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Annual Report

(2007-08)

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4. A Phase IV study to expand access while assessing the safety and efficacy of paromomycin IM injection in an outpatient setting for the treatment of visceral leishmaniasis (VL) in India (Study No. VLPM03).

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Meetings/ Seminars/ Trainings organized

Meetings/ Seminars/ Conferences/ Training attended

Publications

Intramural Studies

1. PCR based diagnosis of visceral leishmaniasis from suspected cases of kala-azar in Bihar.

Objectives:

To develop a new gene target of ITS region of rRNA for the diagnosis of visceral leishmaniasis and to compare PCR result of blood with the result of conventional diagnostic method using aspirates (SA/BM).

Progress:

Peripheral blood (200µl) was collected from 100 suspected cases of kala-azar patients and DNA was isolated from all blood samples using a QIAamp DNA blood mini kit (Qiagen). Imprinted smear of the aspirates (SA/BM) were examined after Giemsa staining for the presence of amastigotes.

A nested PCR was done to amplify the internal part of ITS region of rRNA gene from previously amplified PCR products to increase the sensitivity and specificity. Amplicons were subjected to electrophoresis in 1.2% agarose gel at 50 volts in 1XTBE buffer for 2 hrs and results were visualized under ultra-violet light after staining for 15 min in ethidium bromide (0.5ug/ml). All samples were tested; PCR was also done for 10 positive & negative control samples. All the positive samples showed 600bp band and whereas negative samples showed no band.

Out of 100 suspected VL cases 76 (76%) were positive by PCR from blood and 68 (68%) cases were found to be positive by microscopy. Among these 8 samples detected by PCR but was not found in microscopy. Rests of 24 samples were negative of both tests.

2. Molecular characterization of SAG responsive and Unresponsive kala-azar isolates of Bihar.

***Objective*:**

- To demonstrate, if any variation exists in SAG responsive & unresponsive isolates of kala-azar cases of Bihar using molecular tools.

Progress:

After primary isolation & culture adaptation, mass cultures of different clinical isolates of SAG responsive (n=2) & unresponsive (n=10) strains were carried out in monophasic media. DNA was isolated from these isolates by chemical method (i.e. proteinase K, SDS and CTAB/NaCl) and ITS region of the rRNA gene was amplified from all isolates. PCR products were analyzed in 1.5% agarose gel and a band of 1100bp (approx.) was found in all.

In previously experiments four restriction endonucleases i.e. *Hha* I, *Rsa* I, *Hae* III and *Taq* I, were used, out of which only *Taq* I demonstrated the differentiation in banding patterns among the SAG (R) and SAG (UR) isolates. This time amplicons were digested with *Mae* II (*Tai* I), *Hpy* F1OVI (*Mwo* I), *Tru*1 I (*Mse* I), and *Tas* I (*TspE* I) restriction endonucleases. PCR─RFLP patterns showed 3 restriction cutting sites (i.e. 140, 250, & 561bp) with *Mae* II (*Tai* I), 2 restriction cutting sites (i.e. 360 & 647bp) with *Hpy* F1OVI (*Mwo* I) and 2 restriction cutting sites (i.e. 769 & 820bp) with *Tru*1 I (*Mse* I) in all (SAG-responsive/ unresponsive) isolates. But differences were observed among the SAG (R) and SAG (UR) isolates restricted with *Tas* I (*TspE* I) restriction endonucleases. A band of 400bp (approx.) was observed in SAG (R) isolates but not in SAG (UR) isolates restricted with *Tas* I (*TspE* I).

3. Study of Imprint smear microscopy and PCR application on biopsy from dermal lesions for diagnosis of Post kala azar dermal leishmaniasis (PKDL) cases from Bihar.

***Objectives*:**

1. To apply the PCR for diagnosis of PKDL in comparison to the conventional microscopy of skin biopsy imprint smear.
2. PCR application in PKDL cases after treatment and during follow up.

***Progress* :**

Biopsies were collected aseptically in Tris buffer solution from different skin lesions (i.e. macular, papulo-nodular and erythematous) of the PKDL cases (n=38) after obtaining informed consent. Imprint smears on clean glass slides were prepared for detection of leishmania parasites under microscope and blood sample were collected for haematological tests.

PCR detection of whole ITS region of the ribosomal RNA (rRNA) gene was done in all skin biopsies. Samples were scored as positive when a PCR product of 600 bp could be detected as in the positive controls. For negative control, 5 biopsies were collected from cases with fungal skin lesions and leprosy. Leishmania parasites culture isolates from PKDL cases were used as positive control. The results obtained by PCR technique were compared with microscopy result of the imprint smear.

Out of 38 PKDL cases, 19 cases had only hypopigmented macular lesions and the other 19 cases had mixed lesions of papulonodular-erythematous with or without macular lesions. Past history of kala azar was present in all with duration of 1 to 16 years.

In imprint smear microscopy, Parasite positivity was found in 17 (89.47%) papulonodular cases and only in 7 (36.8%) macular cases with presence of mononuclear cells, histiocytes and lymphocytes in the smear. The biopsy smear from skin lesions of fungal infection and leprosy cases were negative for the Leishmania parasite with few mononuclear cells.

PCR result gave positivity in 17 (89.47%) papulonodular cases of PKDL. **PCR positivity was found to be significantly increased in the hypopigmented macular cases of PKDL** where it was positive in 16 (84.21%) cases, whereas the microscopy could detect only 36.8 % positivity in macular lesions. These cases were positive for leishmania parasites in their imprint smear also. Both PCR and imprint smear were negative in the negative control cases. The study will be conducted in 50 fresh cases of PKDL for final interpretation.

4. Study of clinical and laboratory parameters as a predictive value for treatment failure with different anti-leishmanial drugs.

***Objectives*:**

1. To determine predictors of treatment failures by different anti-leishmanial drugs based on clinical and laboratory parameters.
2. To compare initial cure and final cure by different parameters at 6 month follow up.

***Progress*:**

Fresh and parasitologically confirmed VL case from both the sexes admitted to the indoor ward were taken for this study. The clinical parameters used in this study were demography, duration of illness before the start of anti-leishmanial drugs, AmphotericinB. Size of spleen was measured through USG at day 0, weekly and at the end of therapy as well as follow up at 1 and 6 month.

Laboratory parameters include Hb%, Total and differential W.B.C. count, platelet count, serum albumin, amylase, liver and renal function tests along with few additional parameters such as CRP, serum folate, ferritin, transferrin, iron, apolipoprotein A1 and ApoE and triglyceride were assessed at different time points (Table 1).

Table 1: Laboratory parameters at different time points (N=10)

Parameters	0 Day	7 Day	14 Day	21 Day	EOT	1-month Follow up	Normal
Albumin	1.0 – 3.2	2.9 – 4.1	3.4 – 4.4	3.2 – 4.6	3.7 – 4.7	3.5 – 4.9	3.2 – 5.0
Amylase	61.0 – 176.0	58.0 – 189.0	80.0 – 182.0	70.0 – 261.0	63.0 – 169.0	70.0 – 150.0	<100
Transferrin	93.0 – 170.0	90.0 – 166.2	99.7 – 171.6	117.1 – 168.3	120.5 – 158.2	165.0 – 230.0	200.0 – 380.0
Iron	43.9 – 64.0	49.2 – 68.2	48.9 – 69.0	48.9 – 68.0	52.2 – 70.2	60.5 – 95.0	65.0 – 175.0 (M) 50.0 – 170.0 (F)
Apo A1	72.0 – 131.9	88.0 – 125.1	92.0 – 128.2	98.8 – 128.9	101.2 – 138.8	110.1 – 140.6	122.0 – 161.0
Triglyceride	84.0 – 183.0	58.0 – 250.0	40.0 – 168.0	55.0 – 177.0	54.0 – 148.0	72.0 – 146.0	<150
TIBC	133.3 – 243.5	129.0 – 237.0	142.5 – 254.2	147.9 – 244.1	162.2 – 231.2	176.5 – 252.3	200.0 – 400.0
Transferrin saturation %	22.1 – 48.0	26.1 – 50.0	24.8 – 45.7	26.3 – 44.7	26.9 – 39.9	27.8 – 41.0	-
Hb%	6.2 – 10.2	7.6 – 10.0	8.8 – 10.6	9.0 – 11.2	10.0 – 11.4	11.8 – 13.0	10.0 – 14.0
TC	1750 – 9930	4230 – 8400	4800 – 10500	5200 – 9600	6300 – 10960	6350 – 10980	4000 – 11000
Platelet	80000 – 180000	78000 – 210000	120000 – 250000	200000 – 260000	215000 – 278000	216000 – 292000	150000 – 350000

After administration of Amphotericin B, it was observed that albumin, transferrin, iron, Apo A1, TIBC, Hb% and platelet count was down regulated at the start of the therapy. Alpha-amylase was found to be increased in few VL patients which suggest about the involvement of pancreas during VL infection. The entire above mentioned laboratory parameters improved during the course of therapy. It was observed that transferrin, albumin, iron which was down regulated can act as a

predictive parameter in the assessment of drug response, while alpha amylase can be suggestive of complication related to pancreas and can lead to drug failure in latter course.

The study of predictive value of clinical and laboratory parameters on Miltefosine need to be studied further in order to assess its treatment failure and target those parameters.

5. Susceptibility to Visceral Leishmaniasis (Kala-azar) in human beings - the role of Testosterone.

***Objectives*:**

To evaluate the levels of testosterone in relation to Visceral Leishmaniasis (VL) infection in males.

***Progress*:**

Under this observational pilot study, 20 fresh and parasitologically confirmed VL male cases (age group: 15-45 years) and 20 healthy controls within same age group range were included. After obtaining informed consent, each subject was subjected for estimation of testosterone levels by CIA& RIA technique. Blood samples were collected in fasting stage. Anti-testosterone antibodies were immobilized on microwell plates. This followed further incubation of serum samples from patients and control with HRP labeled testosterone. This was done to allow testosterone in the sample to compete with HRP labeled testosterone for binding to the immobilized antibodies. After washing, enzymes substrate was added and colour development was monitored. It was observed that the amount of testosterone in the samples was inversely proportional to the enzyme activity. The absorbance was measured at 450 nm on ELISA plate reader.

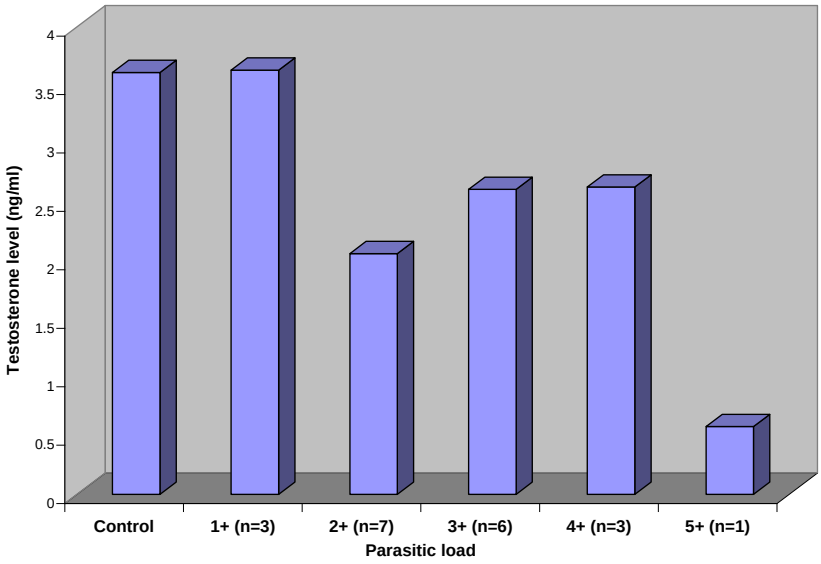
It was observed that testosterone level in control and VL cases with 1+ parasitic load was almost the same, but in VL cases with 5+ parasitic loads it was sharply decreased as compared to control. Preliminary results showed that the level of testosterone in cases and controls do not differ significantly (p-value > 0.05). However, further studies with more number of samples as well as incorporation of cytokine profile are required to arrive at any definite conclusion.

Table: Testosterone level (mean ± S.D.) in VL patients compared to healthy control

Parameters (ng/ml)	VL cases (n=20)	Control (n=20)	p-value
Testosterone	2.475 ± 1.8	3.610 ± 2.55	>0.05

*Normal range: 1.8 – 9.0 ng/ml in male

Figure: Testosterone level in VL cases and its relation to parasitic load



6. Identification of sibling species of *Phlebotomus argentipes* population in Bihar

***Objectives*:**

1. To identify sibling species of *Phlebotomus argentipes* in Bihar
2. To find out relevance of sibling species vis-a-vis Kala-azar endemicity

***Progress*:**

In continuaton with establishment of sibling species in *P. argentipes* population, the work has been extended with the study on bionomics of diferent forms of *P. argentipes*. The biology, morphology and life table including different developmental stages were studied. The egg laying pattern was found similar in all forms as in bunch/scattered/ in row. The egg laying capacity of type III was more i.e.

