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.

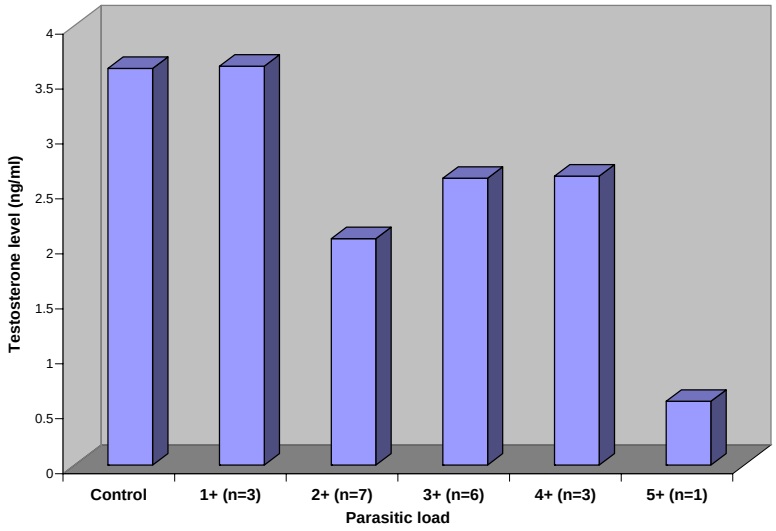
It was observed that testosterone level in control and VL cases with 1+ parasitic load was almost the same, but in VL cases with 5+ parasitic loads it was sharply decreased as compared to control. Preliminary results showed that the level of testosterone in cases and controls do not differ significantly (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.

