M, and Chaicumpa W, 1998. Indirect haemagglutination test using monoclonal antibody-affinity purified antigens for diagnosis of human paragonimiasis due to Paragonimus heterotremus. Tropical Medicine and Internal Health. 3(1):57-9.

Mannen K, 1984. Methods of increasing the sensitivity of the haeinagglutination inhibition test for rabies antibodies. Bulletin of the World Health Organization. 62: 883-892. Geneva. Switzerland.

Parija SC and Ananthakrishnan N; 1985 Evaluation of stabilised cells in the indirect haeinagglutination test for echinococcosis. Journal of Medical Microbiology. 19(1):95-8.

Parija SC, Mishra S.R. and Rao RS. 1986; Sensitized chick cells in the indirect haeinagglutination test for echinococcosis. Journal of Medical Microbiology. 22(3):237-9.

Parija SC, Sasikala A and Rao RS. 1987. Serological survey of hydatid disease in Pondicherry, India. Transactions of Royal Society Tropical Medicine Hygiene. 81(5):802-3.

Parija SC, Kasinathan S and Rao RS. 1989. Rapid indirect haemagglutination (rapid-lHA) using sensitized duck cells for serodiagnosis of amoebiasis at primary health centre level. Journal of Tropical Medicine Hygiene. 92(3):221-6.

Rai GP, Agarwal GS and Vankateswaran KS. 1992. Indirect haeinagglutination test for the serodiagnosis of brucellosis using stabilised sheep red blood cells. Zentralblatt fur Bakteriologie. 277(2): 188-92.

Ray K, Sharma MC, Sivaraman CA and Rai Chowdhuri AN. 1983. Evaluation of IHA tests using *Plasmodiiim falciparum* from in vitro culture & its comparison with IIF & ELISA. Indian Journal of Medical Research. 78:624-30.

Rigano R, loppolo S, Ortona E, Margutti P, Profumo E, Ali MD, Di Vico P, Teggi A and Siracusano A. 2002. Long-term serological evaluation of patients with cysiic echinococcosis treated with benzimidazole carbaniates. Clinical Experimental Immunology. 129(3):485-92.

Sawada T, Rimler RB and Rhoades KR. 1982. Indirect hemagglutination test that uses glutaraldehyde-fixed sheep erylhrocyles sensitized with extract antigens for detection of Pasteurella antibody; Journal of Clinical Microbiology. 15(5):752-6.

Scngupta G, Mohapatra TM and Sen PC. 1986. lmmunodiagnosis of filariasis. Journal of Tropical Medicine and Hygiene. 89(6);313-7.

Soliman M, Nour-Eldin MS, Einaggar HM, El-Ghareb ME and Ramadan NI. 2001. Toxoplasma antibodies in normal and complicated pregnancy. Journal of Egyptian Society of Parasitology. 31(2):637-46.

Sreenivasamurthy GS, D'souza PE and Jagannath MS. 1999. Enzyme linked immuno electro transfer blot in the diagnosis of *Taenia solum* cysticercosis in pigs. Journal of Parasitic Diseases, 23; 85-88.

Van Gool T, Vener H, Vervoort T, Doenhof FMJ, Wetsteyn J and Overbosch D. 2002. Serodiagnosis of imported schistosomiasis by a combination of a commercial indirect hemagglutination test with *Schistosoma mansoni* adult worm antigens and an enzyme-linked immunosorbent assay with *S. mansoni* egg antigens. Journal of Clinical Microbiology. 40(9):3432-7.

Wiwanitkit V. 2002. A note on indirect hemagglutination (1HA) antibody liters among hospitalized patients in Thailand with amebic liver abscesses. Medscape General Medicine. 4(3):5.

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Stage-specific expression of resistance and behavioural response to deltamethrin and DDT selection pressure in Indian strain of *Culex quinquefasciatus* Say

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The larvae and adults of *Culex quinquefasciatus* Say were selected with deltamethrin, deltamethrin with synergist, piperonyl butoxide (PBO) in 1:5 ratio and DDT to study the susceptibility and development of resistance to these insecticides. The adults emerged from larval-selected strains and the larvae derived from adult-selected strains were tested against deltamethrin and DDT to validate the presence of stage-specific expression of deltamethrin resistance. The adult knockdown and irritability studies were performed to study their behavioural response against deltamethrin and DDT. Deltamethrin was confirmed as highly effective larvicide and adulticide against *Cx. quinquefasciatus.* Continuous larval selections with deltamethrin for 40 generations caused 1449-fold larval resistance, but only 1.4-fold in emerging adults. Adult selections with deltamethrin resulted in only 4.3-fold resistance after 40 generations indicating differential response to deltamethrin selection in two developmental stages of the mosquito. Larval selection pressure with synergised deltamethrin on parental and deltamethrin-resistant strain could cause a temporary suppression of the development of deltamethrin resistance. The development of high levels of resistance to DDT and deltamethrin in deltamethrin and DDT-resistant strains, respectively indicates a close link between deltamethrin and DDT resistance mechanisms. The parent as well as the selected adults of *Cx. quinquefasciatus* exhibited significant knockdown and irritability response against deltamethrin and DDT. These investigations suggest the exquisite use of deltamethrin as an adulticide than a larvicide. The results are discussed in terms of the strategies to design control methods and resistance management programs.

Keywords: C*ulex quinquefasciatus,* Deltamethrin, Insecticides, Resistance, Synergist

INTRODUCTION

Insecticide resistance has become a formidable problem in the management of vector mosquitoes by insecticides. Pyrethroid insecticides, however, are widely accepted and are in great demand for vector control programs in indoors due to their rapid toxicity, excito-repellent properties and safety margins against humans. In India, the pyrethroids, allethrin and deltamethrin are in effective use as residual domestic sprays, mosquito mats, coils and impregnated bed nets. The advantage of using pyrethroids, however, is under serious threat due to the selections derived from any one of the above mentioned usages. Effective selection of resistance to pyrethroids has been reported in African strain of *Anopheles gambiae* as a result of use as domestic insecticide (Elissa *et al*, 1993) and in bed nets (Vulule *et al*, 1994)

The different species of *Culex* are increasingly developing resistance to pyrethroids in Asian countries and Tropical world (Jinfu, 1999; Chandre *et al.,* 1999). The possible reasons may be the past continuous usage of Dichloro diphenyl trichloroethane (DDT) in agriculture and domestic areas on a large scale, leading to inheritance of

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pyrethroid resistance from DDT resistance and strong selection pressure of pyrethroids themselves. Deltamethrin is among the six pyrethroid insecticides recommended by WHO in the framework of WHO pesticide evaluation scheme for the treatment of mosquito nets (Zaim *et al.,* 2000). The efficacy of deltamethrin as a larvicide and adulticide against *Cx. quinquefasciatus* has already been documented (Das and Kalyanasundaram, 1984). Preliminary tests have been done to evaluate the development of deltamethrin resistance in *Cx. quinquefasciatus* (Chandre *et al.,* 1998). Resistance to deltamethrin in *Cx. quinquefasciatus* has been reported due to the involvement of mono-oxygenases, hydrolytic esterases or a *kdr*-based mechanism caused by mutation in Na channel gene with cross-resistance to DDT (Sahgal *et al.*, 1994; Bisset *et al.,* 1998; Chandre *et al.,* 1998). Therefore, the present studies were aimed to assess the susceptibility of *Cx. quinquefasciatus* larvae and adults against deltamethrin and DDT, development of resistance and behavioural responses to these insecticides. These studies are of extreme significance in designing effective strategies for vector control and implementing resistance-management programs against *Cx. quinquefasciatus*.

MATERIAL AND METHODS

Mosquito Culture: The present investigations employed the adult *Cx. quinquefasciatus* Say field-collected from Delhi and established in an insectary. The culture was maintained at 28±1 °C and 80 ± 5 % RH with a photoperiod of 14 h daylight and l0h darkness. The larvae were reared in enamel trays (15" x 10") filled with dechlorinated water and fed on finely ground biscuits and yeast in the ratio of 3:2. Adults were fed on water-soaked split raisins. The female adults were provided with rat blood meal on alternate days.

Insecticides/chemicals: The technical grade deltamethrin with a purity of 98.8% and the synergist, PBO were obtained from Roussel Uclaf, New Delhi, India, while p,p'-DDT (99.0% pure) was procured from Ralph N. Emanuel Ltd., Wembley, England. Insecticide-impregnated papers of deltamethrin (0.025%) and DDT (4%) obtained from World Health. Organization, Geneva were stored at 4 °C and were not used more than three times.

Larval Susceptibility Tests/Selections: Larval Susceptibility tests employed early IV instar larvae in accordance with the standard WHO method for mosquito larvae (WHO, 1981 a). The larvae were exposed to ethanolic solutions of the insecticide in batches of 25. Controls were treated with ethanol alone and control mortality, if any, were corrected using Abbott's formula (Abbott, 1925).

In order to assess the development of resistance to the respective insecticide, the early IV instar larvae were exposed to the selection pressure of deltamethrin, deltamethrin + PBO (1:5) and DDT at LC_{90} dosage, separately for successive generations, (Kumar *et al.,* 2002). The mortality data was subjected to regression analyses of probit-mortality on log dosage and their LC_{50}, LC_{90}, slope and heterogeneity (χ^2) about linear regression line were computed (Finney,1971). The extent of resistance developed was expressed as resistance ratio by dividing the LC_{50} value of the insecticide selected strain by that of the parent strain.

Adult Susceptibility Tests/Selections: The adult susceptibility tests were carried out by using standard WHO test kits (WHO, 1981b). Sets of 20, freshly blood-fed 3 day old mosquitoes were exposed to impregnated papers of deltamethrin (0.025%) and DDT (4%) separately for varying durations with at least 4 replicates per bioassay. The adults were held for 24h and mortalities were recorded and corrected for control mortality. Adult selection of the parent strain of *Cx. quinquefasciatus* was performed using the WHO adult susceptibility kits. Several batches of 20-25 fully blood-fed mated females were exposed to 0.025% deltamethrin (former discriminatory dosage recommended by WHO) for different time intervals in order to induce 90% mortality in the adults. The treatment was followed by a 24 h holding period, after which the surviving females were separated and transferred to cloth cages. The eggs laid by the females that survived were reared to adults. The selection pressure was continued for 40 successive generations and the resistance ratios were calculated to assess the development of deltamethrin resistance.

Strains of *Cx. quinquefasciatus:* For the present

study, the adults were drawn from the following strains of *Cx. quinquefasciatus* developed in the laboratory:

a) Parent strain: Field-collected strain maintained without insecticide selection pressure.

b) Deltamethrin-selected strain:

i) Parent strain selected with deltamethrin alone in the laboratory for 40 successive generations at the larval stage.

ii) Parent strain selected with deltamethrin at the adult stage for 40 continuous generations.

c) Deltamethrin + PBO-selected strain :

i) F_{24} deltamethrin larval-selected strain sub-selected with deltamethrin + PBO (1:5) till 40 generations at larval stage.

ii) Larvae of parent strain selected with synergised deltamethrin for 20 generations.

d) DDT-selected strain: Parent strain selected with DDT at larval stage for 40 successive generations.

Cross and Stage-specific resistance studies: The larvae derived from deltamethrin-larval selected strains were tested with DDT and that from DDT-larval selected strains were tested with deltamethrin to assess the development of cross-resistance levels. In addition, the larvae derived from deltamethrin-adult selected strains were tested with deltamethrin and DDT and their resistance to respective insecticide was computed. The adults emerged from various larval selected strains were also tested with 0.025% deltamethrin-impregnated paper and 4% DDT-impregnated paper to determine the levels of resistance developed to these insecticides.