18.33/female than others I & II i.e. 13.11 and 10.93/ female respectively inside insectorium. The measurement of eggs was similar for each type.The morphological differences in larval stages for length and breadth of head and correlation of length of anal bristles with the body length were not significant. The life cycle of type I was found shorter than the others. The courtship during mating experiment was observed among type II and III independently where as cross mating was observed between type I & II showing the sympatric relationship. The relationship of these two forms with type III is to be proved. The biological samples of different developmental stages have been preserved for further Electron Scanning Microscopy and Molecular study to distinguish at micromorphological and gene level.

7. Control of Indian Kala-azar by genetic changing of symbiotic bacteria of the vector, *P. argentipes*.

***Objectives*:**

1. To identify the symbiotic bacteria from the gut of *Phlebotomus argentpes*
2. To transform the bacteria genetically and to ensure the paratransgenic transmission of GM bacteria
3. To ensure development/nondevelopment of *Leishmania donovani* in the presence of modified bacteria.

***Progress*:**

In continuation with the isolation of the symbiotic bacteria from the gut of *Phlebotomus argentipes*, the isolated bacteria were identified and modified genetically. The genetically modified bacteria are being fed to the different developmental stages of *P. argentipes* to see the uptake and their transmission from one developmental stage to the others.

8. Impact of DDT on Kala-azar vector.

***Objective*:**

- To find out the DDT resistant vector

***Progress*:**

DDT spraying was conducted under the direct supervision in 300 households in a village of Mahua PHC under Vaishali district. Prior to spraying, the spray men were trained for adherence to the WHO standard spray procedures encompassing preparation of 5% DDT paste with 50 % wdp, checking the nozzle discharge and methods of spraying.

After four month of spray the DDT sensitivity was assessed using WHO standard tube method. Test results showed the 24-hour mortality in the range of 70-90% (Table 1). The community acceptance was 89% and after explaining the importance of spray 6% head of the households, who were previously reluctant, agreed for spraying. The remaining 5 % HH were either locked or refused for spray.

Table 1: Bio-assay test

Sl. No	No. of sandflies exposed	Knocked down after one hour	Died	24 hr mortality %	Corrected mortality %
R1	20	Nil	16	80	75
Control	10	Nil	2	20	
R2	16	Nil	13	81.25	79.9
Control	15	Nil	1	6.66	
R3	10	Nil	10	100	100
Control	15	Nil	2	13.33	
R4	15	Nil	11	73.33	70.37
Control	10	Nil	1	10	
R5	10	Nil	7	70	65.38
Control	15	Nil	2	13.33	

10. Crucial role of plants' extract in propagation of *Leishmania donovani* promastigotes.

***Objective*:**

- To explore the possibilities of some plants' extract
  - as a source for replacement of blood/blood products/FCS/serum in routine culture of *L. donovani* promastigotes.
  - as a source of antileishmanial compound, if show lethal effect.

***Progress*:**

Based on initial suggestive observations, fresh lot of plants' extract of some plants' (n=13) that belong to different families having different characteristics such as habit, habitat, occurrence, flowering season etc. were used for this study. Three different culture media (2 commercially available i.e. RPMI-1640 and Schneider's insect medium; and another one LGPY) were supplemented with 20% plant's extract. The plain medium was taken as negative control and medium with 10% FCS was taken as positive control. Adequate proliferation was exhibited by 9 plants' extract as supplement in LGPY medium for 74 sub-passages ensuring long term maintenance of promastigotes.

To find out more choice in selection for getting plants' extract easily & economically in different seasons and places, 19 new plant's extract were screened in LGPY medium, out of which 8 exhibited adequate proliferations of promastigotes more than 24 successive sub passage. One plant's extract showed lethal effect on promastigotes even after addition of 10% FCS. (Table 1).

Out of total 21 plants' extract, 2 exhibited thermal stability at 121°C for 20 minutes as they supported promastigotes proliferation in long-term successive sub passaging over 33 sub passages. Plants' extract kept at -20°C for 3 years, at 4°C for 1 year and at room temperature for 6 months exhibited luxuriant growth indicating long storage stability/ self life. Three different plants' extract (20%) were supplemented to 1% agar base in LGPY medium and inoculated with 3 cells/ plate. Whitish mucoid colonies (size 3-4 mm approx.) were observed on 10^{th} day in each plate indicating the capability of these plants' extract to promote cell growth from single cell.

Promastigotes were cryo-preserved in LGPY medium supplemented with 20% of 4 different plants' extract and 20% FCS to elucidate suitability as a serum-free glycerol cryo-medium. After thawing medium supplemented with plants' extract as well as FCS showed luxurious growth on 5^{th} – 6^{th} day.

As a pilot experiment to demonstrate the use of plant extracts' in primary isolation of *L. donovani*, LGPY medium, supplemented with FCS (10 & 20%) and different plants' extract (n=6), were inoculated with splenic/bone-marrow aspirate. Except one, all the five plants' extract supported primary isolation of parasite within 4-5 days. More sets of experiments are in process to authenticate the observations.

Newly isolated promastigotes adapted well in medium supplemented with plants' extract.

Establishment of infection in Balb/c mice was not observed with two new isolates, after 15 sub-passaging in LGPY supplemented with plants' extract.

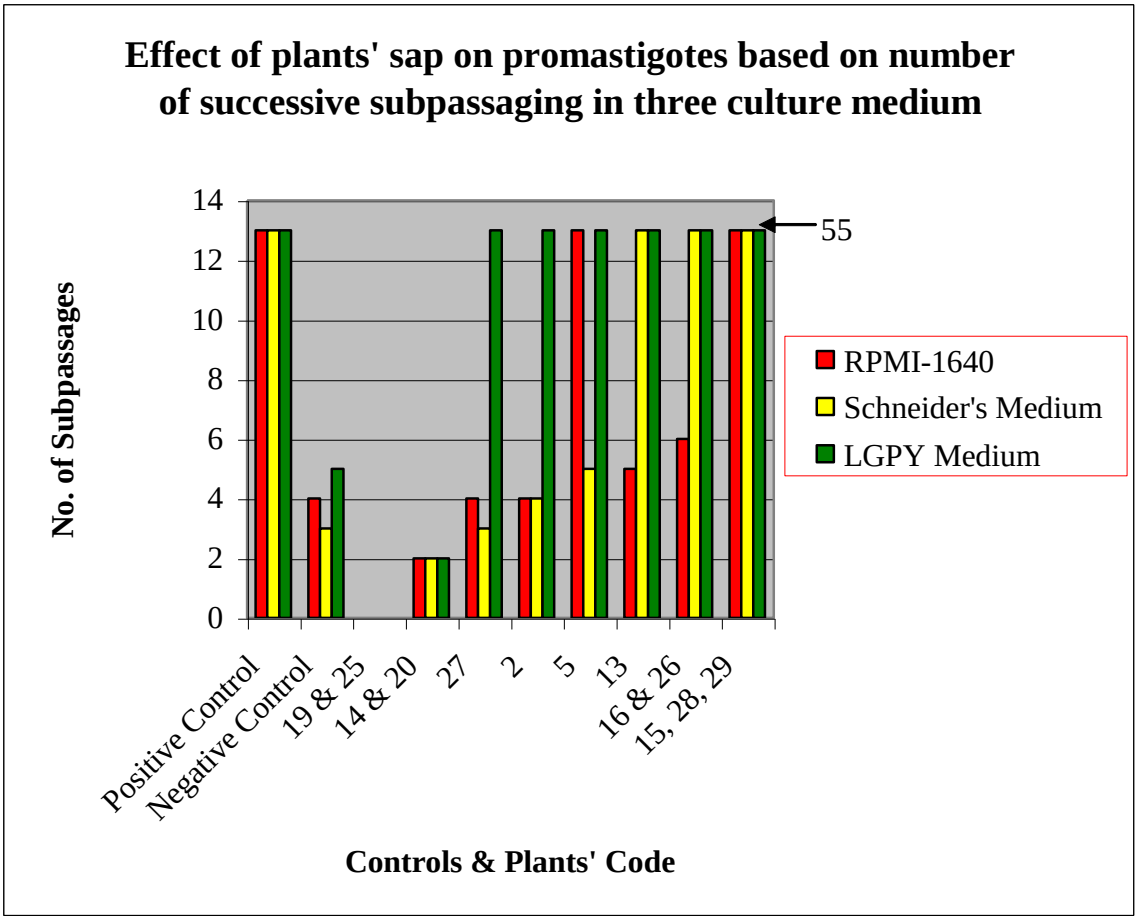


Table 1: Effect of plants' extract on promastigotes based on number of successive sub-passages.

Effect on promastigotes	Description of effect	Survival in No. of sub-passages	PE code/Total No.
Lethal	Non-motile / deformed cells	0	32 /1
Harmful	Cells sustainment < control	1 to 4	33, 34, 36, 45, 46, 49/6
Short-term propagation	Cells sustainment = control	5	35, 37, 38, 39 /4

Long-term propagation	Cells sustainment > control	>24	31, 40, 41, 42, 43, 44, 47, 48 /8
Positive control	Cells sustainment > control	>24	
Negative control		5	

11. Establishment of repository for Leishmania parasites and sera bank.

***Objectives*:**

1. To isolate and maintain Leishmania parasites from different clinical materials as well as from the vector of Kala-azar, *P. argentipes.*
2. To cryopreserve different isolates of Leishmania of different geographical areas and WHO referral centers.
3. To characterize the various isolates of Leishmania.
4. To preserve sera samples of Kala-azar and PKDL cases; other diseases; and healthy controls.

***Progress*:**

Leishmania parasites from splenic/ bone marrow aspirates and dermal lesions of kala-azar and PKDL cases respectively, hailed from different endemic areas, have been isolated and maintained *in vitro* in biphasic media. Out of 30 cropreserved isolates, 8 were revived and 20 new isolates (Kala-azar 19; PKDL 1) are being maintained for its cryopreservation in liquid nitrogen at -20^{o}C. The Amphotericin B unresponsive (N=2) and PKDL (N=1) isolates have been adapted in culture for inoculation in Balb/c mice for *in-vivo* maintenance. The study is in progress.

12. In vitro, role of antigen of Leishmania isolates of SAG responder and non-responder patients in IFN-γ & IL-4 production by similar sets of T-cells.

***Objective*:**

To evaluate cytokines (IFN-γ & IL-4) production in two similar sets of T-cells collected from visceral leishmaniasis cured subjects and stimulated with whole antigen of SAG responder and non-responder isolates of L.donovani.

***Progress*:**

The mononuclear cells from collected samples were stimulated with whole antigen of SAG responder and non-responder isolates and stained with anti-human CD4PE monoclonal antibodies. The cell accumulated IFN-γ & IL-4 were detected with anti-human IFN-γ labeled FITC monoclonal antibodies and anti-human IL-4 labeled APC monoclonal antibodies respectively. The images of 24 samples were acquired by Flow Cytometer and data were analyzed by BD cell quest software. The work on IFN-γ & IL-4 detection in T-cells of VL cured and healthy subjects stimulated with PMA, whole antigen of SAG responder and non-responder has been completed.

The data of IFN-γ production suggested approximately two fold-increased production of IFN-γ in responder than non-responder parasites against T-cell of cured patients.

Table: Cytokine profile of $CD4^+$T-lymphocytes differentiated in response to non-stimulation, stimulation with PMA and Leishmania from different sources.

Groups	**Cytokines**	**Stimulants source and % of T-cells +ve to cytokine**			
		Non PMA	**PMA**	**Non-responder Parasites**	**Responder Parasites**
Antimony Responder VL after treatment (n=7)	IFN-γ	1.85±0.61	9.80±3.75	6.88±3.12	12.19±4.41
	IL-4	1.30±0.63	4.15±1.56	8.01±3.27	4.53±1.89
Antimony Non-Responder VL after treatment (n=7)	IFN-γ	1.18±0.61	7.39±3.72	3.74±1.89	7.75±3.51
	IL-4	3.02±1.21	8.43±5.0	10.22±5.32	7.19±3.19
Control (n=10)	IFN-γ	0.73±0.63	4.17±1.73	2.75±1.13	3.08±0.90
	IL-4	0.60±0.30	3.10±1.20	3.16±1.57	2.41±1.45

13. Role of CD2 Antigen in T-cell signal Transduction pathway in Visceral Leishmaniasis:

***Objectives*:**

- To understand the role of CD2 deficiency in VL and its consequences on CD4 subpopulation of T-cells
- To find out the possible means for modulation of this pathway as a mechanism to ensure protective cytokines in patients.