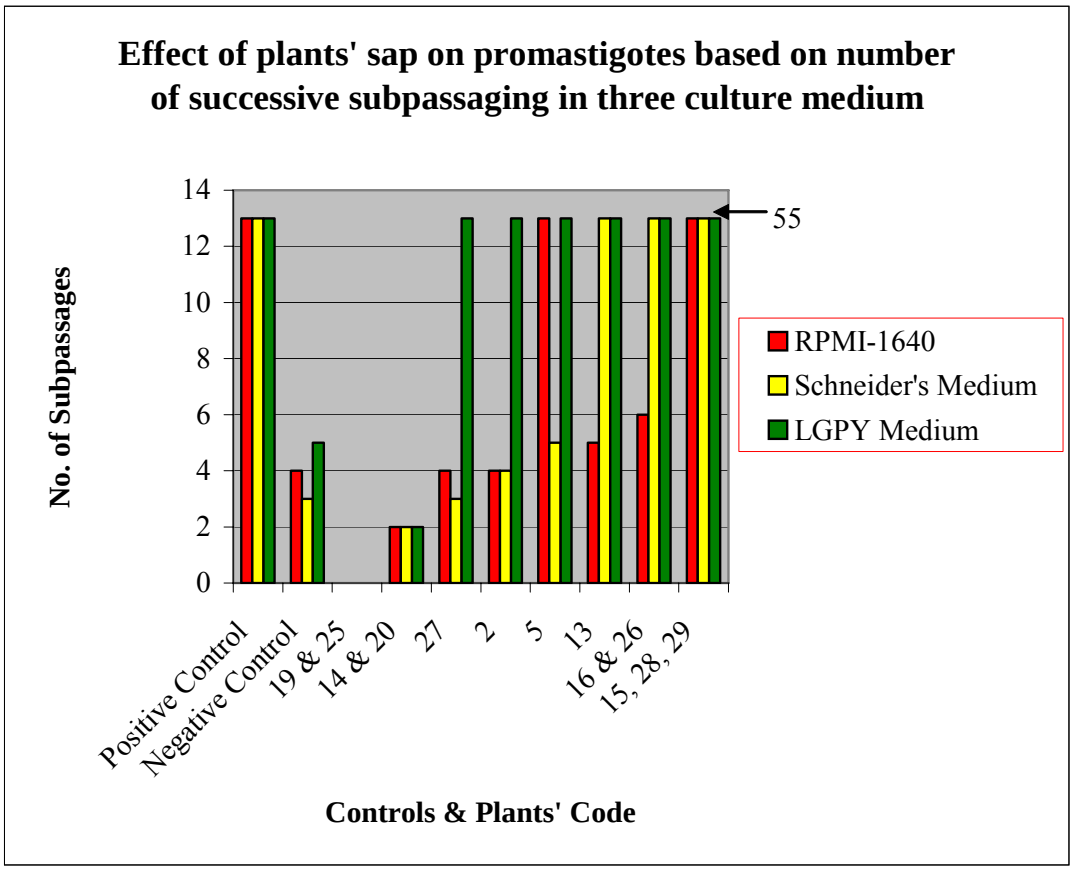


Table 1: Effect of plants' extract on promastigotes based on number of successive sub-passages.

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).

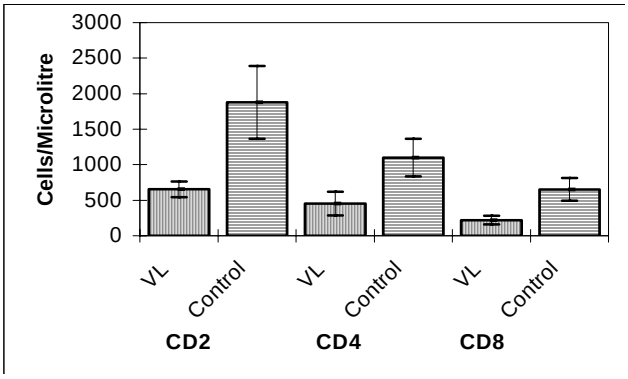
- **Predominance of a well-characterized Th2 cell associated mechanism during VL infection reverts and more T-cells show increased sensitivity for IFN-γ production after anti-CD2 stimulation**

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.



b.

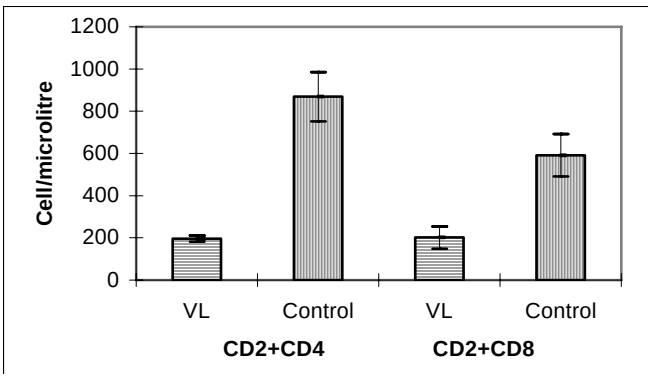


Fig. 1. Immunophenotyping of CD2, CD4 and CD8+ T-cells in VL patients and control. (a) CD2 down regulation is associated with CD4+ Tcell expression during Visceral Leishmaniasis. (b) Analysis of CD2+ CD4+ and CD2+ CD8+ cells in T-cell (CD3+) in patients compared to control.