Knockdown Experiments: The knockdown tests were performed on the adult mosquitoes of *Cx. quinquefasciatus* according to the method of Verma and Rehman (1986). For each experiment; 20 freshly blood-fed 3 day old adult females emerging from the parent and insecticide-selected populations were pre-conditioned for 1h in control tubes followed by exposure to 0.025% deltamethrin-impregnated papers in experimental tubes. The mosquitoes unable to fly but alive were counted at regular intervals as knocked down mosquitoes, till all the mosquitoes had been knocked down. These mosquitoes were regularly removed from the exposure tubes and transferred to the control tubes to observe the recovery. The knockdown and mortality data was subjected to regression analyses and the knockdown resistance ratio to deltamethrin was calculated by dividing the KT_{50} value of resistant strain by that of susceptible population.

Identical knockdown experiments were performed on the adult females of *Cx. quinquefasciatus* with 4% DDT-impregnated papers. The knockdown resistance to DDT was computed by so obtained KT_{50} values.

Irritability Studies: Irritability responses of adult female mosquitoes of *Cx. quinquefasciatus* were assayed using the modified WHO method (Bhatia and Deobhankar, 1962). A Perspex funnel with a hole on the top was kept inverted, separately over the insecticide-impregnated papers of deltamethrin and DDT on a glass plate. Tests were performed on the parent as well as the selected strains. The single adult was allowed to settle on the paper for three min after which the time taken for the first take-off was recorded. The experiment was continued for 15 min during which the total number of flights undertaken by each mosquito was scored. Parallel control tests were performed with silicone oil-impregnated papers. Each treatment had 25 replicates. Data was analysed using Student's t-test. The relative irritability was calculated by dividing mean number of take-offs stimulated by insecticide by that stimulated by control, whereas irritability ratio was determined by dividing relative irritability of insecticide-selected population by that of susceptible population.

RESULTS

Larval Susceptibility Tests/Selections: The larvae of *Cx. quinquefasciatus* were highly susceptible to deltamethrin; however selection of the susceptible larvae of *Cx. quinquefasciatus* with deltamethrin caused a high level of deltamethrin resistance (1449-fold) after 40 generations of selection. Synergised selections of the parent and F_{24} deltamethrin-resistant larvae with deltamethrin + PBO (1:5) initially reduced the resistance levels and the speed of the development

of resistance to deltamethrin but later increased the resistance levels considerably (Table I).

Adult Susceptibility Tests/Selections: Deltamethrin was found to be highly effective against the adults of *Cx. quinquefasciatus.* The selection pressure of deltamethrin exerted at the adult stage for 40 successive generations caused only a slight increase in resistance. When tested against deltamethrin and DDT the adults emerged from the larval-selected strains of *Cx. quinquefasciatus* did not show any significant rise in resistance to these insecticides as compared to the parent strain (Table II).

Cross and Stage-specific resistance studies: The larvae arising from strains selected with deltamethrin at adult stage did not develop any significant level of resistance to deltamethrin. Also, the development of high cross-resistance levels with DDT in the deltamethrin-larval selected strains and with deltamethrin in DDT-larval selected strains is an interesting result (Table I). On the contrary, the adults of deltamethrin-selected strains did not show any significant levels of cross-resistance to DDT, while the adults derived from DDT-larval selected strain, though gained high levels of DDT-resistance, yet did not exhibit any cross-resistance to deltamethrin (Table II).

Knockdown Experiments: The adults of *Cx. quinquefasciatus* exhibited a pronounced knockdown response towards deltamethrin, as the parent adults took only 22 min. exposure to deltamethrin to exhibit 50% knockdown effect. The maximum knockdown resistance was mere 2.5-fold in the adults emerged after the 40 generations of the larval selections with deltamethrin. The other insecticide-selected strains exhibited even lower KT_{50} values confirming the efficacy of deltamethrin as a good knockdown agent (Table III). None of the mosquitoes recovered from the knocked down state.

The exposure of adult *Cx. quinquefasciatus* to 4 % DDT-impregnated papers elicited a much slower knockdown response as compared to deltamethrin (Table IV). The parent strain took a very high 50 % knockdown time of 265 min. against DDT. This knockdown resistance increased 2.4-fold after 20 generations of deltamethrin larval selection further augmenting to 3.6-fold in 40^{th} generation. Selections with synergised deltamethrin registered comparatively low level of knockdown resistance to DDT. Maximum increase in knockdown resistance of 5-fold was exhibited by DDT-resistant F_{40} adults of *Cx. quinquefasciatus.* The knocked down mosquitoes did not recover from this state.

Irritability Studies: Deltamethrin also elicited a highly effective irritability response in the adult *Cx. quinquefasciatus* (Table V), causing less than half the time to fly first in the parent strain as against control paper. Larval/adult selections with deltamethrin alone or synergised deltamethrin did not reduce the irritability response of the adults, to deltamethrin, as the time elapsed before the first flight ranged from 4.4 to 4.7 min as compared to 4.1 min in the parent strain. Larval selections with DDT also resulted in identical irritability response, taking 4.5 min. before first take off (Table V).

The mean number of flights recorded by adult *Cx. quinquefasciatus* also established the efficacy of deltamethrin as an irritant pyrethroid. Exposure to deltamethrin for 15 min caused 4.5 to 8-fold increased irritability in different strains (Table V). However, the larval/adult selections with deltamethrin/synergised deltamethrin/DDT resulted in slight decrease in the irritability status of adult mosquitoes with irritability ratio ranging from 0.55 to 0.8 (Table V).

The irritability responses of female adults of *Cx. quinquefasciatus* to 4% DDT evinces the superiority of deltamethrin over DDT as an irritant insecticide, as DDT exposure caused increased time before first take-off as compared to deltamethrin (Table VI). The insecticide-selected strains exhibited slight decreased irritability response in the adults shown by the longer time duration elapsed before the first flight and the reduced number of flights incurred by the females as compared to the parental strains. The adults of DDT-resistant strain were found to be the least irritant to DDT-impregnated papers.

DISCUSSION

Deltamethrin has already established its high insecticidal activity against various species of *Culex,*

Table I : Larval LC_{50} (ppm), Larval LC_{90} (ppm), Resistance Ratios (RR) and Cross Resistance (CR) to Deltamethrin and DDT in Various Strains of *Cx. quinquefasciatus.*

Strain	LC_{50} to Deltamethrin	LC_{90} to Deltamethrin	RR to Deltamethrin	CR to DDT
Parent	0.000121 (0.000109 - 0.000135)	0.000460 (0.000382 - 0.000555)	-	-
F_{20} Deltamethrin Larval-selected	0.016581 (0.014371 - 0.019129)	0.070437 (0.067999 - 0.072963)	137.0	175.0
F_{40} Deltamethrin Larval-Selected	0.175350 (0.125671 - 0.244667)	1.491458 (1.169434 - 1.610642)	1449.2	342.2
F_{20} Deltamethrin plus PBO-larval Selected	0.005124 (0.004149 - 0.006328)	0.019972 (0.014438 - 0.027628)	51.2	152.4
F_{40} Deltamethrin plus PBO-larval selected	0.023737 (0.019306 - 0.029184)	0.100738 (0.069454 - 0.146111)	196.2	344.0
F_{20} Deltamethrin Adult-Selected	0.000167 (0.0000511 - 0.000747)	0.002140 (0.001718 - 0.003136)	1.4	3.0
F_{40} Deltamethrin Adult-Selected	0.000338 (0.000261 - 0.000437)	0.002055 (0.001277 - 0.003308)	2.8	5.5
	LC_{50} to DDT	LC_{90} to DDT	RR to DDT	CR to Deltamethrin
Parent	0.1414 (0.1196 - 0.1671)	0.6521 (0.4887 - 0.8707)	-	-
F_{20} DDT larval-Selected	13.2862 (11.2888 - 15.6370)	46.6445 (34.7704 - 62.5734)	94.0	21.3
F_{40} DDT larval-Selected	28.2322 (24.2136 - 32.9179)	76.6136 (61.8964 - 94.8302)	199.7	27.1

Values in parentheses indicate the lower and upper 95% fiducial limits.

Table II : Adult LT_{50} (min), LT_{90} (min), Resistance Ratios (RR) and Cross Resistance (CR) to 0.025% Deltamethrin and 4% DDT in the Adults Emerging From Larval/Adult - Selected Strains of *Cx. quinquefasciatus.*

Strain	LC_{50} to Deltamethrin	LC_{90} to Deltamethrin	RR to Deltamenthrin	CR to DDT
Parent	5.731 (4.580 - 7.170)	35.172 (23.610 - 52.397)	-	-
F_{20} Deltamethrin Larval-selected	7.498 (5.460 - 10.295)	62.975 (31.282 - 93.818)	1.3	0.8
F_{40} Deltamethrin Larval-Selected	8.299 (5.833 - 11.807)	82.221 (40.060 - 168.749)	1.4	0.9
F_{20} Deltamethrin plus PBO-Larval Selected	6.613 (4.701 - 9.304)	60.245 (31.261 - 116.103)	1.2	0.8
F_{40} Deltamethrin plus PBO-Larval Selected	8.903 (5.655 - 11.583)	71.004 33.753 - 149.365)	1.4	0.8
F_{20} Deltamethrin Adult-Selected	15.499 (11.942 - 20.116)	80.606 (49.609 - 130.991)	2.7	0.8
F_{40} Deltamethrin Adult-Selected	24.654 (20.012 - 30.372)	85.733 (60.876 - 120.739)	4.3	0.8
	LC_{50} to DDT	LC_{90} to DDT	RR to DDT	CR to Deltamethrin
Parent	74.444 (62.051 - 89.312)	222.504 (150.889 - 328.108)	-	-
F_{20} DDT Larval-Selected	90.488 (68.542 - 119.460)	272.704 (182.013 - 463.177)	1.2	0.7
F_{40} DDT Larval-Selected	94.767 (80.566 - 111.472)	302.321 (224.885 - 406.420)	1.3	0.8

Values in parentheses indicate the lower and upper 95% fiducial limits.
0.025% deltamenthrin is the former and 4% DDT is the present discriminatory dosage recommended by WHO.

Table III : Adult KT_{50} and KT_{90} (in min) of the Parent and Insecticide - Selected Strains of *Cx. quinquefasciatus* on Exposure to 0.025 Deltamethrin - Impregnated Papers.

Strain	KT_{50}	KT_{90}	Slope ± S.E.	Heterogeneity c2 (df)[a]	Resistance ratio
Parent	22.460 (21.601 - 23.354)	29.308 (27.543 - 31.186)	11.090 ± 1.13	7.84(4)	-
F_{20} Deltamethrin Larval-Selected	46.372 (43.050 - 49.950)	77.746 (66.088 - 88.775)	5.710 ± 0.67	1.90(4)	2.1
F_{40} Deltamenthrin Larval-Selected	55.987 (50.257 - 62.534)	92.262 (71.752 - 118.635)	4.238 ± 0.70	4.64(5)	2.5
F_{20} Deltamethrin plus PBO Larval-Selected	39.408 (30.102 - 51.591)	87.004 (57.274 - 132.167)	3.726 ± 0.96	1.22(4)	1.8
F_{40} Deltamethrin plus PBO Larval-Selected	42.058 (37.443 - 56.989)	99.264 (85.569 - 115.151)	9.213 ± 1.82	1.70(4)	1.9
F_{20} Deltamethrin Adult-Selected	40.683 (30.102 - 51.591)	52.030 (57.274 - 132.167)	11.995 ± 1.20	4.88(5)	1.8
F_{40} Deltamethrin Adult-Selected	45,920 (41.409 - 51.381)	68.473 (53.455 - 78.681)	3.627 ± 0.66	2.51(5)	2.0
F_{20} DDT Larval-Selected	32.470 (30.623-34.429)	51.574 (47.074 - 56.527)	6.378 ± 0.62	4.83(6)	1.4
F_{40} DDT Larval-Selected	41.407 (30.985 - 53.606)	101.869 (65.063 - 159.496)	3.278 ± 0.80	0.30(5)	1.8

0.025% deltamethrin is the former discriminatory dosage recommended by WHO.
Values in parentheses indicate the lower and upper 95% fiducial limits.
[a] Degrees of freedom.