***Progress*:**

- **Quantitative T-cell abnormalities due to an inadequate CD2 expression in VL patients**

T-cells from VL patients showed less CD2 expression accompanied with markedly lower CD4+ and CD8+ cell count compared to control. Although both sub-sets showed a reduction in CD2 cell expression compared to control ($P < 0.01$), the reduction in CD2 on CD4+ T-cells was more pronounced (Fig. 1a & b). The impact of this CD2 deficiency was reflected on many T-cells activation parameters. T-cells of VL patients were mostly in G0/G1 stage of the cell cycle (98.20%) with little or no activity of protein kinase C-α (PKC-α) isoform. However, pre-incubation with activating anti-CD2 monoclonal antibody (MAb) resulted in a corresponding increase up to 2.52-fold in T-cells of G2/M population supported by both activity and expression of PKC-α isoform (Table-1, Fig. 2).

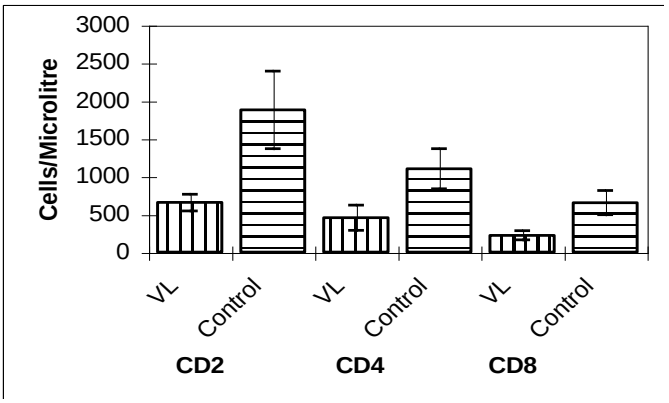
- **Predominance of a well-characterized Th2 cell associated mechanism during VL infection reverts and more T-cells show increased sensitivity for IFN-γ production after anti-CD2 stimulation**

Activation of endogenous CD2 antigen by anti-CD2 triggered a higher frequency of cells to produce IFN-c (3.2-fold) compared to *ex vivo* (Fig. 3). Even in the culture condition, when the T-cells from patients were depleted of APC, IFN-γ production was noticed after CD2 activation (Fig. 4). On the other hand, IL-4 production became low in the anti-CD2 antibody supplemented peripheral blood mononuclear cells (PBMNCs) culture. Prior treatment of PBMNC with anti-CD2 did not permit CD4+

cells to be properly stimulated by recombinant IL-4 and IL-4 production by CD4+ cells, even in presence of r-IL-4, were very low. On the other hand, IFN-γ production did not reduce even in presence of r-IL-4 and it increased when stimulated with anti-CD2 (Fig. 5).

The significance of these findings is that CD2 activation has a strong impact on CD4+ cells, which once activated, accounts for the majority of the IFN-γ producing cells during VL infection. It is also shown that T-lymphocytes enhances their activation for the secretion of IFN-γ in the absence of APC and that a stimulation of CD2 is required for this effect. Hence, evidence for the role of CD2 antigen in immunity to L. donovani infection shown in this study is worth pursuing

a.



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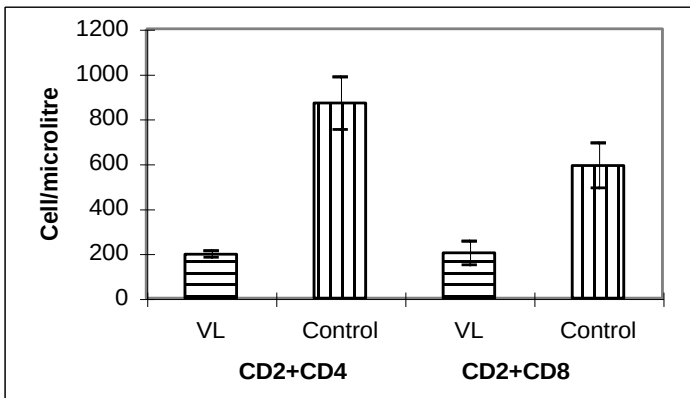


Fig. 1. Immunophenotyping of CD2, CD4 and CD8+ T-cells in VL patients and control. (a) CD2 down regulation is associated with CD4+ Tcell expression during Visceral Leishmaniasis. (b) Analysis of CD2+ CD4+ and CD2+ CD8+ cells in T-cell (CD3+) in patients compared to control.

Table 1: Inability of T-cells to enter into cell cycle was altered after anti-CD2 stimulation.

Categories	Stimulants	G_0/G_1	S	G_2M
VL patients	Ex vivo	98.202±0.48	1.472±0.83	0.327±0.39
	Ld	97.918±0.75	1.178±0.97	0.906±1.33
	Ld + anti-CD2 Ab	95.366±0.22	2.343±0.23	2.291±0.39
Control	Ex vivo	97.87±1.44	1.07±0.67	1.03±0.87
	Ld	97.710±1.35	0.665±0.94	1.625±2.29
	Ld + anti-CD2 Ab	96.16±1.02	1.045±0.06	2.89±0.94

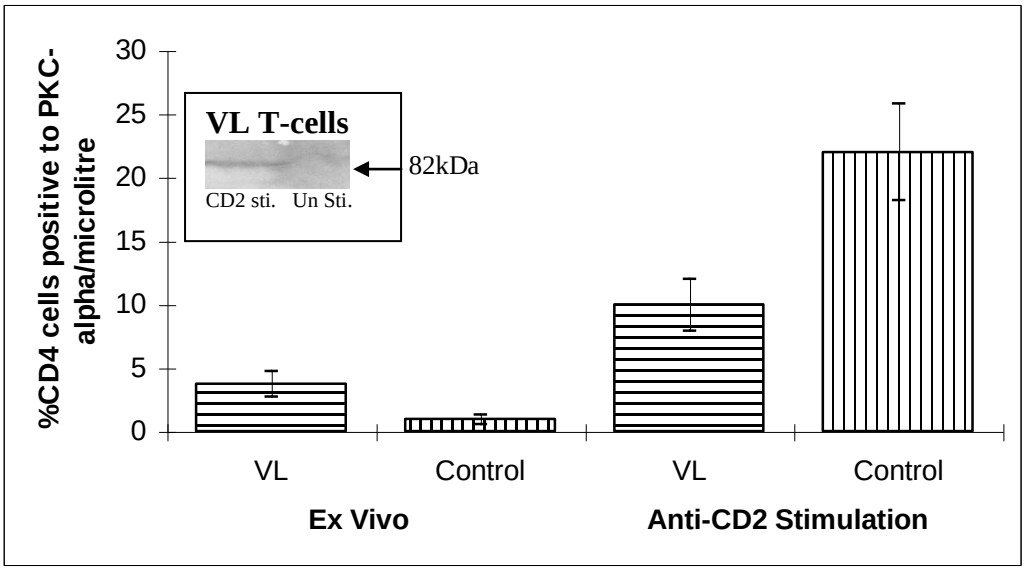


Fig. 2. Effect of CD2 activation on cell signaling mechanism in T-cells of VL patients. Total amount of PKC-a produced by cells stimulated and not stimulated with anti-CD2 antibody evaluated through FACS-Calibur. Immunoblotting of VL T-cells (inset) with PKC-a antibody shows that anti-CD2 induced significant phosphorylation of a protein migrating at 82 kDa.

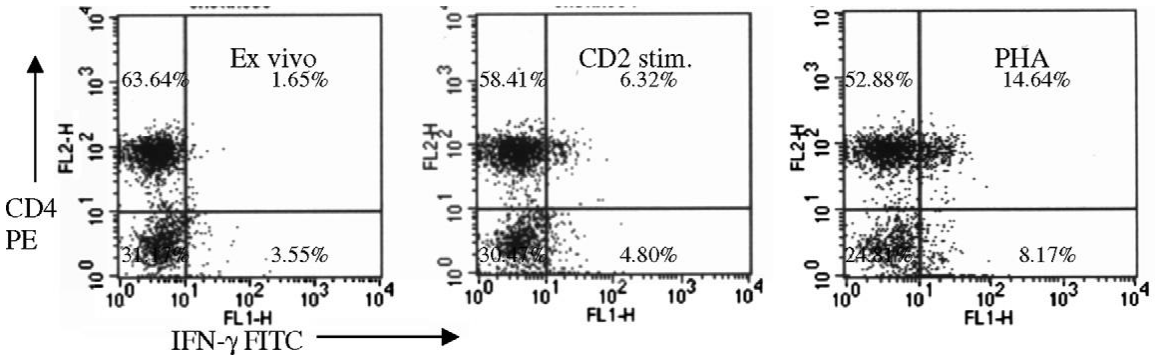


Fig. 3: Increase in frequency of CD4 cells for IFN-γ after activation of CD2 antigen in a VL patient: flow diagram of cultured PBMNCs stimulated with or without anti-CD2 or PHA in presence of brefeldin-A and stained with anti-human CD4 PE and anti-human IFN-c FITC of a VL patient showing increase in frequency of IFN-c producing CD4+ cells (upper right of the quadrant) and IFN-c producing CD4_ cells (lower right of the quadrant).

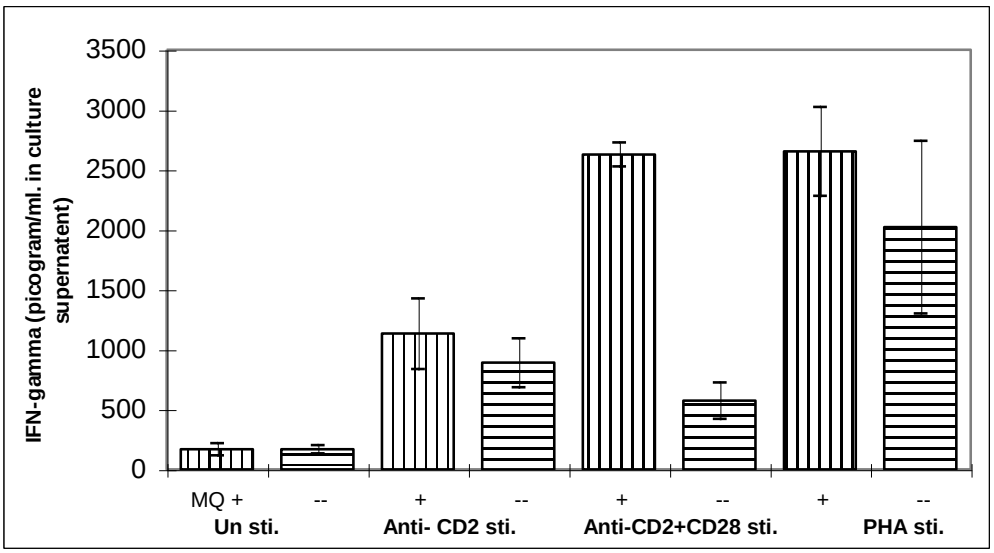


Fig. 4. CD2 activation in VL patients stimulates T-lymphocytes to produce IFN-γ even in absence of APC.

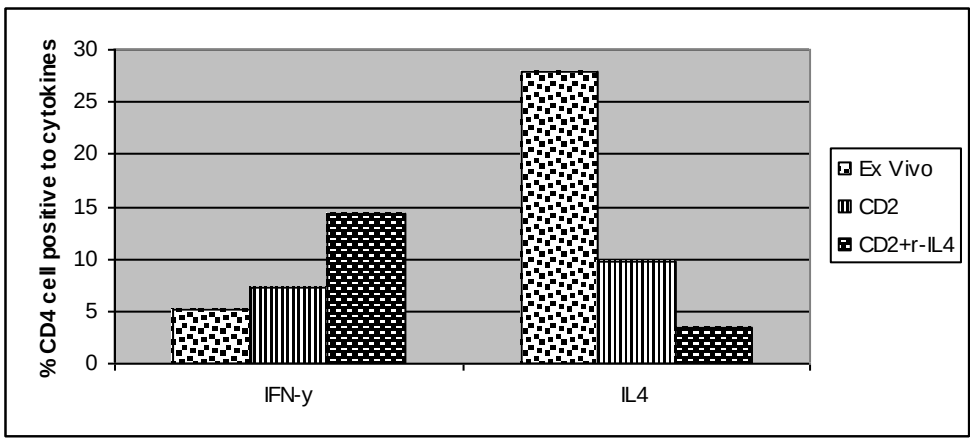


Fig. 5: CD2 activation in VL patients influences IFN-c responsiveness in T-lymphocytes by down regulating IL-4 production.

14. Protective efficacy of purified membrane antigen (Phosphoproteins vs lipophosphoglycan) isolated from *Leishmania donovani* metacyclic promastigotes.

***Objectives*:**

1. To isolate, purify and characterize proteins of *L. donovani* promastigotes with and without Glyco-phospholipid anchors.
2. To elucidate the role of these antigens in *Leishmania* infectivity and survival through their association with multidrug resistance associated proteins.
3. To elucidate the role of these antigens on TGF-β and IL-10, which, *Leishmania* might use as immune escape mechanism.
4. To explore the immunological potential of these antigens in protection against VL.