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

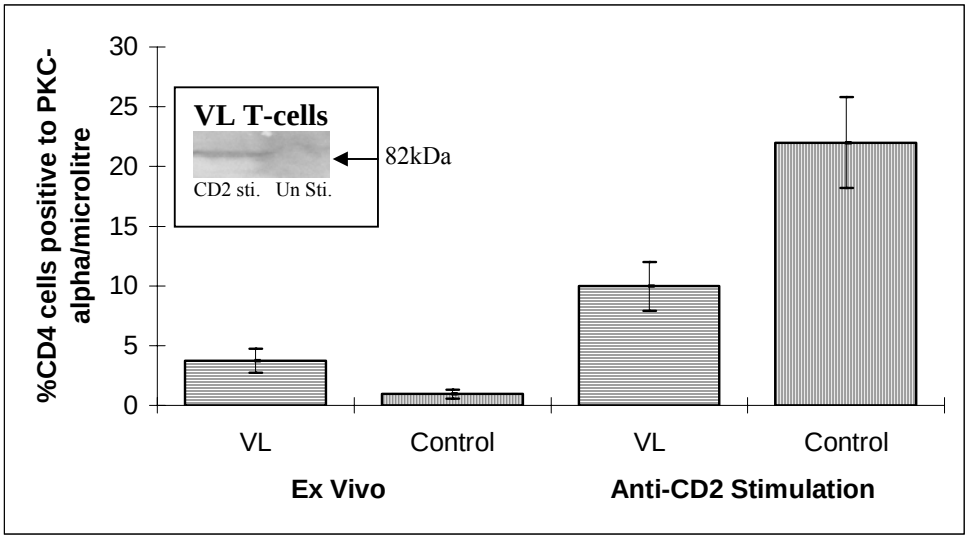


Fig. 2. Effect of CD2 activation on cell signaling mechanism in T-cells of VL patients. Total amount of PKC-a produced by cells stimulated and not stimulated with anti-CD2 antibody evaluated through FACS-Calibur. Immunoblotting of VL T-cells (inset) with PKC-a antibody shows that anti-CD2 induced significant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

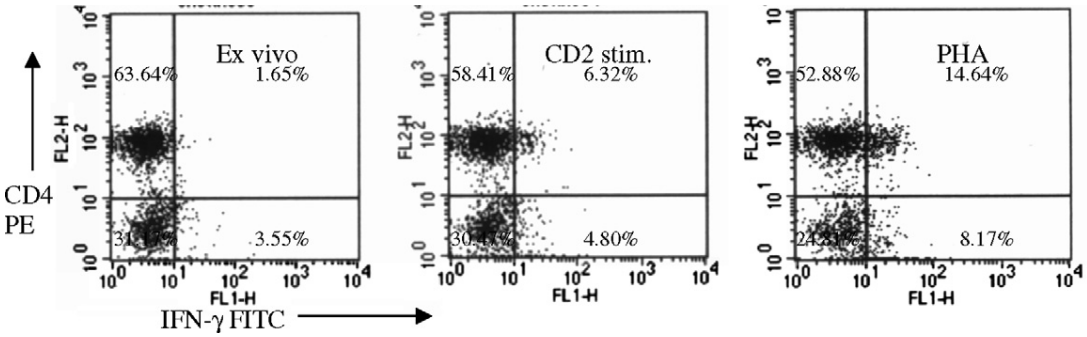


Fig. 3: Increase in frequency of CD4 cells for IFN-γ after activation of CD2 antigen in a VL patient: flow diagram of cultured PBMNCs stimulated with or without anti-CD2 or PHA in presence of brefeldin-A and stained with anti-human CD4 PE and anti-human IFN-c FITC of a VL patient showing increase in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