Table IV : Adult KT_{50} and KT_{90} (in min) of the Parent and Insecticide - Selected Strains of *Cx. quinquefasciatus* on Exposure to 4% DDT - Impregnated Papers.

Strain	KT_{50}	KT_{90}	Slope ± S.E.	Heterogeneity c2 (df)[a]	Resistance ratio
Parent	264.99 (232.98 - 301.40)	497.02 (397.76 - 576.87)	4.984 ± 0.77	1.72(6)	-
F_{20} Deltamethrin Larval-Selected	632.78 (599.85 - 669.65)	1143.08 (978.32 - 1305.72)	4.273 ± 0.62	1.83(7)	2.39
F_{40} Deltamenthrin Larval-Selected	946.46 (901.86 - 997.64)	1451.84 (1107.65 - 1630.25)	3.809 ± 0.53	2.61(9)	3.57
F_{20} Deltamethrin plus PBO Larval-Selected	405.38 (377.25 - 441.53)	745.53 (701.31 - 782.57)	4.823 ± 0.72	1.75(5)	1.53
F_{40} Deltamethrin plus PBO Larval-Selected	578.23 (502.98 - 657.91)	873.10 (813.36 - 950.43)	4.925 ± 0.69	2.99(3)	2.18
F_{20} Deltamethrin Adult-Selected	499.17 (475.03 - 530.23)	803.07 (745.99 - 869.88)	5.321 ± 0.52	2.72(5)	1.88
F_{40} Deltamethrin Adult-Selected	612.35 (545.72 - 682.27)	1035.09 (983.17 - 1082.52)	4.821 ± 0.75	1.97(6)	2.31
F_{20} DDT Larval-Selected	809.48 (775.45 - 847.72)	1556.62 (1465.44 - 1665.67)	5.027 ± 0.72	4.72(7)	3.5
F_{40} DDT Larval-Selected	1314.92 (1275.62 - 1357.42)	1815.45 (1776.20 - 1956.75)	3.598 ± 0.51	2.35(4)	4.96

4% DDT is the discriminatory dosage recommended by WHO

Values in parentheses indicate the lower and upper 95% fiducial limits.

[a] Degrees of freedom.

Table V : Responses of 3-Day Old Adult Females of Parent and Insecticide-Selected Strains of *Cx. quinquefasciatus* in Irritability Tests When Exposed to 0.025% Deltamethrin - Impregnated Paper.

Strain	Mean Time Lapse before first Take-off (in min)		Mean time of take-offs for female (in 15 min)		Relative irritability (irritability ratio)
	Control	Experimental	Control	Experimental	
Parent	8.7 ± 1.13[x] (3.3 - 18.0)	4.09 ± 0.22 a (3.02 - 7.45)	2.4 ± 0.44 (0-7)	20.52 ± 1.58 a (11 - 42)	8.274
F_{20} Deltamethrin Larval-Selected	9.4 ± 2.18 (3.28 - 18.0)	4.67 ± 0.14 ab (3.0 - 6.2)	3.2 ± 0.81 (0-7)	16.72 ± 1.42 b (12 - 42)	5.225 (0.631)
F_{40} Deltamethrin Larval-Selected	10.2 ± 2.20 (3.2 - 18.0)	4.71 ± 0.14 ab (3.0 - 6.5)	3.4 ± 0.81 (0-7)	15.53 ± 1.92 b (12 - 43)	4.568 (0.552)
F_{20} Deltamethrin plus PBO Larval-Selected	9.8 ± 1.10 (3.3 - 19.0)	4.42 ± 0.46 ab (3.0 - 7.48)	3.7 ± 0.32 (0-8)	18.53 ± 0.11 b (11 - 43)	5.000 (0.604)
F_{40} Deltamethrin plus PBO Larval-Selected	10.4 ± 1.14 (3.0 - 18.0)	4.43 ± 0.09 a (3.0 - 4.58)	3.6 ± 0.23 (0-8)	17.74 ± 0.45 ab (14 - 30)	4.917 (0.594)
F_{20} Deltamethrin Adult-Selected	11.1 ± 1.90 (3.0 - 18.0)	4.41 ± 0.07 b (3.0 - 4.58)	3.4 ± 0.86 (0-8)	19.92 ± 0.96 a (14 - 30)	5.859 (0.708)
F_{40} Deltamethrin Adult-Selected	12.5 ± 1.90 (3.26 - 18.0)	4.63 ± 0.77 ab (3.0 - 5.0)	3.6 ± 0.86 (0-8)	19.72 ± 0.94 a (14 - 32)	5.478 (0.662)
F_{20} DDT Larval-Selected	14.2 ± 1.61 (3.20 - 18.0)	4.44 ± 0.11 b (3.0 - 5.17)	2.6 ± 0.91 (0-7)	17.52 ± 1.11 a (11 - 32)	6.738 (0.814)
F_{40} DDT Larval-Selected	(14.8 ± 1.12) (3.3 - 17.0)	4.56 ± 0.08 ab (3.0 - 5.28)	3.0 ± 0.95 (0-7)	16.87 ± 1.43 b (14 - 36)	5.623 (0.680)

0.025% Deltamethrin is the former dosage recommended by WHO.

x Mean ± S.E.M.

Figures in parentheses indicate the range.

Figures in each column followed by the same letter are not significantly different at p=0.05 (Students' t-test).

Table VI : Responses of 3-Day Old Adult Females of Parent and Insecticide-Selected Strains of *Cx. quinquefasciatus* in Irritability Tests When Exposed to 4% DDT-Impregnated Paper

Strain	Mean Time Lapse before first Take-off (in min)		Mean time of take-offs for female (in 15 min)		Relative irritability (irritability ratio)
	Control	Experimental	Control	Experimental	
Parent	11.3 ± 0.86[x] (9.2 - 18.0)	7.34 ± 0.11 a (3.03 - 11.53)	1.9 ± 0.43 (0-4)	16.52 ± 1.34 a (10-22)	8.684
F_{20} Deltamethrin Larval-Selected	12.9 ± 2.18 (3.2 - 18.0)	8.47 ± 0.57 b (3.15 - 11.38)	2.0 ± 0.52 (0-5)	12.62 ± 1.56 bc (6-20)	6.300 (0.725)
F_{40} Deltamethrin Larval-Selected	13.8 ± 1.72 (8.2 - 18.0)	8.95 ± 0.98 a (3.75 - 11.45)	1.9 ± 0.42 (0-4)	11.55 ± 1.43 ab (7-18)	6.053 (0.697)
F_{20} Deltamethrin plus PBO Larval-Selected	12.5 ± 1.62 (4.1 - 19.0)	8.78 ± 0.72 b (5.05 - 11.32)	1.8 ± 0.98 (0-6)	13.58 ± 1.43 c (6-22)	7.500 (0.864)
F_{40} Deltamethrin plus PBO Larval-Selected	12.8 ± 1.43 (5.3 - 18.2)	8.52 ± 0.03 a (5.21 - 11.75)	1.8 ± 0.42 (0-4)	12.84 ± 0.98 a (7-23)	7.111 (0.819)
F_{20} Deltamethrin Adult-Selected	14.3 ± 1.14 (8.5 - 18.0)	8.19 ± 0.69 ab (3.08 - 10.3)	1.5 ± 0.40 (0-4)	13.22 ± 1.50 ab (7-20)	8.800 (1.013)
F_{40} Deltamethrin Adult-Selected	14.7 ± 2.01 (7.5 - 19.3)	8.65 ± 0.57 b (3.92 - 12.31)	2.2 ± 0.81 (0-5)	13.00 ± 1.48 b (6-24)	5.909 (0.680)
F_{20} DDT Larval-Selected	15.0 ± 1.32 (4.2 - 18.0)	9.73 ± 1.02 b (4.20 - 13.03)	1.8 ± 0.49 (0-4)	9.72 ± 0.76 c (6-14)	5.389 (0.621)
F_{40} DDT Larval-Selected	15.7 ± 1.15 (9.8 - 18.5)	10.12 ± 1.13 ab (4.81 - 14.43)	1.9 ± 0.52 (0-4)	8.90 ± 0.85 a (6-18)	4.684 (0.539)

4% Deltamethrin is the former dosage recommended by WHO.
x Mean ± S.E.M.
Figures in parentheses indicate the range.
Figures in each column followed by the same letter are not significantly different at p=0.05 (Students' t-test).

along with knockdown and excito-repellence properties (Finot *et al.,* 1997; Chandre *et al.,* 1998). Even in areas, where mosquitoes are resistant to deltamethrin, bed nets treated with deltamethrin remain effective and can be considered as a method of personal protection (Darriet *et al.,* 1998).

The present investigations also confirm the larvicidal and adulticidal activity of deltamethrin against the Indian strain of *Cx. quinquefasciatus.* However, intense larval selection of the parent strain of *Cx. quinquefasciatus* with deltamethrin resulted in a high level of deltamethrin resistance. In 1998, Chandre *et al.* reported 9 to 38-fold resistance to deltamethrin in the strains of *Cx. quinquefasciatus* from Cote d'lvoire and Burkina Faso after 42 generations of selection. Laboratory selection of *Cx. pipiens pallens* with deltamethrin in South East China caused 80 to 203-times larval resistance after 12-15 generations of selection pressure (Jinfu, 1999). Earlier, in the field trials of 2 years application of deltamethrin in China, a 30-fold resistance to deltamethrin was observed in the larvae of *Cx. quinquefasciatus* (Sinegre, 1984).

Our investigations revealed the inability of the development of deltamethrin resistance in adults even after 40 generations of selection. The use of million of deltamethrin-treated bed nets in Sichuan, China since 1987 also did not cause any resistance in the adults-of *Anopheles sinensis* and *An. anthropophagus* (Kang *et al.,* 1995). However, adult selection of a BKK strain of *Aedes aegypti* with permethrin raised its tolerance to permethrin by 7 to 10-fold (Chadwick *et al.,* 1984). The absence of resistance expression in adults may be significant in management of deltamethrin resistance problem in *Cx. quinquefasciatus* by using deltamethrin as an adulticide, but can be ascertained only after field trials.

The use of synergist PBO along with deltamethrin could cause an incomplete .suppression of deltamethrin resistance in the larvae of *Cx. quinquefasciatus.* PBO is a well known inhibitor of microsomal mono-oxygenases, which are involved in all the metabolism and detoxification of virtually all insecticides (Feyereisen, 1999). Our results suggest the probable involvement of microsomal oxidase-based mechanism in the development of deltamethrin resistance in *Cx. quinquefasciatus,* however, the incomplete block of resistance indicate presence of other mechanisms, which need to be explored at biochemical and molecular levels in this Indian strain. Involvement of different mechanisms conferring resistance to deltamethrin in *Cx. quinquefasciatus* exists in literature (Sahgal *et al.,* 1994; Bisset *et al.,* 1998; Chandre *et al.,* 1998; Chandre *et al.,* 1999), which can cause resistance management problems against *Culex* population.

A close association of deltamethrin and DDT resistance in the larvae of *Cx. quinquefasciatus* was quite evident in the present study. Earlier, Plapp and Hoyer (1968) suggested that cross-resistance to pyrethrins in DDT-resistant *Cx. tarsalis* is due to the pleiotropism of *kdr* gene. Halliday and Georghiou (1985) reported significant contribution of *kdr* towards development of permethrin and DDT resistance in permethrin-resistant strain of *Cx. quinquefasciatus.* In an African strain of *Cx. quinquefasciatus,* Chandre *et al.* (1998) indicated the involvement of target site insensitivity *(kdr)* associated with DDT and permethrin cross-resistance. However, there are no reports stating direct involvement of *kdr* in deltamethrin resistance in *Cx. quinquefasciatus* till date.