***Progress*:**

The glycoinositolphospholipid anchors (GPI) on proteins of *L. donovani* promastigotes were investigated as a potential target of such cellular mechanism, which the *Leishmania* parasite can utilize to manipulate the microenvironment for their own survival advantage. This study further investigated the prospect of these proteins as a potential target for protection against VL.

L. donovani promasigotes (10^8) were equilibrated with 2 ml TBS and 2ml Triton-X 114. Following incubation, suspension was pelletted and supernatant suspended in ice-cold PBS was subject to 37^{o}C water bath. The centrifuged material containing soluble proteins (upper phase) and trans-membrane protein anchored by Glycoinolphosholipid structure (lower phase) were collected. NCP-blotted Polypeptides of these proteins were probed with mouse anti-human pgp-1 and MRP-1 and on the basis of reactive bands in a western blot, relevance of these proteins with drug resistance was established. In a separate experimental set-up, Ficoll separated

PBMC (2x $10^{6/}$ ml) suspenstion from VL patients (n=5) were re-stimulated in 96 well round bottom micro-titre plate in presence and absence of these proteins for 48-72 hrs. The TGF-β and IL-10 level in cells in the presence or absence of the Leishmania proteins were measured using cytokine based ELISA.

***Results*:**

I. Part played by *Leishmania* antigens with parasite infectivity and survival:

In this study we demonstrate that 36kDa polypeptide of both glycoinositolphospholipid (GPI) anchored and non-GPI anchored proteins of *Leishmania* promastigotes phosphorylates pgp-1, an antibody of ABC transporter chain. This may be a feature, which can favor parasite replication in patients.

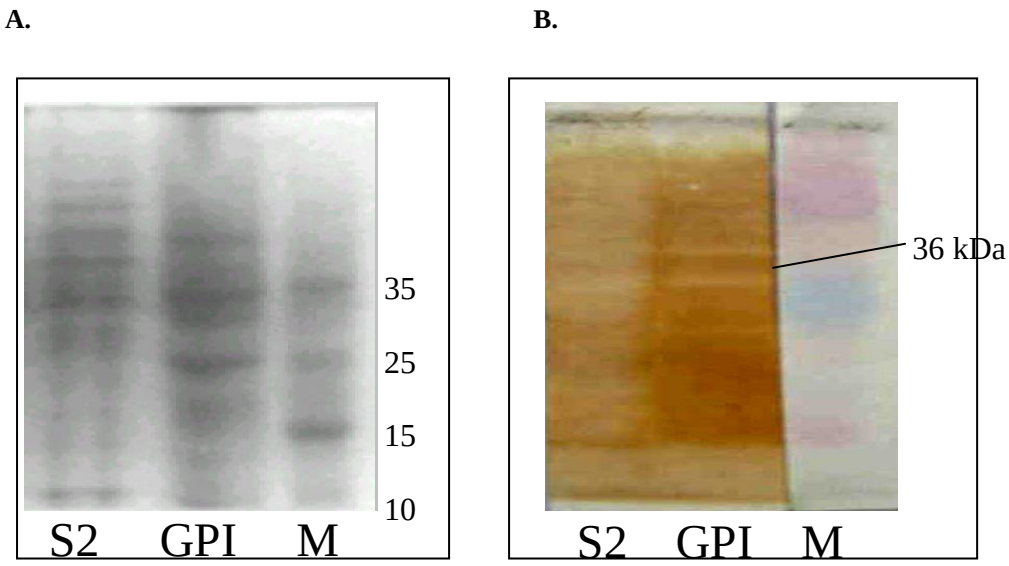


Fig. 1: A. Antigenic profile soluble protein (S2) and GPI anchored protein. B. Immunobloting of the antigen with anti pgp-1 antigen.

The differences in the two types of *Leishmania* antigens are noticed when the pattern of immunological response shown by VL patients are compared. The *Leishmania* protein antigens with a GPI anchored induces higher TGF-β production than produced after sensitization with protein antigens without GPI anchore. The stimulation of PBMNCs with protein antigens without GPI anchor, however, allows the cells to inhibit TGF-β release.

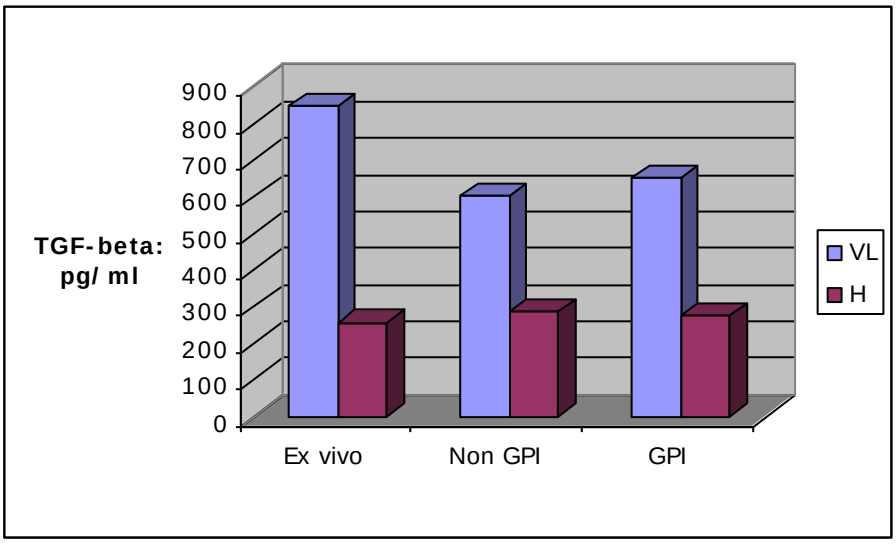


Fig. 2: Expression of TGF-β from macrophages in VL patients before and after stimulation with *Leishmania donovani* proteins with or without glycoinositol phospholipid anchor.

During VL, macrophage releases huge TGF-β and it is anticipated that glycoinositolphospholipid anchored on protein antigens might be used by *Leishmania* to escape immune protection mechanism.

II. Part played by *Leishmania* antigen in immunity to infection:

The magnitude of impact made on immune response by these antigens of *Leishmania* displayed wide diversity. We demonstrate here that antigens with GPI anchor which earlier triggered TGF-β production induces an elevation in baseline value of $CD4^+$ SP cells and $CD4^+CD8^+$ DP cells. Thus, the priming of mononuclear cells of the VL patients with *Leishmania* antigens with GPI anchor can be tested for protection studies. Our results also indicated that protein antigens without a GPI anchor, which earlier negatively regulated TGF-β production, inhances $CD8^+$cells and $CD4^-CD8^-$ DN cells which all may be used by *Leishmania* to create the clinical situation.

15. Study on Immunopathology of Post Kala azar Dermal Leishmaniasis (PKDL): T-cell subsets.

***Objective*:**

To observe the changes in T cell subsets in PKDL lesions and in circulation in relation to VL cases and to understand its role in the Pathogenesis of PKDL

***Specified Objective*:**

- To determine the level of T helper and T suppressor cell in skin lesions and in the peripheral circulation of PKDL cases..
- To measure the cytokine (IL-2, IFN-ϒ, IL-4 and IL-10) in PKDL cases and compare it with VL and control subjects.

  Study will be conducted in 30 PKDL cases.

***Progress*:**

The Cytokine study has been conducted in 6 PKDL cases and 5 normal controls. It was found that cytokine response pattern in PBMNC of PKDL cases does not show much difference from kala azar cases as there is downregulation in IFN-y producing abilities of CD-4 cells with an almost 2-fold rise in the frequency of CD-4 cells positive for IL-4. The result is summarized as below:

Percentage (%)CD-4 cells positive for

Group	**IFN-y**		**IL-4**	
	Before Ag	**After Ag**	**Before Ag.**	**After Ag.**
	%	**%**	**%**	**%**
PKDL cases (no=5)	1.506	7.73	4.904	11.86
Normal control (n=5)	3.73	5.1	3.60	4.36

16. Studies on some nutritional factors in severity of Visceral Leishmaniasis.

***Objectives*:**

1. To identify and assess the nutritional markers/factors in the malnourished VL patients

2. To evaluate the correlation between malnutrition factors and VL
3. To assess the nutritional factors predisposing to severity in VL.

***Progress*:**

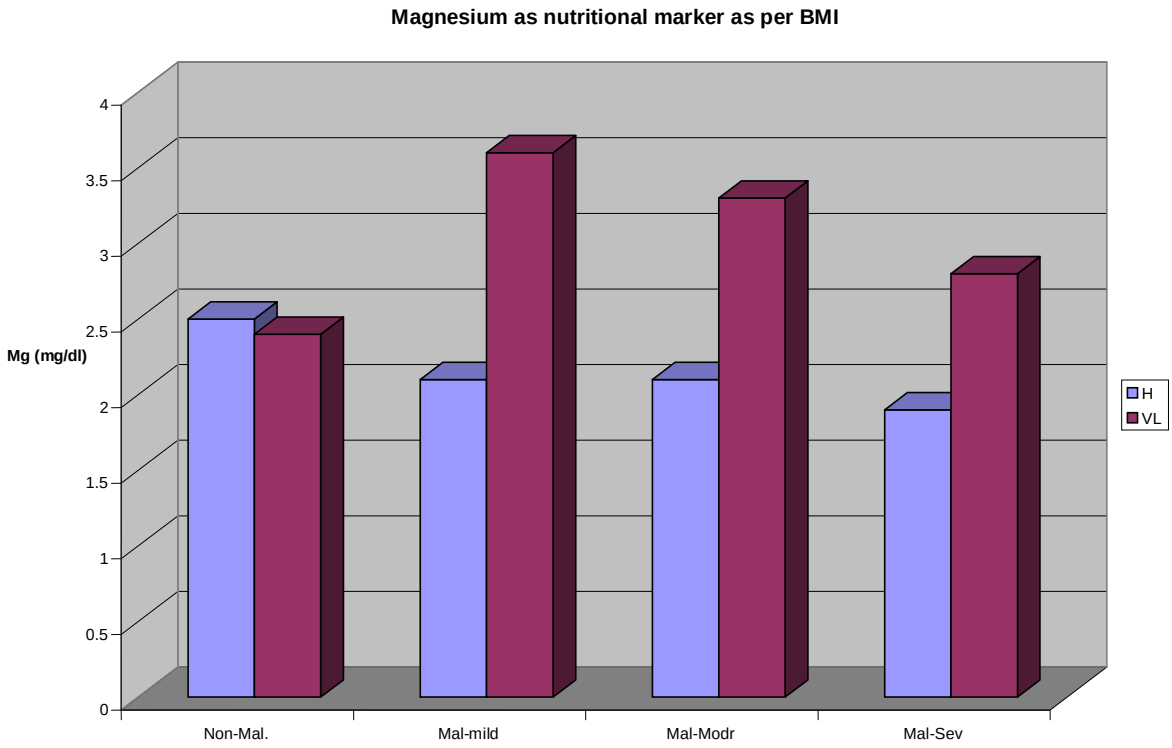
Out of several nutritional- related biochemical tests, few biochemical markers were assessed in different malnourished VL patients according to their BMI along with normal nourished subjects. The biochemical markers investigated were Total cholesterol, High-density lipoproteins, low-density lipoproteins, Triglycerides, Apolipoprotein A1, Apolipoprotein B, Zinc, copper, magnesium, albumin, Iron, transferrin and TIBC.

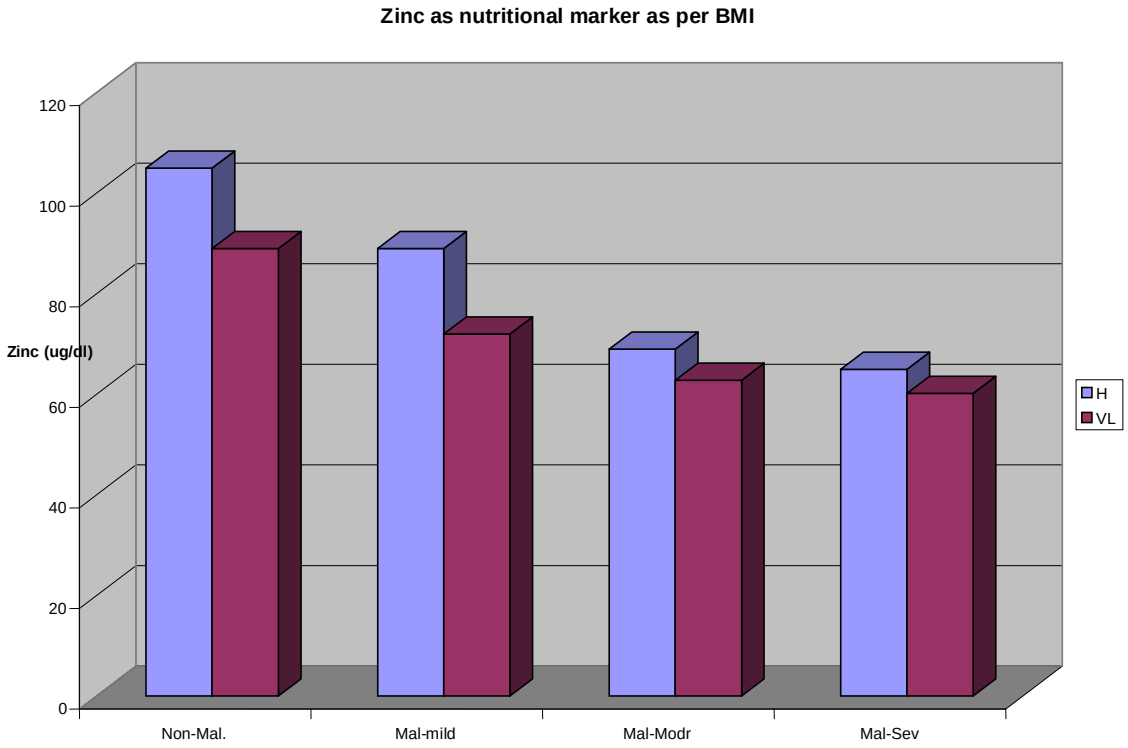
Hypocholesterolemia and increased triglyceride was observed in malnourished VL infection. According to parasitic load, the triglyceride concentration was observed to be directly proportional. Iron and transferrin were observed to be down regulated in VL infection.