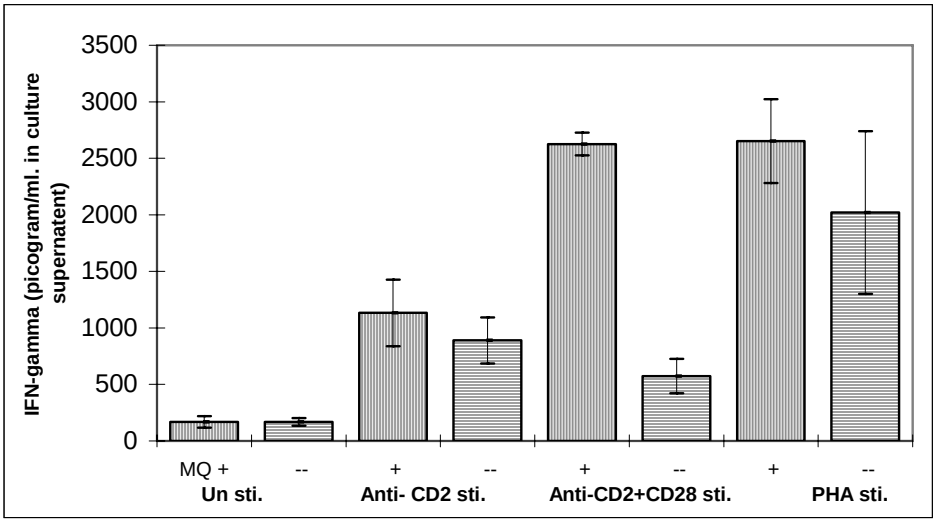


Fig. 4. CD2 activation in VL patients stimulates T-lymphocytes to produce IFN-γ even in absence of APC.

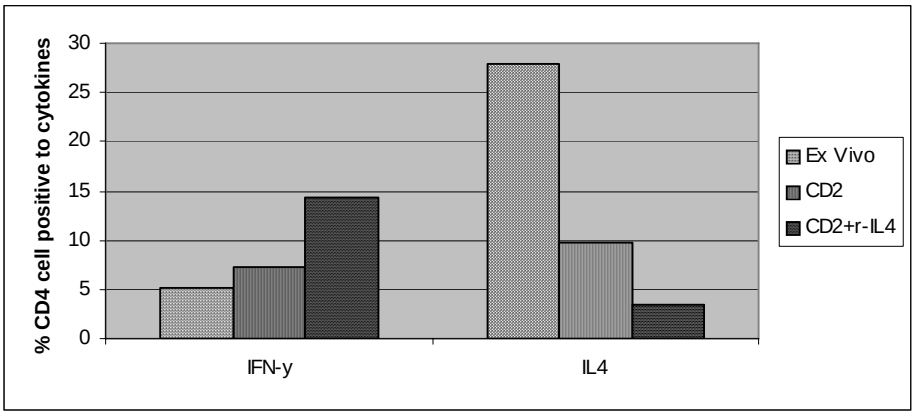


Fig. 5: CD2 activation in VL patients influences IFN-c responsiveness in T-lymphocytes by down regulating IL-4 production.

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.

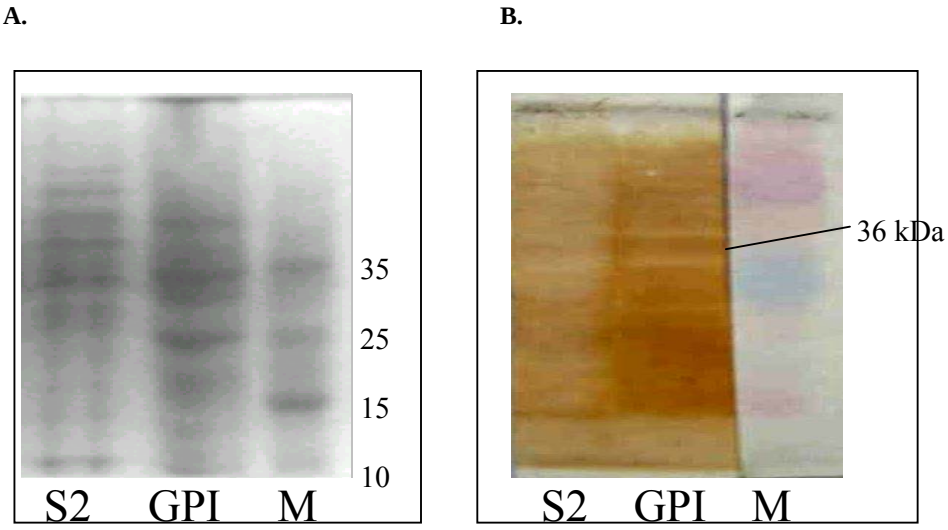


Fig. 1: A. Antigenic profile soluble protein (S2) and GPI anchored protein. B. Immunobloting of the antigen with anti pgp-1 antigen.