The differential stage-specific expression of resistance to deltamethrin/DDT in Indian strain of *Cx. quinquefasciatus,* suggests the possibility of different resistance mechanisms operating in the larvae and adults. Our results agree well with that of Chitra and Pillai (1984), in Indian strain of *An. stephensi* to various carbamates and organophosphates but conflict with that of Priester and Georghiou (1980) who reported 11-28 fold permethrin resistance in the adults derived from permethrin larval-resistant strain of *Cx. quinquefasciatus.* Similar results were reported by Omer *et al.* (1980) in a Pakistani strain of *An. stephensi,* where adults emerged from 144-fold DDT-resistant larvae exhibited 23-fold resistance to DDT and 8.7-fold tolerance to permethrin. The failure of the expression of the high larval resistance in the adult stage enables the use of deltamethrin as an adulticide from a resistance management perspective.

The efficacy of deltamethrin as an effective an early

knockdown agent against the adults of *Cx. quinquefasciatus* is confirmed in the present study. Hougard *et al.* (2003) have reported similar values of KT_{50} (29.3 minutes) in a susceptible strain of *Cx. quinquefasciatus*, on exposure to 0.025% deltamethrin impregnated papers. Earlier studies have shown that knockdown time is a good indicator for an early detection of reduced susceptibility (Chandre *et al.*, 1999). Present studies showed an increase in knockdown time with the increase in resistance levels to deltamethrin and DDT, the exposure to DDT causing slightly higher knockdown resistance as compared to deltamethrin. Earlier, Kang *et al.* (1995) had shown considerable knockdown and mortality in *Cx. quinquefasciatus* that had been exposed to pyrethroids for 1.5 to 2.5 years indicating that knockdown might be a more sensitive method of incipient resistance. Later, in a Californian strain of *Cx. quinquefasciatus*, Hougard *et al.* (2003) reported almost no knockdown effects with deltamethrin. These results again recommend the effective use of deltamethrin as an adulticide rather than larvicide.

Further efficacy and usage of deltamethrin against adults is supported by the high irritability response of *Cx. quinquefasciatus* against deltamethrin and DDT. The slight decrease in irritability response of resistant strains towards deltamethrin/DDT suggests the effective and prolonged usage of deltamethrin against adults of *Cx. quinquefasciatus* as an irritant insecticide. Our results matched with that of Hougard *et al.* (2003) reporting reduced irritability response in deltamethrin-resistant strains of *Cx. quinquefasciatus.* Very few reports are available in the literature on the irritability response of *Cx. quinquefasciatus.* Much attention has been paid to the behavioural responses of *Anopheles* species. This increases the justification for implementing a large-scale study of deltamethrin resistance management against the adults of *Cx. quinquefasciatus.*

These investigations are of extreme significance in this strain of *Cx. quinquefasciatus* from its management perspective, however, the laboratory studies can not justify and ascertain the usage of deltamethrin as an effective adulticide in the outdoors. Therefore, further research is urgently needed to design resistance management strategies in the fields against this pest and to identify the resistance mechanisms.

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REFERENCES

Abbott WB. 1925. A method for computing the effectiveness of an insecticide. Journal of Economic Entomology *18:* 265-267.

Bhatia SC and Deobhankar RB. 1962. Irritability of Susceptible and resistant field populations of *Anopheles culicifacies* in Maharashtra state, India. Indian Journal of Entomology*24:* 36-52.

Bisset J. Dr, Maria L, Rodriguez M, Diaz LC and Soca T A. 1998. Estudio de la resistencia en una cepa de *Culex quinquefasciatus*, procendente de medellin, Colombia. Review of Cubana Medicine Tropica *50 :* 133-137.

Chadwick PR, Slatter R and Brown M J. 1984. Cross-resistance to pyrethroids and other insecticides in *Aedes aegypti.* Pesticide Science *15:*112-120.

Chandre P, Darriet F, Darder M, Cuany A, Doannio JM. Pasteur N and Guillet P. 1998. Pyrethroid resistance in *Culex quinquefasciatus* from West Africa. Medical Veterinary Entomology *12:* 359-366.

Chandre F, Darriet F, Manga L, Akogbeto M, Faye, O, Mouchet J and Guillet P. 1999. Status of pyrethroid resistance in *Anopheles gambiae* sensu lato. Bulletin of World Health Organization 77: 230-234.

Chitra S and Pillai MKK. 1984. Development of organophosphorous and carbamate resistance in Indian strain of A*nopheles stephensi* Liston. Proceedings of Indian Academy Sciences. *93 :* 159-170.

Darriet F, Guillet P, N Guessan R, Doannio JM, Koffi A, Konan LY and Larner P. 1998. Impact of resistance of *Anopheles gambiae* s.s. to permethrin and deltamethrin on the efficacy of impregnated mosquito nets. Medical Tropica *55 :* 349-354.

Das PK, and Kalyanasundaram M. 1984. Evaluation of K-Othrine, a synthetic pyrethroid for insecticidal efficacy against mosquito vectors. Indian Journal of Medical Research *80 :* 74-77.

Elissa N, Mouchet J, Riviere F, Meunier JY and Yao K. 1993. Resistance *of Anopheles gambiae* s.s. to pyrethroids in Cote d'lvoire. Annals de la Societe Belge de Medecine Tropicale *73:* 291-294.

Feyereisen R. 1999. Insect P_{450} enzymes. Annual Review of Entomology *44:* 507-533.

Finney DJ. 1971. Probit Analysis, III (Ed.), Cambridge University press, United Kingdom, pp 339.

Finot L, Duchon S, Chandre F, and Guillet P, 1997. Laboratory investigations on the activity of bifenthrin against mosquitoes. Unpublished document, WHO, Geneva, Switzerland.

Halliday WR, and Georghiou GP. 1985. Cross-resistance and dominance relationships of pyrethroids in a permethrin-selected strain of *Culex quinquefasciatus* (Diptera: Culicidae). Journal of Economic Entomology *78:* 1227-1232.

Hougard JM, Duchon S, Darriet F, Zaim M, Rogier, C, and Guillet P. 2003. Comparative performances under laboratory conditions of seven pyrethroid insecticides used for impregnation of mosquito nets. Bulletin of World Health Organization *81:* 324-333.

Jinfu W. 1999. Resistance to deltamethrin in *Culex pipiens pallens* (Diptera: Culicidae) from Zhejiang, China. Journal of Medical Entomology *36:* 389-393.

Kang W, Gao B, Wang H, Yu T, Xu B, and Curtis CF. 1995. Test for possible effects of selection by domestic pyrethroids for resistance in culicine and anopheline mosquitoes in Sichuan and Hubei, China. Annals of Tropical Medicine and Parasitology *89:* 677-684.

Kumar S, Thomas A, Sahgal A, Verma A, Samuel T and Pillai MKK. 2002. Effect of the synergist Piperonyl Butoxide, on the development of deltamethrin resistance in yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae). Archives of Insect Biochemistry and Physiology *50 :* 1-8.

Omer SM, Georghiou GP, and Irving SN. 1980. DDT/pyrethroid resistance inter-relationships in *Anopheles stephensi*. Mosquito News *40:* 200-209.

Plapp FW Jr. and Hoyer RF. 1968. Possible pleiotropism of a gene conferring resistance to DDT, DDT analogues, and pyrethrins in the house fly and *Culex tarsalis*. Journal of Economic Entomology *11:* 761-765.

Priester TM and Georghiou GP. 1980. Cross-resistance spectrum in pyrethroid-resistant *Culex quinquefasciatus*. Pesticide Science *1:* 617-624.

Sahgal A, Kumar S and Pillai MKK. 1994. Microplate assay of elevated esterase activity in individual pyrethroid-resistant mosquitoes. Journal of Biosciences *19:* 193-199.

Sinegre G. 1984. La résistance des Dipteres Culicides en France. In Colloque sur la reduction d'efficacité des trâitments insecticides et acaricides et problemes de resistance. Paris 20 mars 1984, Sociètè Francaise de Phytiate et de Phytopharmacie: 47-57.

Verma KVS and Rehman SJ. 1986. Development of knockdown resistance *(kdr)* against fenvalerate in a DDT-resistant strain of *Anopheles stephensi*. Current Science 55: 914-916.

Vulule JM, Beach RF, Atiele FK, Roberts JM, Mount D1 and Mwangi RW. 1994. Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin-impregnated bednets and curtains in Kenya. Medical and Veterinary Entomolgy *8:* 71-75.

World Health Organization. 1981 a. Instructions for determining the susceptibility or resistance to mosquito larvae to insecticides. Unpublished Document WHO/VBC/81.807.pp7.

World Health Organization. 198Ib. Instructions for determining the susceptibility or resistance of adult mosquitoes to organochlorines, organophosphates and carbamates insecticides. Establishment of the baseline. Unpublished Document WHO/ VBC/ 81.805.pp7.

Zaim M, Aitio A, and Nakashima N. 2000. Safety of pyrethroid-treated nets. Medical and Veterinary Entomology *14:* 1-5.

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Short Communication

Evaluation of Triclabendazole and Tetramisole control packages against paramphistomiasis and G.I. nematodiasis in cattle and buffaloes

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Triclabendazole @ 10 mg/kg b.wt. and Tetramisole @ 15 mg/kg b.wt., given along with supportive drugs (liver stimulants, haematinics, antidiarrhoeals etc.) to control natural paramphistomiasis in cattle and buffaloes and G.I. nematodiasis in cattle were found to be 100 percent effective from 10th DPT and 7th DPT onwards, respectively. The inclusion of supportive drugs in the control package was found to be very useful in restoring general health and milk production in milch cattle and buffaloes.

Keywords : Buffaloes, Cattle, Nematodiasis, Paramphistomiasis, Tetramisole, Triclabendaole

Paramphistomiasis and gastro-intestinal (G.I.) nematodiasis are the commonest chronic parasitic diseases affecting grazing cattle and buffaloes in villages nearby Ranchi (Bharti, 2000). The subclinical course of the infections usually remain uncared for by the farmers but considerable economic losses incurred in terms of ill health and reduced growth and milk production (Biondani and Steffan, 1988). Regular treatment of the affected animals was found to be useful in protecting their health and production capabilities. This paper reports the studies carried on the animals constantly acquiring natural infections with variety of helminths under the village conditions.

Twelve adult crossbred milch cattle of local village farmers naturally infected with *Paramphistomum/ Fasciola spp*. were selected after stool examination and divided into two groups (I & II). They were maintained by the farmers at their own sheds with adequate feeds and greens. The first group animals were given anthelmntic treatment with Triclabendazole @ 10 mg/kg body weight orally while the second group cattle were left as untreated infected control.

Similarly, twelve milking buffaloes having the same nature of infection were selected for the trials at farmers' doorstep. Six of them were treated with Triclabendazole while the other six were left as untreated infected control.

In the other set of trials, twelve cattle infected naturally with common G.I. helminths such as *Oesophagostomum, Strongyloides, Trichostrongylus, Haemonchus, Bunostomum* and *Trichuris* spp. were selected for the trial at farmer's premises. Six of them were given Tetramisole orally @ 15 mg/kg b.wt. while the remaining six were kept as untreated infected control. All the treated and untreated control cattle and buffaloes in both set's of trials were attended upto 20 days post-treatment (DPT) for pre- and post-treatment EPG on zero day and thereafter twice weekly upto 20 days. Supportive therapy consisting of antidiarrhoeal, liver stimulants, haematinics, minerals, vitamins, antibiotics and others of the type were also given as and when required by any of the animals. The readings recorded were statistically analysed (Snedecor and Cochran, 1968).

Triclabendazole along with supportive therapy was found 100 percent efficacious upto 10th day treatment in cattle and buffaloes suffering from natural chronic paramphistomiasis (Table 1). The transient diarrhoeal reaction, reduced appetite and reduced milk yield

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Table 1: Efficacies of Triclabendazole and Tetramisole against Fascioliasis/Paramphistomiasis and G.I. nemadodiasis in cattle and buffaloes.

