ANNUAL REPORT

[2009-10]

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Preface

During the period 2009-10, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Patna widened its research scope by inducting new initiatives such as establishment of ART centre, Tuberculosis centre, virology laboratory including Swine flue lab. and field station at Sadar Hospital, Motihari. Various intramural and extramural projects, focused on diagnosis, treatment, basic research, vector control and epidemiology, were conducted. The institute has been identified by WHO as reference centre for Leishmania parasite and Sera Bank. The repository has 89 isolates of different categories of leishmania isolate and 443 sera samples from kala-azar and control subjects.

Development of a non-invasive test for diagnosis of kala-azar using sputum is the major achievement as possible translation research. Application of PCR as diagnostic test for Kala-azar and PKDL has shown better result than conventional microscopy of bone marrow/ splenic aspirate and slit kin/ biopsy. Various clinical drug trails for treatment of VL, PKDL and co-infection were undertaken; few of them completed and some are still ongoing.

Role of nutritional factors in relation to severity of VL revealed that zinc and albumin level was down regulated and magnesium up-regulated with increase in severity of malnourishment. Two plants' extract exhibiting lethal effect on promastigotes have been explored.

It has been observed that TGF-β triggers apoptotic death of lymphocytes through up-regulation of tyrosine phosphatase activity and the use of sodium orthovanadate (NaOVa, a tyrosine phosphatase inhibitor) reduces the apoptotic frequency. TGF-β not only induces PTPase but also serine/threonine phosphatase activity, as evident by PP2a activity measurement by ELISA. TGF beta follows smad independent pathway and thus induces PP2a by phosphorylating TAK1 molecule. Trypanothione Reductase (TR), Tryparedoxin (PXN) & tryparedoxin peroxidase (CTP) showed up-regulation in resistant strain, compared to sensitive indicating involvement of thiol metabolic Pathway genes in conferring resistance. ABC Transporter MDR showed ~ 3 fold up-regulation in the resistant strain in comparison to sensitive, but PgPA expression is almost similar. Drug efflux rather sequestration is involved in drug resistance. It was observed that GPI proteins are possibly responsible for up-regulation of innate immune response via the TLR signaling pathway in VL patients. The action of the GPI-anchored proteins was more prominent on the macrophages in respect to up-regulation of TLR expression.

In-vitro study to assess the significance of KMP-11 molecule in protection from VL revealed that the primed T-lymphocytes responded to KMP-11 resulting in release of IFN-γ but not IL-4 in healthy donors together with the induction of super-oxide radicals in macrophages of the patients. The findings suggest that the KMP-11 molecule can be examined further as vaccine candidate for Kala-azar. It was observed that natural T-regulatory cells which are a source of IL-10 and TGF-β expands in response to *Leishmania* antigen. Comparatively a higher % of CD4 Natural T-regulatory cells were observed in active cases of VL. It was decreased in absence of compatible antigen presenting cells and increased in presence of *Leishmania* impregnated macrophages in *in vitro* experiments.

It was observed through in-vitro experiments that vector salivary gland homogenate (SGH) exhibits dual mode of action as it helps in providing immunity as well as infection to the host. The pheromone study revealed presence of chemical communication, mediated by host, and semiochemicals released by male sandfly that helps sandflies to aggregate on the host animal for mating and blood feeding. CDC light trap was evaluated as the most efficient method for monitoring *P. argentipes* population in the Indian subcontinent. Cluster-wide provision of Long lasting nets (LN) significantly reduced the GM LT total *P. argentipes*/house by 24.9%. A new monitoring and evaluation toolkit for IRS has been developed. Application of Remote Sensing & GIS for validation of sandfly distribution and Kala-azar prevalence revealed strong relationship between NDVI and total sandfly density as well as density of *P. argentipes* in both endemic and non endemic foci.