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.

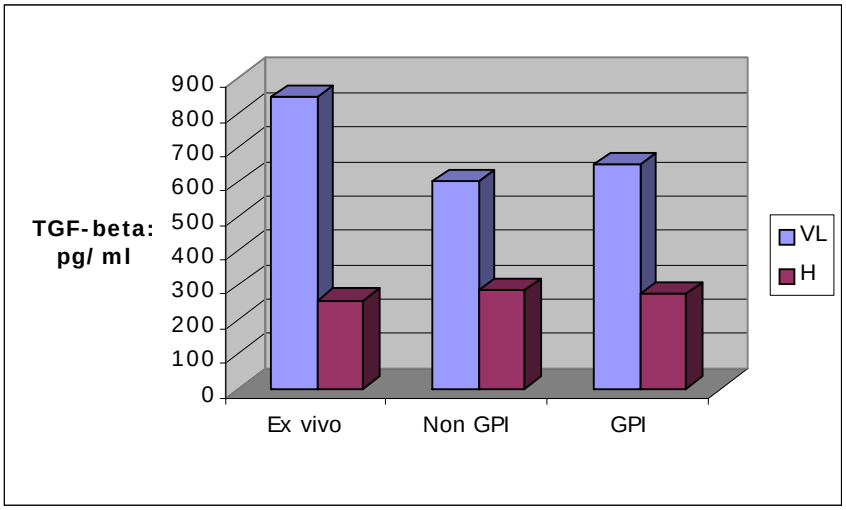


Fig. 2: Expression of TGF-β from macrophages in VL patients before and after stimulation with *Leishmania donovani* proteins with or without glycoinositol phospholipid anchor.

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.

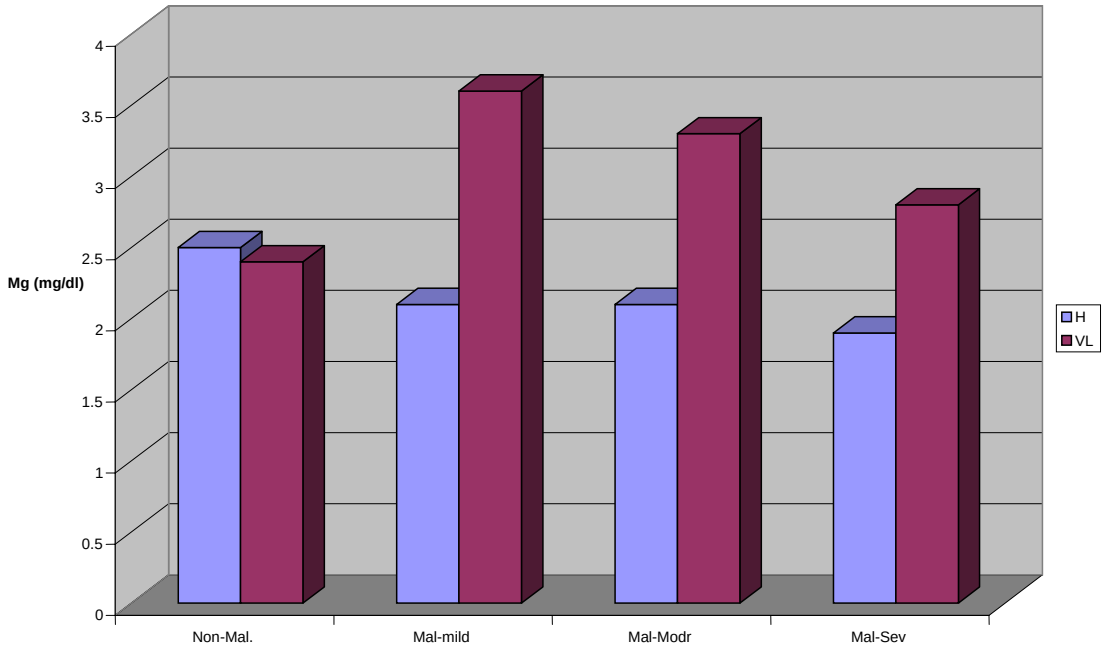
Hypocholesterolemia and increased triglyceride was observed in malnourished VL infection. According to parasitic load, the triglyceride concentration was observed to be directly proportional. Iron and transferrin were observed to be down regulated in VL infection.