Zinc, copper and albumin were down-regulated while magnesium was observed to be increased as the malnourished index increases in VL. The results of few nutritional markers are shown in different figs.

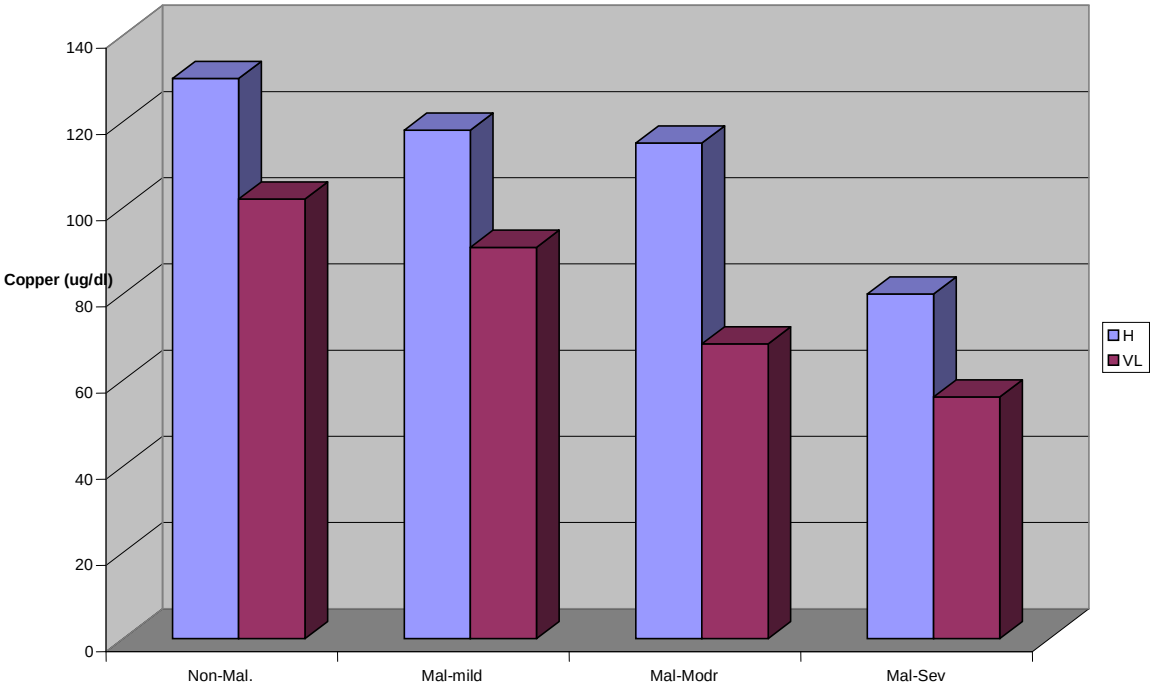
Table I: Healthy vs Mal-nourished & Non-mal-nourished VL

Parameters	-Ve Control	+ Ve Control	VL cases		
			Mild	Moderate	Severe
		Body Mass Index			
	(N 25)	>20 (N 25)	18-20(N 25)	15-18(N 20)	10-15(N 13)
Cholest. (mg/dl)	186.40	110.6	56.30	58.74	49.49
Trigly. (mg/dl)	166.40	142.9	90.35	142.16	212.37
Urea nitrogen (mg/dl)	10.83	10.9	11.40	13.43	11.74
Creatinine (mg/dl)	0.68	0.41	0.42	0.55	0.42
HDL	16.80	23.0	25.40	23.00	22.00
LDL	134.09	63.0	56.00	48.40	34.00
Albumin (g/dl)	4.58	2.8	2.30	2.30	1.90
Electrolytes (mmol/L)					
Na+	136.50	138.0	136.00	142.00	137.00
K+	4.40	3.6	3.20	3.90	3.50
Apolipoprotein A1	128.80	62.0	59.00	59.00	58.50
Apolipoprotein B	84.20	70.0	67.00	72.10	66.70
Zn (μg/dl)	105.00	89.0	72.00	62.80	60.20
Cu (μg/dl)	130.00	102.0	90.80	68.40	56.10
Mg (mg/dl)	2.50	2.4	3.60	3.30	2.80
Iron (μg/dl)	115.00	54.6	48.3	46.4	46.1
Transferrin (mg/dl)	256.00	121.0	111.6	93.0	86.2

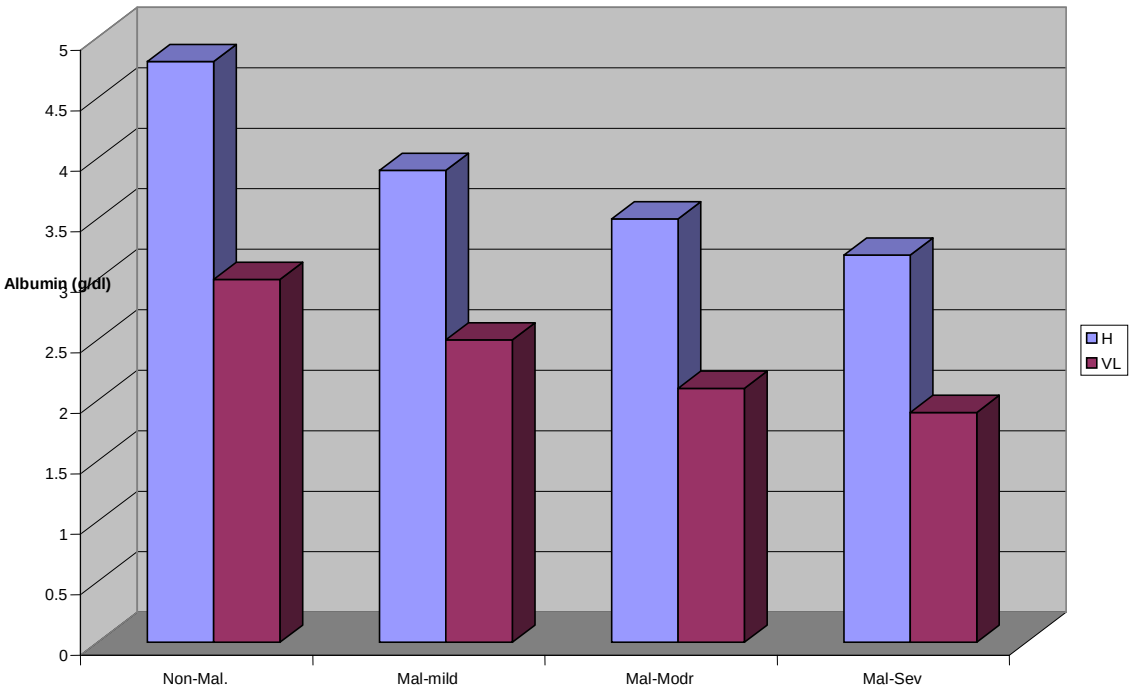

Magnesium as nutritional marker as per BMI
Mg (mg/dl)
4
3.5
3
2.5
2
1.5
1
0.5
0
Non-Mal.
Mal-mild
Mal-Modr
Mal-Sev
H
VL


Zinc as nutritional marker as per BMI
Zinc (ug/dl)
120
100
80
60
40
20
0
Non-Mal.
Mal-mild
Mal-Modr
Mal-Sev
H
VL

Copper as nutritional marker as per BMI



Albumin as nutritional marker as per BMI



17. Magnitude of under-reporting of Visceral Leishmaniasis (VL) cases in Bihar, India.

***Objectives*:**

1. To determine the proportion of under-reporting of VL cases.
2. To estimate actual annual incidence of VL cases.

***Progress*:**

The study was carried out in two VL endemic PHCs (Lalganj and Garaul) of Vaishali district. Based on the high incidence rate of VL in the year 2006 as per the Govt. record, 3 sub-centres of Lalganj (Banthu, Purkhauli and Rekhar) and two sub-centres of Garaul (Bawaria and Kuari) were selected. Door-to-door survey was carried out in all the villages under the selected sub-centres for population enuramation as well as find out the past history of VL in the year 2006. Till date, 17 villages have been surveyed, out of which data of 12 villages have been entered into computer for data analysis.

The interim data analysis revelaed that out of 22,937 surveyed population (targeted sample size: 32,000), 99 cases were encountered having past history of VL in 2006 whereas as per the Govt. record there were 85 cases reported for the base year in the respective health faciltiy centres. Data entry for five more villages as well as field survey in more villages to cover the target population is in progress.

18. Hospital based surveillance for Kala-azar.

***Objective*:**

The main objective of this Institutional project is to:

a) monitor changes in disease patterns including therapeutic response and to collect other relevant information,
b) provide a data base on Kala-azar for researchers to generate and test hypothesis and to carry out clinical and epidemiological research,
c) provide a regular report to the government and other relevant agencies on kala-azar from a systematic sample of all kala-azar patients attending the hospital,
d) to develop an early warning system for forecasting an epidemic,

e) to improve care and introduce better preventive measures.

***Progress*:**

The parasitologically confirmed VL patients, admitted in Indoor ward of this institute, were interviewed through pre-tested questionnaire to collect the information on their demographic, socio-economic status, current and past history of case, besides the therapeutic response based on clinical and laboratory parameters.

Since the inception of this study (Jan. 2001) to March 2007, a total of 1901 (Male 1245, Female 656) were taken into this study. Maximum VL patients were in the age group 5-14 years (39.2%) and male patients (65.4%) were higher as compared to the females (34.6%). About 90% of the patients were from the rural areas of nearby endemic district and 75.2% of the patients hailed from the poor socio-economic strata (Income range Rs. 1000-5000 per month). Nearly 56.4% patients were residing in mud and thatched houses with very poor light condition inside the bedroom and more than 52.1% of the patients kept domestic animals in their houses. Vegetations around the households were reported from 74.5% of the patients.

Clinical and laboratory characteristics were compared between two age groups i.e. ≤12 years (Gr. I) and >12 years (Gr. II). Fever >100^{o}F with chill and rigor was recorded in nearly 50.2% and 38.0% of cases in Gr. I and II respectively. Splenomegaly (>5 cm) was recorded in 70.0% of cases in Gr. I and 66.5% in Gr. II, whereas hepatomegaly (>5 cm) was in 41.1% and 36.1% of cases respectively. Leucopenia was recorded in nearly 74.1% and 51.8%; severe anemia (Hb <6.5g/dl) in 37.2% and 26.2%; SGPT within normal range in 84.3% and 72.8%; and SGOT within normal range in 64.2% and 54.2% of cases in Gr. I and II respectively.

Out of various therapeutic options viz. Sodium antimony gluconate (SAG), Pentamidine, Amphotericin B, Amphotericin B lipid complex, Miltefosine and Paromomycin, SAG and Pentamidine had a cure rate of about 58.5% and 66.7% respectively at our Indoor setting and hence, it is no longer in practice currently. Cure rate with Amphotericin B, Miltefosine and Amphotericin B lipid complex were 93.9%, 97.5% and 100% respectively. Under Phase III clinical trial of Paromomycin and Phase II clinical trial of Sitamaquine, initial cure rate observed were 93.6% and 100% respectively.

Table: Regimen wise cure rate

Regimen	Treatment completed	Cured	% cured
SAG	118	69	58.5
Pentamidine	9	6	66.7
Amphotericin B	1424	1338	93.9
Miltefosine	161	157	97.5
Amphotericin B Lipid complex	3	3	100
Paromomycin	110	103	93.6
Sitamaquine	7	7	100

Extramural Studies

1. Early identification of asymptomatic cases of Kala-azar in endemic foci of Bihar, India: An epidemiological and socio-behavioral study.