LEISHPROT, a web based repository database for all known *Leishmania donovani* proteins, was developed to provide easy access to voluminous data related to sequences and annotation with linkage facility to other data source. *LeishMICROSATdb*, a database of di to hexa nucleotide repeats of three Leishmania species i.e. *L. major*, *L. infantum* and *L. braziliensis*, has been developed using genome sequence data available in NCBI. Structural models of twelve different proteins of various *Leishmania* strains have been developed and tested for its ligand protein interaction with diverse sets of ligands by computational tools of discovery studio. Anticancer drug sulforaphane has been indicated for leishmaniasis by *in-silico* ligand protein interaction studies of a surface protein of *Leishmania* i.e. KMP11. Three compounds have shown best fit score to act as potential drug candidates against P-glycoprotein of *Leishmania donovani*.

Director

Contents

INSTITUTIONAL STUDIES

1. Hospital based surveillance.

Objectives:

- To capture patient data at all the doorsteps.
- To interlink the patients' data, generated at various levels, in a comprehensive database
- To assist the Institutional scientists by providing patients related information instantly as per their research requirement.

Progress:

During the period April 2008 – Oct. 2009, a total of 12,224 new patients were registered in OPD, out of which 1097 (9%) were admitted in ward for confirmatory diagnosis and/or treatment for Kala-azar (VL)/ PKDL. The total number of admission in ward reached to1219 including 122 re-admission for follow up, relapse or any other complain.

With a view to optimize the bed occupancy, OPD-level VL case screening using rk39 was initiated prior to advice for admission. Out of 12,224 new registered cases, 1592 (13%) were subjected to rK39 and 547 (34.4% of tested) were found positive and accordingly admitted in ward. Comparatively high number of new admission records (1097 vs 547; about 2-fold) may be attributed to referral by MSF, outside-diagnosed/ treated, relapse or non-responsive etc. who were not subjected for rk39 test.

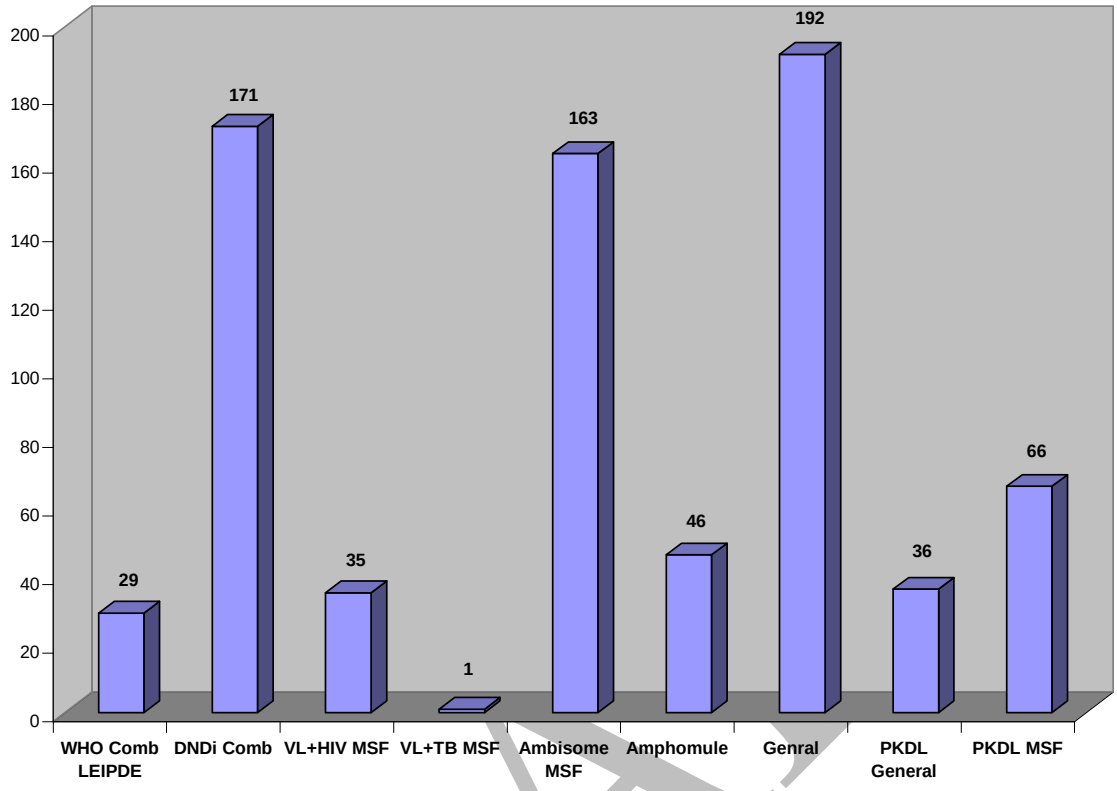
Out of 1219 admitted cases, 739 (60.6%) were either enrolled in the then undergoing different clinical drug trials or treated as per the standard regimen of amphotericin B; rest were non-Kala-azar/ PKDL cases (18.6%), referred or left against medical advice (7.1%), just for follow up investigations (13.2). A total of 5 patients (0.4% of total admission) died in the indoor. On an average, new registration in OPD, total admission in ward and confirmed cases put on treatment came to 643, 64 and 38 respectively per month.