Species of animals	Groups No. of	Drug used	Dose and route	Av. Pre-treatment EPG (0 day)	Av. Post - treatment EPG and drug efficacy (%) 3rd day	7th day	10th day	13th day	17th day	20th day
Cattle	I(6)	Triclabendazole + Supportive drugs	10 mg/kg b.wt. orally	766.67 ±55.76	133.33 ±42.15 (82.61)	66.66 ±33.33 (91.310)	0.00 ±0.00 (100)	0.00 ±0.0 (100)	0.00 ±0.00 (100)	0.00 ±21.08 (95.65)
	II(6)	-	-	600.00 ±57.72	633.33 ±55.76	700.00 ±57.75	766.66 ±49.43	866.66 ±42.15	950.00 ±49.99	1016.67 ±47.72
Buffaloes	III (6)	Triclabendazole +Supportive drugs	10 mg/kg b.wt. orally	55.00 ±42.82	83.33 ±30.73	33.33 ±21.08	0.00 ±0.00 (100)	0.00 ±0.00 (100)	0.00 ±0.00 (100)	0.00 ±0.00 (100)
	IV (6)	-	-	483.33 ±60.09	616.67 47.73	700.00 ±57.74	783.33 ±47.73	866.67 ±42.16	933.33 ±49.44	1050.00 ±56.27
Cattle	V (6)	Tetramisole +supportive drugs	15 mg/kg b.wt. orally	650.00 ±42.81	83.33 ±30.73 (87.18)	0.00 ±0.00 (100)	0.00 ±0.00 (100)	0.00 ±0.00 (100)	0.00 ±0.00 (100)	0.00 ±0.00 (100)
	VI (6)	-	-	616.67 ±60.08	700.00 ±57.73	783.33 ±60.06	850.00 ±49.98	950.00 ±56.26	966.67 ±42.15	1050.00 ±56.26

Figures in parentheses indicate percent efficacies

observed in some of the treated animals of both the species were found to be restored almost to the previous status within 3-4 days indicating that the supportive treatments would have aided the vital organs in recovering from the ill effects caused by the parasites.

The absolute efficacy of the drug against the experimental and natural *Paramphistomum* spp. were also reported by some of the earlier workers (Mishra *et al*., 1987; Naseer *et al*., 1994). However, none of the above reports indicated the duration of recovery. On the other hand, Waruiru *et al.* (1994) could find 95.90.percent efficacy of the drug only upto 14th day post-treatment.

From 7th DPT, Tetramisole with supportive drugs showed 100 percent efficacy against the common G.I, nematodiasis in cattle (Table 1) while some of the earlier workers (Mishra (1972), Jagannath *et al.* (1988) and Naseer *et al.* (1994) could observe similar effect of the drug on 15th DPT. The early recovery from the chronic infections with improvement in general health and production in cattle indicated that Tetramisole could be a better choice for regular application of therapeutic control of G.I. nematodiasis in milch cattle and buffaloes.

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REFERENCES

Bharti P. (2000). Prevalence, therapeutic control and pathophysiology of common helminths infecting stallfed and grazing cattle and buffaloes in and around Ranchi, Bihar M.V.Sc. Thesis, Birsa Agricultural University, p - 103,

Biondani CA and Steffan PE. (1988). Effects of gastrointestinal parasites on milk production in dairy herds. Veterinaria Argentina, **5** (42) : 116-127. (Cited from CAB abstract C.D. 1987-1989).

Jagannath MS, D'Souza PE and Rahman SA. (1988). Effects of Tetramisole treatment against strongyle infection among dairy cattle: Current Res., Univ. of Agril. Sci., Bangalore, **17** (7) : 95-97.

Mishra SC. (1972). Efficacy of three newer anthelmintics against haemochosis in experimental buffalo calves. Indian Vet. J., **49** (11) : 1088-1090.

Mishra SC, Swain G, Panda DN, Mohapatra PK and Das NB (1987). Efficacy of Fasinex (CIBA GEIGY) against Fascioliasis in cattle, buffaloes and goats. Indian Vet. J., **64** (8) : 701-704.

Naseer MH, Abd-Rabo, TMA and Rabo TMAA. (1994). The efficacy of Fasinex (Triclabendazole) against natural infections of cattle by fascioliasis in Kafer El-Sheikh Governorate. Assiut-Vet-Med-Jour., **31** (26) : 229-236.

Snedecor GW and Cochran WG. (1968). Statistical methods. 7th Edn., Oxford and IBH Publishing Co., New Delhi.

Waruiru RM, Nasen P, Kyrsgaar NC, Thomsborg SM, Munyua WK, Gathuma JM and Bogh HO. (1994). An abattoir survey of gastrointestinal nematode infectious in cattle in the Central Highlands of Kenya. Vet. Res. Communications. **22** (5) : 325-334.

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Short Communication

Prevalence of porcine cysticercosis in south India

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A systematic study was undertaken, during a period of three years (from July, 2000 to August, 2003) to determine the prevalence of *Cysticercus Cellulosae* from pigs in Southern states Viz. Andhra Pradesh, Tamil nadu, Karnataka and Kerala. Conventional meat inspection as well as immunodiagnostic tests like Countercurrent Immunoelectrophoresis (CIEP) and Enzyme Linked Immuno Sorbent Assay (ELISA) were used for the detection of porcine cysticercosis and its seroprevalence. By classical meat inspection, per cent positivities of 3.52, 5.50, 5.73 and 5.38 were recorded in Andhra Pradesh, Tamilnadu, Karnataka and Kerala respectively. By CIEP 6.16, 5.83, 6.04 and 5.69 per cent of sera samples were found positive in Andhra Pradesh, Tamilnadu, Karnataka and Kerala respectively. By ELISA 6.50, 6.22,6.40 and 6.50 per cent of sera samples were found positive in Andhra Pradesh, Tamilnadu, Karnataka and Kerala respectively. Detection of porcine cysticercosis by CIEP and ELISA was higher than conventional meat inspection in all these states of South India

Key Words : CIEP, ELISA, Porcine cysticerocosis , Prevalence

Cysticercosis caused by the intermediate stage or cystic form, *Cysticercus Cellulose of* the tapeworm, *Taenia solium* in pigs is one of the most important zoonotic parasitic diseases that is highly prevalent in India. Cysticercosis causes significant economic losses due to condemnation of infected carcasses (Acha et al, 1994, D'Souza and Hafeez, 1998). A comprehensive idea about the prevalence of cysticercosis in a particular locality is needed not only for the record ofepidemiological data but also for the development of improved diagnostic assays and also for its effective control. Hence, a systematic study was undertaken to determine the prevalence of *C.cellulosae* in certain southern states of India, Viz. Andhra Pradesh, Tamilnadu, Karnataka, and Kerala by routine meat inspection as well as by certain serodiagnostic tests like CIEP and ELISA.

The study was undertaken in various districts of Andhra Pradesh, Tamilnadu, Karnataka and Kerala. The blood of these pigs was collected for the separation of serum and the carcasses were thoroughly screened as per the routine meat inspection procedures (paramanik et al. 1985). A total of 935 pigs in Andhra Pradesh, 345 in Tamilnadu, 366 in Kamataka and 167 in Kerala were screened during their slaughter, to assess the prevalence of porcine cysticercosis (Table 1).

A total of 584 sera samples from Andhra Pradesh, 257 from Tamilnadu, 281 from Karnataka and 123 from Kerala were also collected from pigs and screened for the prevalence of porcine cysticercosis by serological tests like CIEP and ELISA (Table 2). Antigen B was prepared by homogenising the solid parts of C.*cellulosae* Viz, the walls and scolices as per the methods of Bertha et al (1982) and the hyper immune serum was raised in rabbits.

Counter current immuno electrophoresis (CIEP) was performed according to the method of Geerts et al. (1981a) and Kumar and Gaur (1987b) using 1 Per cent Agarose (Loba chemie, Mumbai) prepared in Barbitone buffer (pH 8.6).

Enzyme Linked Immune Sorbent Assay (ELISA) test was conducted as described by Kumar and Gaur (1987b) by coating Immulon plates (Dynatech, Virginia, USA)

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with Antigen-B in phosphate buffered saline, pH 7.2.

In the present investigation out of 1813 pigs examined in four states of south India (Andhra Pradesh, Tamilnadu, Karnataka and Kerala) *C.cellulosae* was observed in 82 pigs and the overall percentage of infection was 4.52. Out of the four states screened, the incidence of *C.cellulosae* was highest in Karnataka (5.73%), followed by Tamilnadu (5.50%), Kerala (5.38%) and Andhra Pradesh (3.52%) (Table 1).

Table 1: Prevalence *of Cysticercus ceilulosae* in pigs in Andhra Pradesh, Tamilnadu, Krnataka and Kerala. (Based on meat inspection)

State	No. of animals observed	No. of animals infected	% infected
Andhra Pradesh	935	33	3.52
Tamilnadu	345	19	5.50
Karnataka	366	21	5.73
Kerala	167	9	5.38
Total	1813	82	4.52

The results of CIEP and ELISA conducted on the above pigs are presented in Table : 2 . In Andhra Pradesh 6.16 per cent (36/584) and 6.50 per cent (38/584) serum samples were found positive in CIEP and ELISA respectively. In Tamilnadu 5.83 per cent (15/257) and 6.22 per cent (16/257) sera samples were found positive by CIEP and ELISA respectively. In Kamataka out of 281 sera samples, 17(6.04%) and 18(6.40%) were found positive by CIEP and ELISA respectively. In Kerala out of 123, 7(5.69%) and 8(6.50%) sera samples were found positive by CIEP and ELISA respectively.

Nageswara Rao (1970) in his survey in Andhra Pradesh only for five months, had reported that 7.61 percent of country pigs and 3.08 per cent of yorkshire pigs were infected with *C.cellulosae. D'souza* and Hafeez (1998) reported 6.35 per cent infection of *C.cellulosae* in the pigs of four districts of Andhra Pradesh and 6.00 per cent in three districts of Karnataka.

The incidence of C.*cellulosae* in pigs has been reported by many workers from different parts of India. The first record of Alwar (1958) from Madras indicated 7.6 per cent infection. Extensive studies have been conducted in Uttar Pradesh where Ahluwalia (1960) had found 8.0 percent infection in Western Uttar Pradesh. Deka and Gaur (1990) observed 15.5 to 17.4 percent pigs to be infected from different places of Western Uttar Pradesh. Pathak et.al (1984) noted 17 percent incidence in Uttar Pradesh. There are only two reports from West Bengal indicating a stray case of infection by Ray et al (1972) and 4.24 percent by Pramanik et.al.(1985). Varma(1982) studied the incidence in Gurgaon in Haryana and reported 7.8 percent infection. Studies at Lakhimpur in Assam indicated 36.7 percent infection in pigs (Dilip Deka 1992 and Deka *et. al.* 1995). The reports of the incidence of *Taenia solium* cysticercosis from different parts of India ranged from 1.8 to 36.7 percent. A high incidence has been found in the North Eastern states including Assam (7.01 to 36.7%) whereas the rate of incidence in many studies from Uttar Pradesh revealed prevalence from 5.35 to 17.4 per cent. The finding of the present study revealed a prevalence 3.52, 5.50, 5.73 and 5.38 per cent infection of C.*cellulosae* in Andhra Pradesh, Tamilnadu, Karnataka, and Kerala respectively, which is considerably low in comparision with other states.