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.

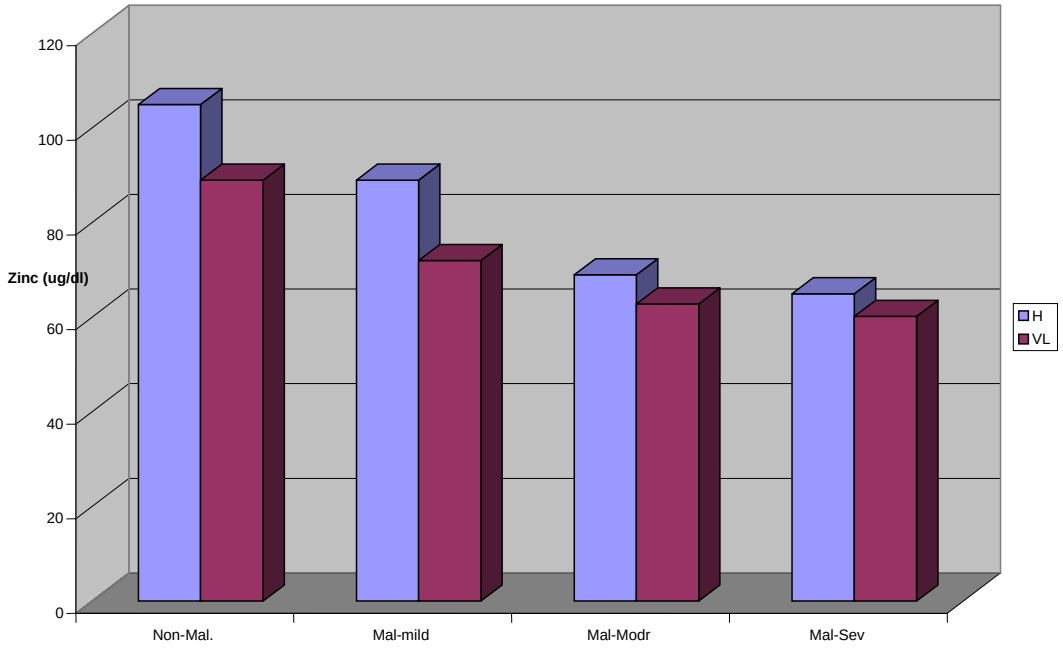
Table I: Healthy vs Mal-nourished & Non-mal-nourished VL