Table 1: Distribution of OPD registered patients and admitted patients

Month	Year	New Reg.	Admission			Indoor Patient Distribution				
			New	Re	Total	Non-KA	Ref/ LAMA	FU	Death	Treated
Apr	2008	691	49	5	54	2	3	7	0	42
May	2008	674	66	5	71	8	6	8	0	49
Jun	2008	722	63	7	70	10	6	12	0	42
Jul	2008	624	55	11	66	9	8	11	0	38
Aug	2008	726	63	5	68	19	6	10	2	31
Sep	2008	782	60	8	68	15	3	15	0	35
Oct	2008	600	46	4	50	5	4	9	0	32
Nov	2008	572	61	3	64	15	2	12	0	35
Dec	2008	468	53	7	60	9	4	15	1	31
Jan	2009	521	52	7	59	7	4	9	1	38
Feb	2009	585	67	5	72	7	4	9	1	51
Mar	2009	606	76	3	79	15	6	3	0	55
Apr	2009	528	58	6	64	11	4	5	0	44
May	2009	593	54	6	60	15	3	4	0	38
Jun	2009	705	73	7	80	20	5	9	0	46
Jul	2009	820	69	8	77	23	12	6	0	36
Aug	2009	726	55	7	62	21	3	3	0	35
Sep	2009	613	41	7	48	10	2	1	0	35
Oct	2009	668	36	11	47	6	2	13	0	26
Total		**12224**	**1097**	**122**	**1219**	**227**	**87**	**161**	**5**	**739**
(%)			**(9.0%)**			**(18.6%)**	**(7.1%)**	**(13.2%)**	**(0.4%)**	**(60.6%)**
Avg/ Month		**643**	**57**	**6**	**64**	**11**	**4**	**8**	**0**	**38**

The clinical trials underwent during this period were two combination therapy trials (one WHO/TDR and another DNDi sponsored), Amphomule study sponsored by Bharat serum and MSF sponsored trails for VL/HIV, VL/TB, Ambisome for VL and PKDL. Near about by the end of this reporting period, patients' enrollment target in both the combination therapies had been achieved. Since PKDL treatment requires repeat course (2 or 3 depending upon the physician discretion), admission occurrence of PKDL reached to 102 (66 as MSF patient and 36 as General) including re-admission for repeat course (Fig. 1).

Fig. 1: Distribution of treatment allocation (Total 739)



2. Establishment of repository *Leishmania* parasites and sera bank at RMRIMS, Patna.

Aim and objectives:

The study aims to establish a repository / national resource of *Leishmania* parasites and sera sample which can cater parasites and sera sample of different groups to scientists/researchers working on various aspects of this disease. The objectives of this study are:

- To isolate *Leishmania* parasites from VL / PKDL patients having different characteristics such as clinical pictures, drug responses, geographical locations, etc., and from vector; their culture adaptation and cryopreservation in liquid nitrogen as well as *in –vivo* maintenance of few selected important isolates.
- To cryopreserve different reference isolates of *Leishmania.*
- To characterize the various isolates.