Objective:

- To determine the asymptomatic cases and their conversion into disease stage.
- To study the socio-behavioral aspect of VL in the community

***Progress*:**

This study was conducted in two villages namely Banthu and Haribanshpur of Vaishali district of Bihar on the basis of high endemicity of the VL cases as per the State Govt. data. Out of 1525 population (in 282 households) enumerated, 1017 were subjected for clinical examination, rk-39 dipstick test and PCR. The current and past history of case, clinical findings, diagnosis and treatment details were recorded.

Out of 1017 screened individuals, 97 were found positive by rK-39 and 31 were PCR positive in 345 blood samples tested so far. A total of 36 individuals were found for past history of kala-azar. All rk39 strip test / PCR positive cases are being followed on first week of every month. After 3-months follow up, out of 61 rk-39 positive (excluding past history of kala-azar), 21 developed as full blown kala-azar case and remaining 40 are still asymptomatic. The follow up has to be extended for one year to assess the proportion of asymptomatic cases and their conversion rate into disease stage.

A separate pre-tested semi structured questionnaire was canvassed to all the head of the households to elicit the information on socio-behavioral aspect in relation to disease, transmission, diagnosis, prevention and treatment seeking behavior. Data analysis on socio-behavioral aspect is under progress.

3. A Phase II, multi-centre, open-label, randomised study to evaluate the safety, tolerability and pharmacokinetics of oral sitamaquine compared with amphotericin B in the treatment of visceral leishmaniasis caused by *L. donovani* in endemic areas. (Sponsor: GlaxoSmithKline; Study No. STQ 105938)

Objectives(s)

Primary objective:

- To characterize the pharmacokinetic profile of multiple doses of sitamaquine with or without food in subjects with visceral leishmaniasis.

Secondary objectives:

- To describe the safety and tolerability of sitamaquine in the treatment of subjects with visceral leishmaniasis, and to compare with amphotericin B.
- To evaluate the efficacy of a 21 day course of treatment with oral sitamaquine.

***Progress*:**

Being one of the clinical center of this multicentric study, 62 patients were screened after obtaining proper written informed consent, out of which 11 were randomized for medication (7 in Sitamaquine group and 4 in amphotericin group). The subjects randomized in sitamaquine group (sequence 1-4, categorized as fed and fasting stage) received target dose of 2mg/kg once daily for 21 days and subjects under sequence 5 were treated with intravenous amphotericin B in the dose of 1 mg/kg every alternate days for 30 days.

Out of 11 enrolled patients, one patient (amphotericn group) withdrew himself from the study in the mid of the treatment and rest (n=10) completed the treatment. Pharmacokinetics samples were collected as per the time point defined in the protocol. All the patients completed 6-months follow up for assessment of final cure, AE/SAE, if any.

The study data analysis, conducted by the sponsor for all the participating clinical centres, revealed the following facts:

Pharmacokinetic parameter:

- No significant food effect was observed on AUC(0-τ) and Cmax values for sitamaquine or its metabolite indicating sitamaquine can be dosed either with or without food.
- Minimal accumulation of sitamquine was observed after repeat dosing for 21 days.
- Sitamaquine was eliminated with mean t1/2 values ranging from 18.3 to 22.8 hours on Day 21.

Efficacy:

	STQ (n=41)	Amphotericin B (n=20)
Initial Parasitological cure 95% CI	39 (95%) 83.5 – 99.4%	19 (95%) 75.1 – 99.9%
Final Clinical cure 95% CI	35 (85%) 70.8 – 94.4%	19 (95%) 75.1 – 99.9%

Safety and tolerability:

A clear advantage of sitamaquine over amphotericin B was demonstrated with regards to tolerability. Only 10% STQ subjects reported an adverse event (AE) compared to 85% amphotericin B subjects. The most frequently reported AEs for STQ were increased urine protein creatinine ratio, decreased neutrophil count and headache whereas for amphotericn B the most frequently reported AE were chills, vomiting and gastritis.

Overall 6 subjects under STQ arm experienced increased protein/ creatinine ration after Day 14 which returned to normal limit by Day 49 and remained normal at Day 180 in all but one case remained just above the upper limit of normal. No safety signals were reported for hepatic or cardiac parameters.

4. A Phase IV study to expand access while assessing the safety and efficacy of paromomycin IM injection in an outpatient setting for the treatment of visceral leishmaniasis (VL) in India (Study No. VLPM03).

***Objectives*:**

- To evaluate the safety of paromomycin IM injection in an outpatient setting in the Vl endemic regions of Bihar [Module 1].
- To evaluate the effectiveness of the Access program to provide outpatinet VL treatment with paromomycin IM injection in progressively more resource-contrained, VL-endemic regions of Bihar [Module 2 & 3].
- To evaluate the safety of paromomycin IM injection in an outpatinet setting in progressively more resource-contrained, VL-endemic regions of Bihar [Module 2 & 3].

***Progress*:**

This open-lebel, multi-center, sigle arm study has been divided in different modules (module 1 – 3). In module 1 at RMRIMS, one of the Kala-azar Centres of Exellance (KACE) in this study, a total of 124 suspected subjects were screened, out of which 65 were enrolled till date against the total target of 67. Four patients are on treatment and rest 61 have completed the treatement. No severe AE was noticed during the course of treatment. Screening and enrollment under module 2 is expected to start very shortly.

5. Safety and efficacy of oral miltefosine in patients with post kala-azar dermal leishmaniasis (PKDL) – dose-finding study caomparing 8 and 12 weeks of treatment. Open, randomized dose ranging multicenter trial (Study No. D-18506-Z015).

***Objectives*:**

Primary objective:

- To assess miltefosine regimens with 8 and 12 weeks treatment duration for their curative potential in PKDL.

Secondary objectives:

- To characterize the safety of oral miltefosine when used for periods up to 12 weeks.
- To assess the rates of initial response in relation to duration of treatment.
- To assess the rates of relapse after intial response.
- To assess the clinical response of facial erythema and mucosal lesions.

***Progress*:**

Altogether 17 subjects having nodular and/or papular leisons, suspected for PKDL, were screened in this study. Out of 17, 12 were enrolled as per the target. After randomization by the Sponsor (Zentaris), 6 patients were enrolled in each arm (8 weeks and 6 weeks) and treatment with miltefsoine was started. Till date, 4 patients have completed treatment, 7 are on treatment and rest one was dropped due to absteinance of the patient from receiving the medicine. Follow up of the treated patients is in progress. No AE or SAE was noticed.

6. The efficacy and safety of a short course of miltefosine and liposomal amphotericin B for visceral leishmaniasis in the Indian subcontinent (Study No. LEI PDE 06 03).

***Objectives*:**

- To evaluate the efficacy and safety of a short course of liposomal amphotericin B (one injection of 5 mg/kg) in combination with Miltefosine (2 weeks) for the treatment of visceral leishmaniasis (Kala-azar) in India.

***Progress*:**

Screening and recruitment under this multi-centric study were desgined with three pauses depending upon the age-group of the subjects as follows:

Age group	**Total Target of RMRI**	**Pause**	**Pause No.**
>18 – 65 years	20	7	I
>12 – 18 years	5	2	II
2 – 12 years	25	7	II
Total	**50**	**-----**	**-----**

At our centre, 1st pause is over and for 2nd pause target has been achieved. After completion of Day 15 treatment, the data will be send to DSMB for its clearance to move ahead in third age group. Altogether, 27 subjects suspected for VL were screened in first age group and 6 in second age group, out of which 10 and 2 patients were enrolled respectively. Out of total 12 enrolled subjects, 10 had completed the treatment and 2 (one in each age group) are under treatment. All the treated patients were examined for parasitological conirmation for initial cure n Day 29 and all of them were initially cured (100%). No AE or SAE were observed during the treatment and during 2-months follow up (in 3 patients). The study is in progress.

7. Exploratory investigations to detect the existence of chemical communication between male and female *Phlebotomus argentipes* (Old-world Sandfly), Indian vector of Kala-azar for mate and host location.

***Objectives*:**

- Developing improved laboratory culturing methods for larger scale fly out put of *P. argentipes.*
- Designing simple laboratory experiments to detect the existence of semiochemical mediated communication for behavioral responses between male & female *P. argentipes.*
- Isolation of semiochemicals released by male *P. argentipes* by solvent extraction or by volatile entrainment technique.
- Behavioral studies for validation of the chemical communication between male and female *P. argentipes* using male extracts in Y-tube olfactometer.

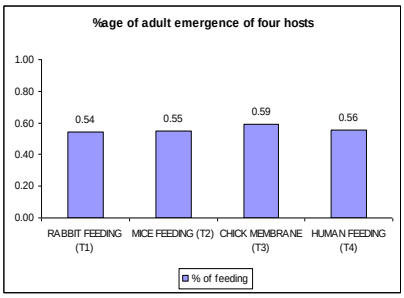
***Specific objectives*:**

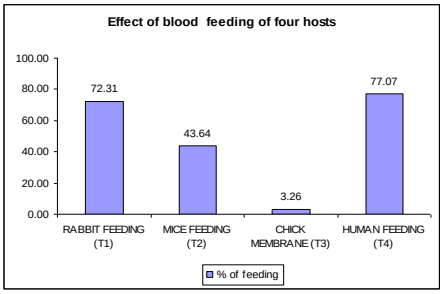
a) Establishing the critical parameters for improving the *P.argentipes* colony with increased fly out put
b) Experimental evidence for the role of chemical communication in *P.argentipes* for host and mate location.
c) Cross mating experiments to establish species homogeneity in *P.argentipes*.

***Progress*:**

To improve the laboratory colony output, wild sandflies were collected from highly endemic Muzaffarpur and Vaishali districts for establishment of colony. Out of 65 collected sandflies, 23 were confined for egg laying. The fly out put from these confinements was 160.

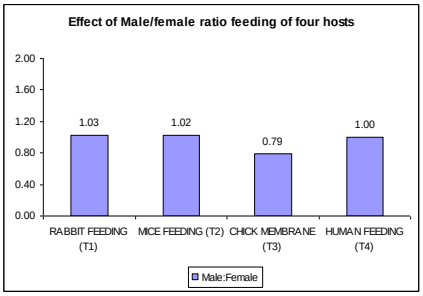
The emerged flies were fed on different animal to analyze the blooding ratio. After feeding, the fecundity, egg hatching, emergence and male female ratio were studied. The blood feeding preference showed significant difference ($p<0.05$) among the different hosts studied. The blood feeding ratio was high on human and rabbit in comparison with mice and it was very low on artificial feeding on chick membrane. No significant difference ($p<0.05$) in adult emergence (%) was observed. Male to female ratio was similar in all four different hosts studied but the egg hatching ratio showed significant difference ($P<0.05$) among hatching (%) of four different hosts studied. Egg laid per female from different animal feeding showed no significant difference ($p<0.05$).

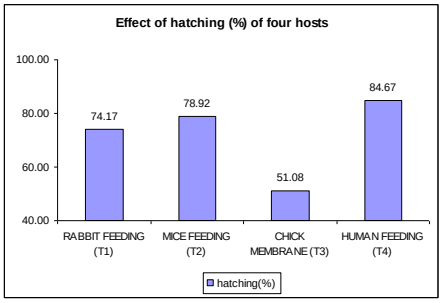




Host	Blood feeding (%)
Rabbit feeding	72.32b
Mice feeding	43.64c
Chick Membrane	3.26d
Human feeding	77.07a
CV	9.95 %
SEM	1.41
CD ($p<0.05$)	4.06
CD ($p<0.01$)	5.45

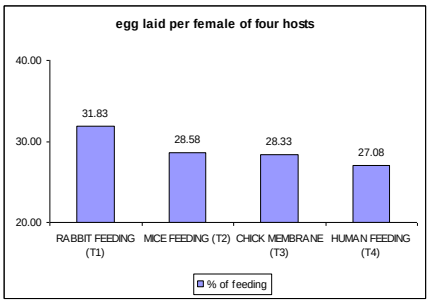
Host	%age of adult emergence
Rabbit feeding	0.5433
Mice feeding	0.5483
Chick Membrane	0.5925
Human feeding	0.5550
CV	23.9 %
SEM	0.04
CD ($p<0.05$)	NS
CD ($p<0.01$)	NS





Host	Male: Female ratio (%)
Rabbit feeding	1.03
Mice feeding	1.02
Chick Membrane	0.79
Human feeding	1.00
CV	30.78 %
SEM	0.08
CD (p<0.05)	NS

Host	Male: Female ratio (%)
Rabbit feeding	74.17
Mice feeding	78.92
Chick Membrane	51.08
Human feeding	84.67
CV	23.26 %
SEM	4.84



Host	%age of adult emergence
Rabbit feeding	31.833
Mice feeding	28.583
Chick Membrane	28.333
Human feeding	27.083
CV	27.24 %
SEM	2.27
CD (p<0.05)	NS
CD (p<0.01)	NS

To find out the existence of semiochemical communication between male and female *P.argentipes*, 100 virgin male flies were collected in a vial and the flies were kept in – 20 ^{0} C for 5 -10 minutes and double the volume of flies n – hexane were added and kept in 4 ^{0} C for overnight. The final extract was reduced to 0.25 ml for final experiment. A small circular filter paper (Watt man No.1) dipped in the final male extract was placed inside the burrowed cage. Another small circular filter paper (Watt man No.1) dipped in n-hexane was placed simultaneously with experiment paper as a control to find out the semiochemical communication. In side the cage virgin female flies (n=25) was released and number of sitting on the experimental and control papers were noted.