Seroprevalence of *C. cellulosae* in pigs of four states of South India (Andhra Pradesh, Tamilnadu, Karnataka and Kerala) revealed 6.02 per cent and 6.42 per cent by CIEP and ELISA, respectively. The prevalence rates by ELISA compared to meat inspection were little higher, indicating that few animals have been exposed to infection with *C.cellulosae* but could not be detected on

Table 2: Seroprevalence of *Cysticercus cellulosae* in pigs in Andhra Pradesh, Tamilnadu Karnataka and Kerala (Based on CIEP and ELISA)

	No. of sera Samples screened	CIEP		ELISA	
		No. found positive	% infection	No. found positive	% infection
Andhra Pradesh	584	36	6.16	38	6.50
Tamilnadu	257	15	5.83	16	6.22
Karnataka	281	17	6.04	18	6.40
Kerala	123	7	5.69	8	6.50
Total	**1245**	**75**	**6.02**	**80**	**6.42**

routine meat inspection.

D'souza and Hafeez (1999) also reported that the results of CIEP and ELISA with sera from pigs found positive on meat inspection and negative on meat inspection in relation to the system of rearing. A certain percentage of pigs found to be negative on meat inspection from the free range system of rearing were found positive by both the serodiagnostic tests indicating that they have been exposed to infection with C. *Cellulosae* but could not be detected on routine meat inspection.

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REFERENCES

Acha WA and Aguilar JF. 1994. Studies on cysticercosis in central America and Panama. Am. J. trop. Med. Hyg., 13:48-53.

Ahluwalia SS. 1960. Some larval helminthic infestations in pigs. Indian Journal of Veterinary Science and Animal Husbandry., 30:235-239.

Alwar VS 1958. Parasites of Pigs *(Sus scrofa domestica)* in Madras, Indian Vet. J., 35:112-116.

Bertha F, Flisser A, Plancarte A and Larralde C. 1982. Immunodiagnosis of Human Cysticercosis: ELISA and Immuno electrophoresis. In; Flisser, A., Williams, K., Laclette, J.P., Larralde, C and Beltra, F., (Eds), Cysticercosis Present state of knowledge and perspectives. Academic Press, New York, pp 163-170.

Deka DK, and Gaur. SNS. 1990. Counter current immunoelectrophoresis in the diagnosis of Taenia hydatigena cysticercosis in goats. Veterinary Parasitology 37: 223-228.

Deka DK, Choudhury S and Chakraborty A. 1995. Parasites of domestic animals and birds in Lakhimpur (Assam). J. of Vet. Parasitology 9:21-25.

Deka Dilip. 1992. Occurrence of metacestode infections in domestic animals of Lakhimpur of Assam. Lakhimpur Vet. Col 4:1-2.

D'Souza PE and Hafeez Md. 1998. Studies on Cysticercus cellulosae in pigs in an organized abattoir in Andhra Pradesh, India, Journal of Veterinary Parasitology. 12 (I): 3-35.

D' Souza PE and Hafeez Md. 1999. Comparision of Routine Meat Inspection, CIEP and ELISA to detect *Cysticercus Cellulosae* Infection in Pigs. Indian vet. J. 76: 285-288.

Geerts S, Kumar V, Aerts N and Ceulemans F. 1981. Comparative Evaluation of Immunoelectrophoresis, Counterimmun-oelectrophoresis and Enzyme Linked Immunosorbent Assay for the Diagnosis of Taenia saginata Cysticercosis. Veterinary Parasitology, 8: 299-307.

Kumar D and Gaur SNS. 1987. Counterimmunoelectrophores the diagnosis of Taenia solium Cysticercosis in pigs using fractionated antigens. Journal of Veterinary Parasitology. 1,43-46.

Nageswar Rao *K.* 1970. A survey of the incidence *of Cysticercus cellulosae* among pigs in certain parts of Andhra Pradesh. Thesis submitted to Andhra Pradesh Agricultural University, Hyderabad for the award of M.Sc (vet) degree.

Pathak KML, Gaur SNS and Garg SK. 1984. Counter current immunoelectrophoresis, a new technique for the rapid serodiagnosis of porcine Cysticercosis. J. of Helminthology 58: 321-324.

Paramanik AK, Bhattacharya HM and Sengupta DN. 1985, Occurrence *of Cysticercus cellulosae* in slaughtered pigs in Calcutta and its public health significance. Indian J. Anim. Hlth., 24: 143-146.

Ray S, Banerjee TP and Banerjee KN. 1972. Extensive *Cysticercus cellulosae* infection in a pig. Indian J. of Animal Health 11:237.

Varma TK. 1982. A note on the incidence of helminth parasites of domestic pigs. Livestock Adviser.7: 56-57.

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Short Communication

Observations on the soil contamination with the zoonotic canine gastrointestinal parasites in selected rural areas of Tezpur, Assam, India

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A total of 130 soil samples were screened for the presence of helminthic ova/egg from different rural areas of Solmara, Tezpur, Assam, India. Of the 130 samples, being examined, 7 (5.4 %) were positive for Toxocara sp., 4 (3.1%) each for *Ancylostoma sp* and *Strongyloides* sp., 16 (12.31%) for both *Toxocara & Ancylostoma* sp. and 9 (6.9 %) for *Toxocara, Ancylostoma* and *Strongyloides* sp.

Key words: *Ancylostoma, Strongyloides, Toxocara,* Zoonoses

The importance of zoonotic transmission of certain canine nematodiasis to humans has already been recognized. In fact, people who are frequently in contact with soil and domestic/pet animals are more vulnerable to zoonotic diseases. Strictly speaking children are at higher risk of infection as they play in unprotected soil and with domestic animals (D' Souza et *al.* 2002; Schantz 1989; Worley,1984; Grover *et al* 2000). Of all the canine nematodes of zoonotic importance , *Toxocara canis* and *Toxocara cati*, the common intestinal parasites of dog and cat are worthy of being mentioned because of their clinical importance and occasional occurrence. The common intestinal parasite of dog *Ancylostoma caninum* and related species of canine hookworm are responsible for zoonotic disease. Larvae of *Toxocara* sp. cause visceral larva migrans ,VLM and the larva of *Ancylostoma* sp of dogs and cats cause cutaneous larva migrans, CLM.

Stray dogs are very common in Balitika, Noorbari, Juglani villages and also in the residential complexes located at Solmara cantonment in Tezpur. Traub *et al* (2002), put on record a case of hydatidosis in a 6 year old child and one case of blindness due to *Toxocara* larva migrans in the tea growing communities in Phulbari, which is very near to the present study area. It has also been reported that promiscuous defaecation (acquired habit of defaecating outdoors) pattern exists in these communities. Dogs play a major role in the dissemination and environmental contamination of infective stages of *A.lumbricoides* as they are living in close association with human habitat (Traub *et al* 2002). Moreover, eggs of *Ascaris* have been shown to adhere to items such as utensils, furniture, money, fruits, vegetables, door handles and fingers in highly endemic areas due to their sticky and resistant nature (Kagei N,1983). Traub *et al* (2002) reported that the most important role played by the dogs in this endemic foci is (tea growing areas of Tezpur, Assam) to contaminate the environment and the soil than to a lesser degree by the known dissemination method as described by Kagei (1983).

He also reported the prevalence of Hookworm(94%), *Toxocara canis*(ll%) and *Strongyloides* sp(2%) in the dog faecal samples of Phulbari, Addabarie and Balipara tea estates of Tezpur, Assam respectively.

Based on the above factual evidences, the present study was undertaken with a view to:

a) screen the presence of *Toxocara* sp in the soil samples in and around Solmara cantonment area.

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b) record the level of soil contamination with other canine GI parasitic zoonoses.

Soil samples 130 nos. were collected from playgrounds and dog frequented areas of Balitika, Juglani, Noorbari villages and Solmara residential complex for the present study.

The samples (20-25 gms) were collected with the help of a spatula from the top layer of the soil upto a depth of 1-3 cm in properly labelled plastic bags and were transported to Parasitology Lab, DRL.The samples were processed to recover helminth egg/ova using the techniques of O'Lorcain(1994),Garcia, (1993) and Yadav A.K.(2000).

Out of 130 soil samples, 40 (30.7%) were found positive for helminth egg larva (Table -1).

Out of 33 soil samples collected and analysed from Solmara, 2, 4 and 6 were found to be positive for *Toxocara sp., Ancylostoma* sp and for both the species respectively. 34 samples were collected from Solmara residential complex of which only 6 were found positive with mixed incidence of *Toxocara sp* and *Ancylostoma* sp. 9 out of 16 samples, collected from Juglani were found positive for *Toxocara* sp + *Ancylostoma* sp. + *Strongyloides* larvae. Only *Strongyloides* larvae were isolated from the soil samples of Juglani and Noorbari villages. A prevalence rate of 6.1-12% of *Toxocara* sp has been observed in the present study which is more or less in confirmation with the observations of O' Lorcain(1994) and D'Souza *et al* (2002), those who had reported prevalence rates ranging from 1-30% in UK, and 2.7 - 28% in Bangalore respectively. The present finding is much higher while compared to 1% in Australia (Lloyd 1998), and 4.16% in Chandigarh (Grover *et al* 2000), however, it is very low in comparision to 19% in USA, 25.5% in Iraq and Canada (0'Lorcain,1994). As far as *Ancylostoma sp* is concerned, 12.1% prevalence in and around Solmara cantonment well within the range of 10.8 -16% as reported by D'Souza *et al* (2002). Highest prevalence of *Toxocara* sp + *Ancylostoma* sp was recorded in this cantonment which may be due to frequent wandering and defaecating of dogs inside the campus. Same is in the case of Juglani where prevalence rate was 56.3% (*Toxocara* sp + *Ancylostoma* sp + *Strongyloides* sp).

It can be inferred from the present study that the isolation of *Toxocara* egg/ova in the Solmara residential complex and the villages, pose a potential risk of infection to inhabitants and those who work in these contaminated areas.

This presumption is further strengthened by the fact that almost all of the villagers seldom use shoes to protect themselves, instead they prefer to remain barefooted. The isolation of the *Toxocara* sp + *Ancylostoma* sp from the childrens' playground

Table 1. Prevalence of Canine gastrointestinal nematodes in and around Solmara Cantonment, Tezpur, Assam, India

Place of Collection	**No. of Samples Collected**	**Percent positive for**				
		Toxocara	***Ancylostoma***	***Strongyloides***	***Toxocara* + *Ancylostoma***	***Toxocara* + *Ancylostoma* + *Strongyloides***
DRL office campus	33	2(6.1%)	4(12.1%)	Nil	6(18.2%)	Nil
DRL old residential complex (with Childrens' Playground)	34	Nil	Nil	Nil	6 (17.6%)	Nil
Balitika	22	2(9.1%)	Nil	Nil	4(18.2%)	Nil
Juglani	16	Nil	Nil	3(18.8%)	Nil	9(56.3%)
Noorbari	25	3(12%)	Nil	1(4%)	Nil	Nil
Total	130	7(5.4%)	4(3.1%)	4(3.1%)	16(12.31%)	9(6.9%)

located inside the residential complex of Solmara suggests the possible risk of transmitting the zoonotic diseases to children. The presence of viable infective stages(larvae) of *Strongyloides* sp and *Ancylostoma* sp in the soil samples of the study area may pose a potential threat to health status of the villagers and may play an important role in contributing incidence of creeping eruptions(visceral or cutaneous larva migrans) among the human population as reported earlier by Traub *et al* (2002).

Predominance of causative organism *Toxocara* sp for VLM and *Ancylostoma* sp for CLM in Solmara cantonment area is of great concern. This warranted a detailed epidemiological study for prevention and control of zoonotic nematodiasis in the affected area.

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REFERENCES

D'Souza PE, Dhanalakshmi and MS Jaganath 2002. Soil contamination with canine hookworm and Roundworm ova in Bangalore J.Parasit Dis *26(2)107-108.*

Garcia Lynne S. 1993. In Diagnostic Medical Parasitology, 2nd Edition, American Society For Microbiology, Washington, P - 542 & 512.

Grover R, Bhati G, Aggarwal A and Malla N. (2000). Isolation of Toxocara eggs in and around Chandigarh, India, J Parasit Dis 24(1) 57-59.