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

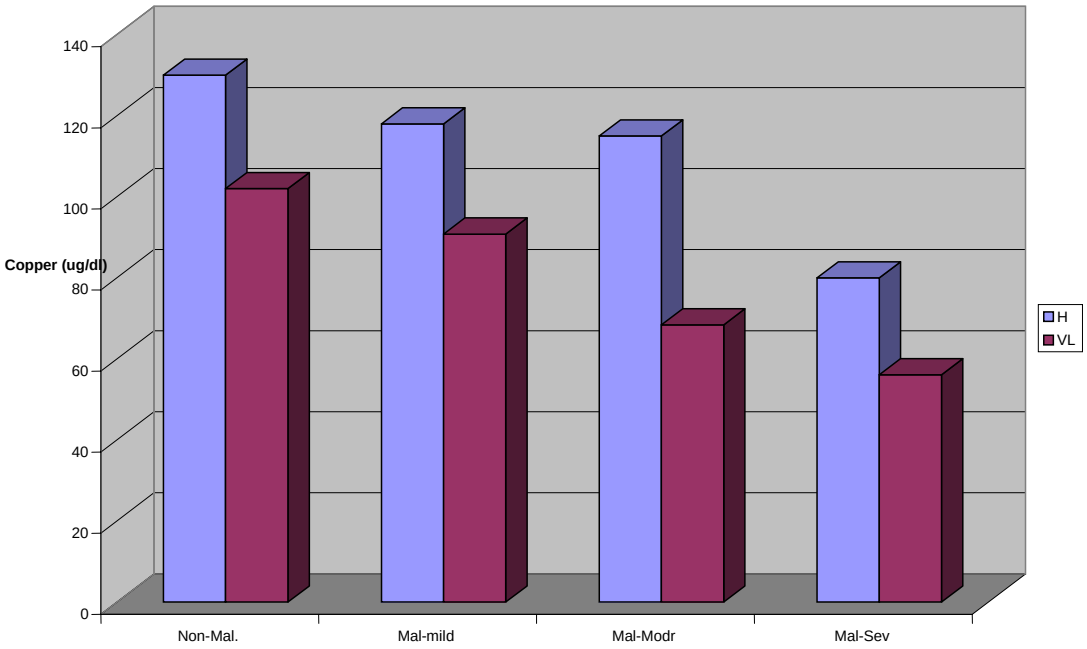
Magnesium as nutritional marker as per BMI



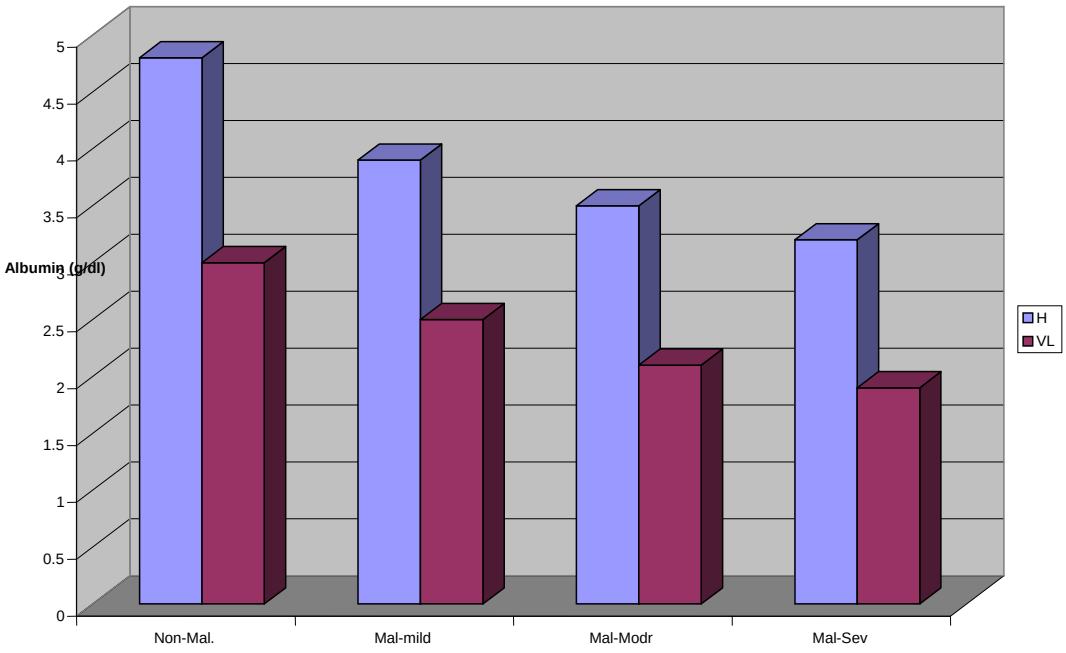
Zinc as nutritional marker as per BMI



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