Date	No. of fly	Time in seconds				No. of sitting on extract		Temperature ^{0}C	Humidity %
		Min		Max		Exp.	Cont.		
		Exp.	Cont.	Exp.	Cont.				
25.10.07	25	7	3	36	17	34	10	27	72
30.10.07	25	2	4	26	20	28	8	28	78
05.12.07	25	7	3	90	24	5	7	23	62
12.12.07	25	4	2	52	21	12	15	22.5	60

08.01.08	25	2	1	600	23	23	12	28	60

8. Cost Effective Integrated Vector Management as a Contribution to the Visceral Leishmaniasis Elimination Initiative on the Indian Sub-continent: a multi-centre study.

***Objectives*:**

- To contribute evidence for more effective vector management in support of the VL elimination initiative.

***Specific objectives*:**

- To evaluate the safety and effectiveness of IRS/ITN/Eco-environmental management in reducing vector density & transmission of VL.
- To evaluate cost of IRS/ITN/eco-environmental management.
- To identify most cost-effective intervention amongst IRS/ITN/eco-environmental management.
- To evaluate acceptability of the three strategies.

***Progress*:**

Based upon the average vector density for the month of November and December in the study area, 6 groups of 4 clusters/villages were identified. The clusters in each group were listed 1 to 4 and randomly allocated for interventions.

Demography of the selected villages was studied for all the three intervention arms and for the control arm. In the selected villages most of the houses were having precarious walls like IRS arm having 67.91, EVM arm 70.85, LLIN arm 72.52 and the control arm having the less number of precarious walls 48.36. The remaining houses were having brick or mud wall which is more suitable for sandfly breeding. Almost all the houses were mud floor and approximately 23 to 40 % of the houses were mixed houses which promotes the sandflygenic condition.

Table 1: Demography of the selected villages

	DEMOGRAPHY	CLUSTER 1-6 IRS	CLUSTER 7- 12 Environmental management	CLUSTER 13-18 LLIN	CLUSTER 19-24 Control
Type of Houses	Total House Holds =	**240**	**295**	**273**	**304**
	Total no. of Houses with Brick wall =	**77**	**86**	**75**	**157**
	Total no. of Houses with precarious wall =	**163**	**209**	**198**	**147**
	Total no. of MD =	**85**	**119**	**85**	**72**
Demography	Total Population =	**777**	**885**	**881**	**898**
	Total House Holds =	**240**	**295**	**273**	**304**
Housing pattern	% of children under 15=	**69.59**	**82.82**	**58.34**	**60.47**
	% of Precarious walls=	**67.91**	**70.85**	**72.52**	**48.36**
	% of Mud floor =	**100**	**100**	**100**	**100**
	% of Mixed Houses =	**35.42**	**40.34**	**31.14**	**23.68**
Village Name		Harpur Ganga Ram, Paswan Tola, Paswan Tola 1, Paswan Tola 2, Harpur Gangaram, Pandit Tola, Baluapur	Bhoraha, Rasoolpur, Paswan Tola, Bannipur, South Tola, North Tola	Bhatauna, Jhakharseokh, Chainpur Mohammed	Dariapur Kaphen, Dariapur Kaphen 1, Chakbhikhi West, Chabhikhi West 1, Funda, Funda 1
	Chainpur Mohammed				
Panchayat	**Marwan**	Rasoolpur Mubarak	Balti Rasoolpur		
PHC		Mahua	Bhochahan	Marwan, Kanti	
District	**Muzaffarpur**	Vaishali	Muzaffarpur	Muzaffarpur	Muzaffarpur

Table 2: Sandfly density in different clusters during pre and post intervention (DDT, LLINs and EVM) and Control

Type of Intervention	Cluster code	House Hold/MIXED DWELLING	Survey	Collection Method	Counts Male	Female	Total
PRE DDT	C01 - C06	30	Baseline	TRAP/ASP	201	141	**342**
POST DDT	C01 - C06	30	FIRST	TRAP/ASP	30	28	**58**
PRE EVM	C07 - C12	30	FIRST	TRAP/ASP	366	461	**827**
POST EVM	C07 - C12	30	Baseline	TRAP/ASP	49	54	**103**
PRE LLINs	C07 - C12	30	Baseline	TRAP/ASP	34	25	**59**
POST LLINs	C07 - C12	30	FIRST	TRAP/ASP	20	13	**33**
CONTROL	C19 - C24	30	Baseline	TRAP/ASP	27	38	**65**
CONTROL	C19 - C24	30	FIRST	TRAP/ASP	109	106	**215**

Eco-environmental Intervention:

The eco- environmental management was carried out in the month of March in 6 clusters of village Bhoraha (Muzaffarpur District). After intervention from each cluster, 5 households were randomly selected after stratification into mixed houses or human houses. In each cluster 2 houses were mixed and 3 were human. Vector density was measured after four month of intervention and the relative density of the sandflies was estimated for each house.

Cost effective analysis

All the three control methods (IRS, EVM & LLIN) were analyzed to find out the effective vector control strategy for the control of Kala-azar vector. The data revealed that the IRS was the cheap and effective method with minimum amount of acceptability in comparison with control arm. EVM and LLIN are the most acceptable control strategy for the control.

9.a. KALANET Study: Vector Biology in Control Trial (WP-5).

***Objectives*:**

To demonstrate whether the blanket use of LLINs in a community provides any mass effect, which would provide protection to those in the community who fail to use LLIN for any reason.

***Specific objectives*:**

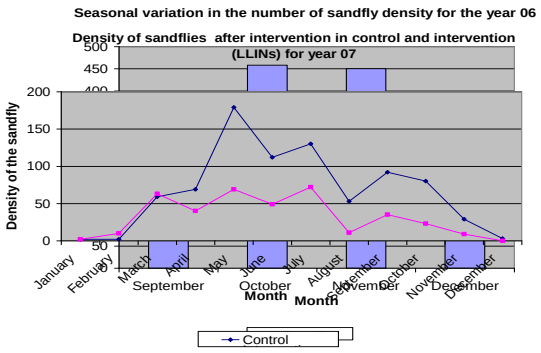
To test whether the community wide usage of LLINs is associated with

- a reduction in the mean *P argentipes* abundance inside houses
- a reduction in the mean *P argentipes* abundance inside cattle sheds
- a reduction in the survival rate (parity rate) of *P argentipes* (collected inside houses and cattle sheds)
- a reduction in the prevalence of *L donovani* infections in *P argentipes* (collected insides houses and cattle sheds).Both parameters were estimated monthly in 3 villages with LLIN and 3 villages without LLIN

***Progress*:**

Six clusters of about 25 households were randomly allocated to the intervention and control arm. Ten households & ten cattle sheds (CS) in 6 clusters were selected randomly and followed one night per month from September to November 2006 and after intervention.

To find out the sandfly density before and after intervention, light traps were set up in 10 selected households and untreated bed nets were provided to household members for the night of capture. There was no significant reduction till March. From April onwards, gradual reduction in sandfly density was observed i.e. 42.03%, 61.45%, 56.25%, 44.61%, 79.24%, 61.96%, 71.25% and 68.96% in April to November respectively. In the month of December, the sandfly density was very low. Community acceptance was 100%. There is a need of long term study to find out conclusive result as the manufacture claims for LLINs impact for 5 years with near about 20 wash.



9.b. Efficacy, acceptibility and cost-effectiveness of long lasting insecticidal treated nets in the prevention of Kala-azar (WP-7).

***Objectives*:**

To measure and compare the efficacy of two types of long lasting insecticidal bet nets

***Progress*:**

The data collected were analysed and presented during the second consortium meeting of KALANET held at London. Regarding the role of LLIN in personal

protection against biting of sandfly was not found significant. There was no significant decrease in the density of sandflies with the use of Olyset and Permanet in comparison to traditional net. The distribution of nets was random in the population. Hence, the manipulation of outdoor sandflies can not be ignored. The blanket use of LLIN in the population can be effective in conrolling the density of sandfly. Further, the susceptibility status of sandflies against the used insecticide will be evaluated. The blood meal analysis of preserved samples will be conducted to see the change in feeding preference, if any. The rate of infectivity of *Leishmania donovani* among *P. argentipes* population will also be evaluated through PCR.

10. Validation of sandfly distribution and Kala-azar disease prevalence through Remote Sensing & GIS in endemic and non endemic foci of Kala-azar to reaffirm the earlier out come and its applicability for the entire Kala-azar endemic region of Bihar.

***Objectives*:**

To correlate geographical distribution of sandfly (*P.argentipes*) in relation to visceral leishmaniasis and the satellite data obtained in respect of macro and micro system and other ground truth in the endemic and non endemic areas evaluate its applicability in entire Kala-azar endemic area and its role as "**epidemic predictor**".

***Progress*:**

On the basis of kala-azar incidence data of last five years (Govt. of Bihar and Jharkhand), five highly endemic blocks i.e. Goraul, Hajipur, Jandaha, Mahua, & Raghopur in Vaishali district and five non endemic blocks in Lohardagga district have been selected for the study. Five test villages have been selected in each PHC randomly on the basis of number of incidence in endemic foci.

Land use land cover information of ground truth validation has been initiated in both foci according to study plan in summer, rainy and winter season so far twice its inception.

Base map with locality vegetation index, water bodies and other landscape elements conducive to vector breeding are being generated and ground truth validation has been completed in two endemic PHCs. This have been subsequently scanned, digitized and plotted considering all parameters of base map. Geometric correlation and classification of these two areas are in progress.

Density of *P.argentipes* (vector) maintains a steady pattern in all the seasons. In rainy season, vector density of 15-22 MHD was found in endemic regions. There is a positive correlation as indicated from preliminary results of abundant water bodies, marshy areas, edible soft stemmed shrubs, agricultural crops with occurrence of high vector density. Alluvial soil with alkaline pH of 7.8 settlement areas with house flooring of loose wet soil with organic debris are indicators of high sandfly density in endemic areas in contrast to less water bodies, marshy areas.

It may be opined from the available data that sandflygenic potential an area arise due to specific environmental composition of water bodies, soil type, peridomestic vegetation, agricultural crops etc. can be assessed for macro stratification for vector abundance, non abundance vis- a- vis kala-azar occurrence non-occurrence.

Meetings/ Seminars/ Trainings organized

1. A Scientific talk on "Paratransgenic approach to vector-borne diseases", delivered by Prof. Ravi Durvasula, Associate Prof. & Chief of Medicine , University of New Mexico, School of Medicine, USA on 07th March 2007.
2. Training to German doctors' belonging to Charite and the Medical Faculty of Humboldt University, Berlin on various aspects of Leishmaniasis was organized on 7th April 2007.
3. The first Scientific Committee meeting of MSF on "Visceral Leishmaniasis Control Programme in Bihar State" was organized on 12th April 2008.
4. Training on "Molecular Immunology & Parasitology" was imparted to Post-Graduate Students of Dept. of Zoology, University of Calcutta from 7th – 10th May 2007.
5. A Scientific talk on "Cell surface molecules of the lesihmania parasites and its biological significance" delivered by Dr. Vahab Ali, Asstt. Professor, Dept. of Parsitology, Gunma University, Japan on 4th June 2007.
6. WHO/TDR sponsored Principal Investigators' meet on "Miltefosine PKDL study" was organized on 08th June 2007.
7. WHO/TDR sponsored Principal Investigators' meeting for protocol discussion of "Combination therapy study" was organized on 09th June 2007.
8. Training to the nominated scientists of ICDDR-B, Dhaka on "Diagnosis and Management aspect of Kala-azar" from 17th – 30th June 2007.
9. A meeting with Dr. D.K. Yadav, Registrar, Magadh University, Bodh Gaya was held on 09th August 2007 to discuss the recognition of the Institute by Magadh University for Ph.D. course.
10. Scientific Review meeting of Field Research of the iOWH sponsored VLFR01 study was held on 28th August 2007.
11. Meeting with Mr. Krishna Kumar, O.S.D. to His Excellency Governor of Bihar, was held on 03rd Oct. 2007.
12. Meeting with Dr. Prema Jha, Vice Chancelor, T.M. Bhagalpur University, was held on 04th Oct. 2007.