Kagei N. 1983. Techniques for the measurement of environmental pollution by infective stages of soil transmitted helminthiasis ; Yokogawa M, Hayashi S, Kobayashi A, Kagei N, Suzuki N, Kunni C, eds. Collected papers on the control of soil transmitted helminthiasis , Vol - II , Tokyo, Japan, Asian Parasite Control organization., 27-46.

Lloyd S. 1998. Toxocariasis in Zoonoses - Biology, Clinical practice and Public health control by Palmer SR, Lord Soulsby, EJL & Simpson, DIH, pp 841-854.

Lorcain P. 1994. Prevalence of Toxocara canis in public playgrounds in the Dublin area of Ireland J Helminthol 68:237-241.

Schantz PM. 1989. Toxocara larva migrans, now J Trop Med &. Hyg 4th (Suppl) : 21-34.

Traub, R.J, Robertson ID, Irwin P, Mencke N and Thompson RCA. 2002. The role of Dogs in transmission of gastrointestinal parasites in a remote tea growing community in North Eastern India, Am J Trop Med Hyg, 67(5)539-545.

Worley H, Green JA, Forthingh JE, Sturner KA et.al. 1984. *Toxocara canis* infection: clinical and epidemiological associations with seropositivity in kindergarten children J Infect Dis 149:591-597.

Yadav AK. 2000. Young Scientists' workshop on the diagnosis and identification of parasites of Bio-medical importance, a training manual.

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Short Communication

Epidemiology of *Taenia solium* cysticercosis in pigs of Northern Punjab, India

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The study was carried out to record the prevalence of swine cysticercosis and to find out the correlation between the disease and various epidemiological factors. The overall prevalence rate was found to be 6.35 per cent among 236 pig carcasses. Age-wise prevalence was more (6.48%) in younger pigs < 1 year of age than those aged > 1 year (6.25%). Prevalence rate was found to be 8.82 per cent in males and 4.48 per cent in females. *Cysticercus cellulosae* infection was thus apparently independent of age and sex of pigs whereas it had significant statistical correlation with the breed and managemental practices adopted. Season wise prevalence was found to be highest (13.46%) in post monsoon and lowest (3.13%) in pre monsoon season but was not found statistically significant. Most common predilection sites were found to be muscles of thigh, forequarter and neck followed by masseter muscles, tongue, heart, diaphragm, liver, oesophagus and kidney.

Keywords: Epidemiology, Pigs, Punjab, Swine Cysticercosis

Taenia solium infection (taeniasis) and cysticercosis is a major public health problem, associated with pork consumption in both developed and developing countries (Kumar and Gaur, 1994). Cysticercosis in animals is of economic importance due to losses caused by condemnation of organs/carcasses of slaughtered animals. Clinical symptoms in pigs are usually absent and animals appear to be perfectly healthy despite heavy infection. Possible infection of man with cysticerci lead to serious disease in the form of dermal, muscular, ocular or even neuro-cysticercosis (Cruz *et al.*, 1989). Swine cysticercosis has been reported by various workers in different parts of India (Deka *et al.*, 1985; Pathak and Gaur, 1988; Varma and Ahluwalia, 1989; Deka and Gaur, 1990). The present investigation was carried out to determine the prevalence of porcine cysticercosis vis-a-vis certain epidemiological and husbandry parameters in a relatively unexplored region of Punjab.

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Regular visits were made to the pig meat shops and backyard/unauthorized slaughter houses in different parts of the Ludhiana City, Punjab throughout the calendar year from March, 2002 to February, 2003. Postmortem examination of 236 pig carcasses was carried out. Carcasses were thoroughly screened for the presence of cysts of *Taenia solium* in different organs and tissues. Samples found positive for cysts were brought to the laboratory on ice and examined in detail for number, size, colour and texture of the cysts. History regarding age, sex, breed and management practices followed for rearing of pigs was taken. The data were statistically analysed using Chi square test.

Out of 236 swine carcasses examined at different pig meat shops, 15 were found positive for presence of C. *cellulosae* showing an overall prevalence of 6.35 per cent. Prevalence of C. *cellulosae* reported in present study is in accordance with the available reports from different parts of the country (Sinha 1970, Pathak and Gaur, 1988, Varma and Ahluwalia 1989). However, it was quite low as compared to studies made by Pathak *et*

al (1984) who reported an incidence of 17 per cent and Deka *et al* (1985) who reported as high as 20.8 per cent. This variation in the prevalence rate in different areas is possibly due to variation in the management practices under which pigs are being reared and ecological variation.

Grossly, the cysts were visible and present on or embedded inside different tissues and organs of the carcasses. The smaller cysts were usually white while the bigger were pink or yellowish in colour. This is probably due to the age of larvae i.e. smaller cysts contain younger while larger ones have older larvae. All the cysts were of cellulose type having thin collagenous capsule. These were typically spherical or oval, white or yellow vesicle with a translucent bladder wall, through which the scolex could be seen like a small solid eccentric granule. In pigs, only cellulose type of cysts are found, whereas in man, in addition to cellulose, racemose type of cysts are also observed which are bigger than cellulose type and scolex is not visible through cyst wall (Rabiela *et al* 1989).

Most common predilection sites were found to be thigh muscles (Fig. 1), forequarters and neck muscles where cysts were found in all the 15 positive cases. Pramanik *et al* (1985) had also reported maximum distribution of the cysticerci in muscles of thigh, neck, shoulder and tongue. Ocular and orbital cysticercosis has also been reported in pigs (Cardenas *et al* 1984) but in the present study, no cyst was found in brain and eye muscles. Out of the 15 carcasses examined 73.79 and 53.34 per cent had cysts in masseter muscles and tongue, respectively. In heavily infected cases, the cysticerci were also seen in heart, liver, diaphragm, kidney and oesophagus and this observation was confirmatory of the studies by Kumar *et al* (1991).

On statistical analysis of the data (Table 1), the breed and management practices were found to have significant correlation ($p < 0.01$; df=l) with the prevalence of parasitosis whereas no statistical relationship could be established with age, sex ($p > 0.01$; df=l) and season($p > 0.01$; df=3). These observations regarding age and sex were in agreement

Table I: Prevalence of cysticercosis in pigs in Ludhiana (Punjab) based on various parameters

Parameter		Observations on meat inspection			
		Positive		Negative	
		Number	Percent	Number	Percent
Age	< 1 year	7	6.48	101	93.51
	>1 year	8	6.25	120	93.75
Sex	Male	9	8.82	93	91.18
	Female	6	4.48	128	95.52
Breed	Desi	13	10.29	114	89.76
	Crossbred	2	1.83	107	98.17
Management	Intensive system	1	094	105	99.06
Practices	Extensive System	14	10.76	116	89.24
Season	Pre monsoon	2	3.13	62	96.87
	Monsoon	3	4.69	61	95.31
	Post monsoon	7	13.46	45	86.54
	Winter	3	5.36	53	94.64

with Pathak and Gaur (1989). The prevalence rate was quite high in pigs reared under extensive system indicating expectedly greater exposure to human excreta in case of free roaming pigs. Prevalence was found to be less in crossbred pigs because most of the crossbred pigs are kept under intensive system of rearing. Martinez *et al* (1997) had reported high prevalence of the infection in the areas where more than 50 per cent of houses had no latrines and most of the householders allow their pigs to roam freely. Vazquez *et al* (2001) observed that pigs under restraint with basic hygiene and sanitary conditions may be effective and practical interventions for the control of *T. solium* in rural communities. Although higher during post monsoon season, no significant statistical relation between occurrence of disease and season could be established.

A large number of pigs are slaughtered clandestinely everyday in Ludhiana and pork is invariably utilized for human consumption. Control of cysticercosis is a difficult task because of local habits, traditions and practices. Restriction of the scavenging habit of pigs that have access to human faecal material might help in the control of this condition. The prompt treatment of infected people will not only reduce the source of infection but will also eliminate the danger of auto-infection with cysticerci. The incidence of swine cysticercosis in a particular area reflects the incidence of taeniosis in that area and Taenia carriers further pose risk of neurocysticercosis to other human beings. Moreover, lack of knowledge regarding the transmission of the disease also plays an important role in its spread. This calls for a need of having a satisfactory control of the disease through proper veterinary inspection of meat and providing basic public health education among the vulnerable sections of society.

REFERENCES

Cardenas RR, Cells SR and Helnandez J P 1984. Ocular and orbital cysticercosis in hogs. Veterinary Pathology 21:164-167.

Cruz M, Davis A, Dixon H, Pawlowski Z S and Proano J, 1989. Operational studies on the control of the *Taenia solium* taeniasis/cysticercosis in Ecuador. Bulletin of World Health Organization 67: 401-407.

Deka D K and Gaur SNS, 1990. *Taenia solium* cysticercosis in pigs in western parts ofUttar Pradesh. Journal of Veterinary Parasitology 4: 53-63.

Deka DK, Borkakoty MR and Lakhar BC, 1985. Cysticercosis in domestic animals in north eastern region of India. Indian Journal of Parasitology 9:83-85.

Kumar D and Gaur SNS, 1994. *Taenia solium* cysticercosis in pigs. Helminthological abstracts 63: 365-383.

Kumar D, Gaur SNS and Varshney KC, 1991. Host tissue response against *Taenia solium* cysticercosis in pigs. Indian Journal of Animal Health 61:270-273.

Martinez MJJ, Aluya A, S-de, Martinez V N, Jaramillo ACJ,, Gemmell M, and De, Aluja AS, 1997. Epidemiology of cysticercosis in pigs in a rural community in the state of Guerrero, Mexico. Veterinaria-Mexico 28:281-286.

Pathak KML and Gaur, SNS 1988. Surveillance of human taeniasis and porcine cysticercosis in certain parts of Uttar Pradesh, Abstracts of the second National Congress of Veterinary Parasitology Bangalore, India pp 5.

Pathak KML, and Gaur SNS 1989. Prevalence and economic implications of *Tania solium* taeniasis and cysticercosis in Uttar Pradesh state of India. Acta Leidensia 57: 197-200

Pathak KML, Gaur, SNS and Kumar D, 1984. The epidemiology of strobillar and cystic phases of *Taenia Solium* in certain parts of Uttar Pradesh (India) Indian Journal of Veterinary Medicine 4: 17-18.

Pramanik AK, Bhattacharya HM and Sengupta, DN, 1985. Occurrence of *Cysticercus cellulosae* in slaughter pigs in Calcutta and its public health significance. Indian Journal of Animal Health 24: 143-146.

Rabiela M **T,** Rivas A and Flisser A, 1989. Morphological types of *Taenia solium* cysticerci. Parasitology Today 5: 357-359.

Sinha BK 1970 Larval cestodes from domestic pigs *(Sus scrofa domestica)* in Bihar, India. Ceylon Veterinary Journal 18: 86-87.

Varma TK and Ahluwalia, SS 1989. Incidence of *Cysticercus cellulosae* in slaughtered pigs and human taeniasis in western and central Uttar Pradesh. Indian Veterinary Journal 66: 673-674.

Vazquez FS, Ballestero RG, Flisser A and Schantz PM, 2001. Hygiene and restraint of pigs is associated with absence of *Taenia solium* cysticercosis in a rural community of Mexico. Salud-Publicade-Mexico 43: 574-576.

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Short Communication

Haematobiochemical variations during mange mite infestation in pigs and its therapeutic management

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The significantly reduced pre-treatment levels of haematobiochemical profiles Hb, PCV, TEC, Cu, Zn, Serum glucose and ALT in *Sarcoptes scabiei* infested pigs were found to have returned to about normal ranges (Hb- 13.69 & 13.62, PCV- 38.61 & 38.21, TEC- 7.28 & 7.28, Zn- 58.00 & 56.33, Cu- 67.66 & 62.66, Serum glucose- 89,00 & 89,00and ALT-37.33 & 37.83, respectively) after Ivermectin and Neem oil + karanj oil + camphor control regimes. Both the mite control packages were observed to be very useful in maintaining the normal health parameters in pigs.

Key words: Camphor, Haematobiochemical profiles, Ivermectin, Karanj oil, Neem oil, Pigs, *Sarcoptes scabiei*

Sarcoptes scabiei mite infestation in pigs causes marked reduction in the health and productivity due to marked alterations in haematobiochemical profiles (Prasad *et al.*, 2001; Minz, 2002). Information regarding haematobiochemical variations-during mite infestation in pigs and its therapeutic management is very scanty. Hence, the present investigation was undertaken to evaluate the haematobiochemical variations during mange mite infestation in pigs and their long-term effective control by herbal and chemical acaricidal packages.

Eighteen desi pigs aged about 2-3 months having natural infestations of *Sarcoptes scabiei* were selected after skin scraping examination (Soulsby, 1982) and split into three groups of six animals each. They were maintained on the usual supply of adequate balanced feeds. Group 1 and II animals were treated respectively with Ivermectin 300 µg/kg body weight single S/C injection and a herbo-mineral preparations (containing Neem oil *(Azadirachta indica)* 50ml, Karanj oil *(Pongamia glabra)* 50 ml and Camphor 10gm applied topically with cotton swab on alternate days upto 12 days. Supportive drugs in the form of liver stimulant, mineral mixture, iron preparations were used as and when needed. Group III were kept as untreated control. Haematological (Hb, PCV, TEC) and Biochemical (Zn, Copper, Glucose, ALT), profiles were recorded on day 0 pretreatment, day 20 post treatment and subsequently at monthly intervals till day 210 post treatment. For the estimation of biochemical profiles blood sera were separated *from* the collected blood samples by centrifugation @ 1000 rpm for 5 minutes. The estimation of Cu, Zn, Serum glucose and Alanine aminotransferase (ALT) were done with the help of standard Diagnostic Kits supplied by Crest Biosystem Pvt. Ltd., Goa (India) taking the observations in Double Beam UV-spectrophotometer, The Hb values were assessed by cyanmethaemoglobin (Digital haemoglobinometer) and PCV & TEC by Jain (1986),

Statistical analysis of the data of different parameters was done by using analysis of variance at 1% and 5% level of significance as per the methods described by Snedecor and Cochran (1994).

The observations in respect of haematobiochemical profiles before and after treatment have been presented in Tables I and II.

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The significantly reduced values of Hb (g%), PCV (%), TEC (Xl0^6/cumm), Cu (µg/dl), Zn (µg/dl), serum glucose (mg/dl) and increased level of ALT (U/ml) before treatment on 0 day were found to have returned towards the normal ranges after treatment on 20th DPT in the Ivermectin group and the average Hb, PCV and TEC values at 210th DPT were also found almost at the normal ranges. Similarly with the herbo-mineral treatment package applied topically, these values got improved significantly during the same period of observations. In contrast, the lowered haematological values remained almost unchanged on the same days of observations in control pigs. Similar findings were also reported by Prasad *et al.*, (2001) and Minz (2002) during mite infestation and treatment with Ivermectin and Doramectin in pigs. The present findings were also in close agreement with the findings of Hirudkar *et al.* (1997) in sarcoptic mange in sheep, Naidu and Rao (1999) in sarcoptic mange in goats and Dalapati and Bhowmik (1996) in psoroptic and chorioptic mange of goats. The marked reduction in haematological profiles in mange affected pigs might have taken place owing to decreased feed intake due to constant irritation caused by the mite infestation leading to deficiency of essential nutrients needed for normal haemopoiesis. Possibly the toxic effects of mite secretion might have caused some degree of disturbances in food assimilation, metabolism and synthesis of these biochemical molecules in different vital organs at cellular levels. The increased level of Alanine aminotransferase (ALT) during mite infestation clearly revealed the increased activity of liver and other vital organs involved in the neutralization of toxic materials (Hoe and Wilkinson, 1973). These values were recouped shortly after application of specific and supportive drugs, which would have restored the normal functions of the different organs involved in digestion, assimilation and synthesis of different haematobiochemical constituents.

The use of neem (*Azadirachta indica)* and its oil in various skin disorders including mange infestations in different animals has been well documented. Likewise, there are several published reports on the acaricidal value of Karanj *(Pongamia* spp) alone or in combination with synergists (Chhabra and Saxena, 1998).

Camphor used in the experiment was effective because it produced numbness and local anaesthetic effect on the body of animals that potentiated the action of other-two oils.

On comparison of both the drugs Ivermectin and herbo-mineral treatment, the recovery was faster in Ivermectin treated groups but both the drugs were 100 percent effective against mite till 14th day post treatment.

Table 1 : Haematological profiles (Means ± S.E.) during *Sarcoptes scabiei* mite infestation and Control packages application

Parameters	Observation periods (days)	Group I	Group II	Group III
Hb (g%)	0	11.05 ± 0.03^a	11.10 ± 0.03^a	11.00 ± 0.03^a
	10	12.58 ± 0.12^b	12.50 ± 0.03^c	10.95 ± 0.03^a
	210	13.69 ± 0.07^b	13.62 ± 0.05^c	10.84 ± 0.04^a
PCV (%)	0	6.54 ± 0.07^a	6.50 ± 0.37^a	31.00 ± 0.33^a
	20	35.50 ± 0.31^b	34.50 ± 0.20^c	30.66 ± 0.40^a
	210	38.61 ± 0.16^b	38.21 ± 0.13^c	29.73 ± 0.14^a
TEC (x10^6/cumm)	0	6.54 ± 0.07^a	6.50 ± 0.11^a	6.52 ± 0.12^a
	10	7.19 ± 0.01^b	7.14 ± 0.10^c	6.51 ± 0.11^a
	210	7.28 ± 0.05^b	7.28 ± 0.02^c	6.48 ± 0.05^a

Gr. I - Ivermectin treated; Gr. II - Neem oil + Karanj oil + Camphor treated.
Gr. III - Infected untreated control.
Figures under same superscripts in a row did not differ significantly.

Table 2 : Biochemical profiles (Mean ± S.E.) during *sarcoptes scabiei* mite infestation and its treatment.

Parameters	Observation periods (days)	Group I	Group II	Group III
Zinc (µg/dl)	0	47.66 ± 0.76[a]	46.83 ± 0.59[a]	46.83 ± 0.59[a]
	20	58.00 ± 0.47[b]	56.33 ± 0.99[b]	45.83 ± 0.59[a]
Copper (µg/dl)	0	51.33 ± 0.60[a]	52.00 ± 0.52[a]	52.50 ± 0.39[a]
	20	67.66 ± 0.75[b]	62.66 ± 0.58[c]	52.00 ± 0.47[a]
Serum glucose (mg/dl)	0	72.16 ± 0.67[a]	71.83 ± 0.51[a]	72.00 ± 0.52[a]
	20	89.00 ± 0.33[b]	89.00 ± 0.33[b]	71.50 ± 0.51[a]
ALT (U/ml)	0	41.16 ± 0.53[a]	42.50 ± 0.39[a]	41.50 ± 0.39[a]
	20	37.33 ± 0.28[b]	37.83 ± 0.25[b]	41.83 ± 0.54[a]

Gr. I - Ivermectin treated; Gr. II - Neem oil + Karanj oil + Camphor treated.
Gr. III - Infected untreated control.
Figures under same superscripts in a row did not differ significantly.

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REFERENCES

Chhabra MB and Saxena MJ. (1998). The use of phytotherapeutic agents in the control of acariasis in animals: a review. J. Vet. Parasitol. 12: 3-8.

Dalapati, MR and Bhowmik MK. 1997. Clinico-haematological biochemical and pathological profiles of Psoroptic and Chorioptic mange in goats. Indian Vet. *J.* 73. 728-733.

Hirudkar US, Deshpande PD, Narladkar BW, Bapat ST and Moregaonkar, SD. 1997. Sarcoptic mange in sheep. Haemato logical and biochemical changes during treatment with herbal medicine. Indian Vet. J., 74: 834-836.

Hoe CM and Wilkinson JS. 1973. Liver function, A review. Aust. Vet. J., 49: 163.

Jain NC. 1986. Schalm's Veterinary Haematology, 4th edn. Lea and Febiger, Philadelphia.

Minz P. 2002. Clinicopathological and biochemical studies of mange in pigs. M.V.Sc. Thesis, Birsa Agril. Univ., Ranchi.

Naidu MM and Rao DV. 1999. Clinical and haematological observations in goats with sarcoptic mange, Indian Vet., *J.*, 76: 730-732.

Prasad KD, Kumar S and Singh SK. 2001. Haematological status of pigs during *Ascaris suum* and *Sarcoptes scabiei* infection and Doramectin treatment J. Vet. Parasitol, 15: 63-65.

Snedecor GW and Cochran WG. (1994). Statistical methods. 8th edn. East West Press, New Delhi, pp-254-268.

Soulsby EJL. 1982. Helminths, Arthropods and Protozoa of Domesticated animals, 7th ed. Balliere and Tindal. London.

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Dated : December 2004

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Prof. Md. Hafeez, Deptt. of Parasitology, College of Veterinary Science, Tirupati has been honoured with 'Dr. G.D. Bhale Rao gold Medal Award' in recognition of his contributions to the field of parasitology. The award was presented to him during the 15th All India Congress of Zoology on 29th October, 2004 at University of Jammu, J&K State, India

BOOK REVIEW

Title: Parasitic Zoonoses

Author: Veer Singh Rathore

Publisher: Pointer Publishers, Vyas Building, S.M.S. Highway, Jaipur 302 003 (Raj.) India.

Year: 2005

Pages: 177

ISBN: 81-7132-399-5

Parasitic zoonotic diseases are of great public health importance and after a long gap a book on 'Parasitic Zoonoses' has been published by an Indian author.

The handbook, is the fruit of the hard work of the sole author who is a teacher-researcher in Veterinary Parasitology at S.D. Agricultural University, Sardar Krushinagar (Gujarat). The handbook compiles information, arranged in logical sequence, in 9 chapters, each of which is followed by up-to-date bibliography and references. In the general section, there are 4 chapters devoted (one each) to Introduction, Epidemiology, Diagnosis and Control of parasitic zoonoses. The other 5 chapters are related to specific aetiological classes viz. Trematodes, Cestodes, Nematodes, Protozoa and Arthropods. The Introduction includes different classifications of zoonoses. The chapter on Epidemiology deals with ecological, human behavioral, migration and socio-economic factors as also the transmission routes. The relatively extensive coverage of Diagnosis of Parasitic Zoonoses, in a separate chapter (50 pages) is a distinct positive feature. This also scores with the initiative of including in its ambit, the latest molecular biology tools such as nucleic acid probes and polymerase chain reaction, citing up-to-date references. Under Prevention and Control, whole spectrum of measures, from hygiene and sanitation, control of intermediate hosts and vectors, reduction in reservoir, veterinarian's role and health education, to immunoprophylaxis and preventive chemotherapy, have been discussed in a lucid manner.

The chapters on various zoonoses covers the essentials like life cycles in easy-to-understand drawings, as well as latest information gleaned from world literature arranged in logical sequence. It is pleasing to find among Protozoan Zoonoses, such emerging and re-emerging entities as toxoplasmosis, sarcocystosis and cryptosporidiosis with precise delineation of human and livestock segments as also diagnosis and control in the light of recent advances. By contrast, Arthropod Zoonoses has been granted a measly 5 pages and pentastomids have been altogether omitted.

The book will be a useful reference material for post-graduate, as well as advanced undergraduate students of both veterinary and human medicine, parasitology, public health and biological sciences. It will also provide valuable source material for research scientists in these fields. Moreover, at the printed price of Rs. 125/- (soft cover) per copy, it is good value for money and is sure to be well-received.

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