13. "WHO Inter-Country Training of Trainers Workshop for Kala-azar Elimination" was organized from 19th – 23rd Nov. 2007.
14. Training to German doctors' belonging to Charite and the Medical Faculty of Humboldt University, Berlin on various aspects of Leishmaniasis was organized on 24th November 2007.
15. One-day training course on various aspects of Kal-azar was imparted to 29 students of M.Sc. Final year from Dept. of Zoology, Patna university on 04th Dec. 2007.
16. WHO, NVBDCP & ICMR sponsored "Intra-Country Training for State/ District Programme Managers Workshop for Kala-azar Elimination", was organized from 24th – 28th December 2007.
17. International Mission Meeting on Kala-azar Elimination Programme was held on 03rd February 2008.
18. A scientific talk on "Understanding Planet Earth" was delivered by Dr. R.N. Singh, Director, Geological Survey of India on 29th February 2008 to mark "National Science Day" celebration at the Institute.

Meetings/ Seminars/ Conferences/ Training attended

Dr. Pradeep Das, Director

- Participated in the 15th meeting of the Scientific Advisory Group, held at ICMR Hqd. on 12th – 13th July 2007.

- Attended meeting with O.S.D. to Chancellor cum Governor at Raj Bhawan on 20th August 2007 for University affiliation of the Institute for Ph.D. Course.

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

- Attended "WHO Inter-Country Training of Trainers Workshop for Kala-azar Elimination" as Coordinator, held at RMRI from 19th – 23rd Nov. 2007.
- Attended WHO, NVBDCP & ICMR sponsored "Intra-Country Training for State/ District Programme Managers Workshop for Kala-azar Elimination", as a facilitator held at RMRI from 24th – 28th Dec. 2007.

Dr. P.K.Sinha, Dy. Director

- Attended 56th ASTMH meeting held in **Philadelphia**, USA from 3rd- 8th Novemebr 2007 and presented papers on i) " Paromomycin IM Injection for the Treatment of Visceral Leishmaniasis"; ii) Oral sitamaquine compared with amphotericin B in the treatment of visceral leishmaniasis (VL) caused by*L.donovani* in endemic areas:Interim Day 90 efficacy/safety results

- Attended **PDT meeting of WHO/TDR** on Miltefosine phase II drug trial in PKDL, ICMR/TDR sponsored Combination therapy, 7th Nov. 2007 in Philadelphia,USA.

- Attended WHO meeting as observer to finalize the guidelines and SOP of clinical diagnosis and treatment of Kala-azar, held at NICED, Kolkata from 16th – 20th April, 2007.

- Attended Principal Investigators' meeting for Study Protocol No. 0207 entitled "A randomized, open-label, parallel-group, 2 sequential step, safety and efficacy study to evaluate different combination treatment regimens (co-administration), of either AmBisome and paromomycin, AmBisome and miltefosine, or paromomycin and miltefosine for the treatment of acute, symptomatic Visceral leishmaniasis (VL)", sponsored by DNDi, Geneva, held at New Delhi from 30th April – 1st May 2007.

- Attended Sitamaquine Advisory Board meeting as consultant for "Drug development of Sitamaquine and Combination therapy strategies", organized by GSK, UK at Hotel Grand, New Delhi on 4th – 5th Sept. 2007.

- Attended meeting on "Bill Milinda Gates Foundation's preparations for an Integrated Kala-azar National Disease Control Programme" at Hotel Chanakaya, Patna on 10th Sept. 2007.

- Attended Expert Group meeting on Leishmania vaccine, held at ICMR Hqr. On 12 Oct. 2007.

- Attended PI meeting of DNDi combination study VL-COMBO-07, held at New Delhi on 15 Oct. 2007.

- Attended iOWH sponsored Study Training Programme of VLPM03 – Module 2 clinical trial, held at Hotel Chanakya on 23rd Jan. 2008.

- Attended the First meeting of DSMB & DMT for the DNDi sponsored combination study (Study No. VL-COMBO-07), organized at Gurgaon, Haryana from 12-13 Feb. 2008.

- Attended "International Microbicides Conference 2008" held at New Delhi from 24th – 27th Feb. 2008.

Mr. Narendra Kumar, Dy. Director

- Attended WHO/TDR meeting on Intervention strategies for the Control of Kala-azar, held at Varanasi from 26th March – 3rd April 2007 and presented the findings of the Project

- Attended WHO/TDR meeting on Intervention strategies for the Control of Kala-azar, held at Varanasi in July 2007 for finalization of tools for the 2nd Phase of the Study.

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

- Attended WHO, NVBDCP & ICMR sponsored "Intra-Country Training for State/ District Programme Managers Workshop for Kala-azar Elimination", as a facilitator held at RMRI from 24th – 28th Dec. 2007.

Dr. Neena Verma, Dy. Director

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

Dr. VNR Das, Asstt. Director

- Attended Training on "Bioinformatics for Medical Doctors" at NIC, New Delhi from 14th – 15th Oct. 2007.
- Attended Training on "Biomedical Information Retrieval" at NIC, New Delhi from 15th – 17th Jan. 2008.
- Attended WHO, NVBDCP & ICMR sponsored "Intra-Country Training for State/ District Programme Managers Workshop for Kala-azar Elimination", as a facilitator held at RMRI from 24th – 28th Dec. 2007.
- Attended a meeting on "Preparation of World Leish 4 Conference" at CDRI in Jan. 2008.
- Attended "WHO Inter-Country Training of Trainers Workshop for Kala-azar Elimination" held at RMRI from 19th – 23rd Nov. 2007.

Dr. Krishna Pandey, Asstt. Director

- Attended Training on "Bioinformatics for Medical Doctors" at NIC, New Delhi from 14th – 15th Oct. 2007.
- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.
- Attended "WHO Inter-Country Training of Trainers Workshop for Kala-azar Elimination" held at RMRI from 19th – 23rd Nov. 2007.
- Attended WHO, NVBDCP & ICMR sponsored "Intra-Country Training for State/ District Programme Managers Workshop for Kala-azar Elimination", as a facilitator held at RMRI from 24th – 28th Dec. 2007.

Mr. A.K. Gupta, Asstt. Director

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

Dr. Vijay Kumar, S.R.O.

- Attended WHO Workshop on the Progress of 1st Stage of IVM Project and the Proposal writing for the 2nd Phase, held at Varanasi from 26th – 28th March 2007.
- Attended WHO, NVBDCP & ICMR sponsored "Intra-Country Training for State/ District Programme Managers Workshop for Kala-azar Elimination", as a facilitator held at RMRI from 24th – 28th Dec. 2007.

Dr. Sanjeev Bimal, S.R.O.

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

Dr. C.S. Lal, S.R.O.

- Meeting with O.S.D. to Chancellor cum Governor at Raj Bhawan on 20th August 2007 for University affiliation of the Institute for Ph.D. Course.

Dr. Nawin Kumar, R.O.

- Attended WHO, NVBDCP & ICMR sponsored "Intra-Country Training for State/ District Programme Managers Workshop for Kala-azar Elimination", held at RMRI from 24th – 28th Dec. 2007.
- Attended iOWH sponsored Study Training Programme of VLPM03 – Module 2 clinical trial, held at Hotel Chanakya on 23rd Jan. 2008.

Dr. SriKant Kesari, R.O.

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

Dr. D.S. Dinesh, R.O.

- Attended the 1st Thematic meeting of European Commission sponsored project entitled "Efficacy, acceptability and cost-effectiveness of long-lasting insecticidal nets in the prevention of Kala-azar – KALANET", held at London School of Hygiene & Tropical Medicine, UK from 17th – 20th Sept. 2007.

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

Dr. N.A. Siddiqui, R.A.

- Attended WHO/TDR meeting on Intervention strategies for the Control of Kala-azar, held at Varanasi in July 2007 for finalization of tools for the 2nd Phase of the Study.

Mr. Anil Kumar, R.A.

- Attended XXVII Laboratory Animal Supervisors' Training Course, held at NCLAS, NIN, Hyderabad from 3rd Sept. – 30th Nov. 2007.

Mr. Subhakar Kr. Singh, R.A.

- Attended "Advance course on Immunology, Vaccinology and Biotechnology applied to Infectious Diseases", organized by UNICEF/UNDP/World Bank/WHO at WHO Immunology Research and Training Centre, Department de Biochimie, University of Lausanne, Switzerland from 4th Sept. – 18th Oct. 2007.

Dr. V.P. Singh, S.T.O.

- Attended "Capacity Building Workshop on Application of Multivariate Mixed Effects Models", organized by The National Institute of Medical Statistics (NIMS), ICMR with support from NACO, WHO-India and CDC, Atlanta at Dept. of Biostatistics, SGPIMS, Lucknow from 10th – 12th Dec. 2007.

Mr. R.B. Verma, T.O.

- Attended meeting with V.C., T.M. Bhagalpur University and the university delegates at University campus on 03rd Nov. 2007.

- Attended "Capacity Building Workshop on Application of Multivariate Mixed Effects Models", organized by The National Institute of Medical Statistics (NIMS), ICMR with support from NACO, WHO-India and CDC, Atlanta at Dept. of Biostatistics, SGPIMS, Lucknow from 10th – 12th Dec. 2007.

- Attended iOWH sponsored Study Training Programme of VLPM03 – Module 2 clinical trial, held at Hotel Chanakya on 23rd – 24th January 2008.

Mr. Brijnath Prasad, ALIO

- Attended Training on "Biomedical Information Retrieval" at NIC, New Delhi from 15th – 17th Jan. 2008.

Mrs. Pushpa Raj, Sister I/c

- Participated in HIV Pre-test Counseling Training for the Phase 4-Module 1 (VLPM03) study, organized by iOWH at Patna on 14th June 2007.

Mr. S.B. Barman, Tech. Asstt.

- Participated in HIV Pre-test Counseling Training for the Phase 4-Module 1 (VLPM03) study, organized by iOWH at Patna on 14th June 2007.

Mr. Umesh Kumar, Lab. Tech.

- Attended iOWH sponsored Study Training Programme of VLPM03 – Module 2 clinical trial, held at Hotel Chanakya on 23rd – 24th Jan. 2008.

Publication (2007-08)

1. R. Dey, N. Majumder, S. Bhattacharjee, S. Bhattacharyya (Majumdar), R. Banerjee, S. Ganguly , Pradeep Das and Subrata Majumdar. *Leishmania donovani* Induced Ceramide As The Key Mediator of Akt Dephosphorylation In Murine Macrophages: Role of PKCζ and Phosphatase. ***Infect & Immun***. May; 75(5):2136-42. (2007)

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3. C.S.Lal, A. Kumar, S. Kumar, K. Pandey, N.Kumar, S. Bimal, P.K.Sinha and Pradeep Das. Hypocholesterolemia and increased triglycerides in paediatric visceral leishmaniasis.***Clinica Chimica Acta***. 382 : 151-153. (2007)

4. K. Pandey, P.K. Sinha, V.N.R. Das, N. Kumar, N. Verma, S. Bimal, C.S.Lal, R.K.Topno, D. Singh, R.B.Verma, S.K.Bhattacharya and Pradeep Das. Wilson Disease with Visceral Leishmaniasis: An Extremely Uncommon Presentation. ***Am. J. trop. Med. Hyg.*** 77(3): 560 – 561. (2007)

5. Chauhan DS, Sharma VD, Parashar D, Chauhan A, Singh D, Singh HB, Das R, Aggarwal BM, Malhotra B, Jain A, Sharma M, Kataria VK, Aggarwal JK, Hanif M, Shahani A, Katoch VM. Molecular typing of Mycobacterium tuberculosis isolates from different parts of India based on IS6110 element polymorphism using RFLP analysis***. Indian J Med Res.*** 125(4):577-81. (Apr. 2007).

6. K Pandey, PK Sinha, S Bimal, CS Lal and P Das. HIV-Leishmania co-infection in India. ***Science and Culture***, 73(5-6): 159-161. (May-June 2007).

7. Shyam Sundar, T.K.Jha, Chandeshwar P. Thakur, Prabhat K. Sinha and Sujit K Bhattachaya. Injectable paromomycin for Visceral Leishmaniasis in India. ***The New England Journal of Medicine***, 356(25); 71-81. (June, 2007)

8. Bhattacharya SK, Sinha PK, Sundar S, Thakur CP, Jha TK, Pandey K, Das VNR, Kumar N, Lal C, Verma N, Singh VP, Ranjan A, Verma RB, Anders G, Sindermann H, Ganguly NK. Phase 4 trial of miltefosine for the treatment of Indian visceral Leishmaniasis. ***J. Infect Dis.*** 2007 Aug 15; 196(4): 591-8. Epub (2007 Jun 29).

9. Kumar N, Pandey K, Das VNR, Sinha PK, Topno RK, Verma N, Lal CS, DasP,Bhattacharya SK. Miltefosine in the treatment of a case of visceral Leishmaniasis with renal dysfunction. ***Ann Trop Med Parasitol***, 101 (7):649-51. (Oct. 2007).

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