- To preserve sera sample of KA/PKDL patients and suffering from different diseases, as well as of normal cases from different areas at -20ºC.
- Proper documentation and archiving of all details.

Progress:

Total 89 different isolates of *Leishmania donovani* are presently being maintained in liquid Nitrogen. One cryovial of all the 17 different isolates of *L. donovani* that were cryopreserved (10-13 vials per isolates) earlier, were revived after 2-3 months of storage and after revival the original cultures were discarded. Currently, 22 isolates are being maintained *in vitro* by sub-passaging in bi-phasic medium at the interval of 2-3 weeks, 2 isolates are *in vivo* maintenance (in BALB/c mice) and 13 isolates are in culture adaptation for cryo-preservation in near future. Altogether 33 isolates have been mass cultured and provided to scientists for their research projects.

In sera bank, 443 sera samples of various categories such as confirmed VL cases, relapsed VL cases, VL with HIV co-infection, PKDL cases, healthy endemic, healthy non-endemic etc have been preserved at -20^0 C in small aliquots that are being utilized for different research works. Recently, 450 rK39 test kits of different lots have been evaluated using sera bank samples.

Table: Details of sera samples stored in Sera bank

Category code	Category	# Sera samples
01	VL cases	48
02a	Healthy endemic with follow-up	39
02b	Healthy endemic without follow-up	79
06	Healthy non-endemic	152
07	PKDL cases	24
03	Relapsed VL cases	13
05	HIV-VL co-infection	05
04	Other disease (TB)	58
08	Others	25
	Total	**443**

INTRAMURAL STUDIES

Epidemiology

3. The economic burden of Visceral Leishmaniasis at the household level in Bihar, India.

A Ranjan et al.

Aims and Objectives:

Qualitative Research

- To identify the factors affecting the income and expenditure pattern at household level in Kala-azar affected households in rural areas through FGDs and in-depth interview of VL affected HH.

Quantitative Research

- To estimate the economic impact at the household level by measuring direct and indirect cost for VL care
- To investigate coping strategies to pay for VL care

Progress:

Paroo PHC was identified as the highest endemic PHC of VL in Muzaffarpur district during 2008 based on the records obtained from the concerned District Malaria Officer. After going through the PHC records of VL cases in the year 2008, it was found that two villages namely, Bangra and Deoria, were most affected and eventually the affected households of these two villages were identified. A sampling frame of potential participants for conducting two focus group discussions (FGD) were created, and 12 participants were selected after narrating the purpose of the study. Date, time and venues were fixed prior to FGDs. Two FGDs were conducted by PI as moderator and two investigators as coordinators. Proceedings were tape-recorded and notes were taken by investigators. Later on, proceedings were transcribed using tape-recorded and notes.

For conducting in-depth interviews, a sampling frame of potential participants was created from the records of these two villages. Apart from this, households having past history of VL in previous year were also identified by the team. Twenty participants were

selected from the two villages after getting their consent. Three investigators conducted in-depth interviews using a common guideline. The study is in progress.

4. Spatial and seasonal distribution of Kala-azar cases in Bihar, India.

NA Siddiqui et al.

Objectives:

- To evaluate the average amplitude of kala-azar cases across the seasons and to observe the seasonal trends.

Specific objectives

- To measure the average annual incidence of Kala-azar.
- To measure the magnitude of seasonal variation of kala-azar incidence.
- To measure the magnitude of geographical distribution of Kala-azar incidence.
- To measure magnitude of patients-visiting rates to public sector facilities in different seasons.
- To identify the possible risk factors for seasonality like average temperature, humidity and rainfall.

Progress:

The district and year wise retrospective data of reported cases and deaths of Kala-azar of all the 39 districts of Bihar for the period of 2001 – 2007, available at the State Health Department, Bihar and National Vector Borne Diseases Control Programme, India were collected, compiled in a database and analyzed. The preliminary data analysis revealed that in seven years period a total of 1,41,650 cases of kala-azar were treated in the Public Sector with 1116 deaths. The annual incidence of kala-azar varied from 1.14 – 4.56 per 10,000 population (Mean 2.4) and the cause specific deaths rate varied from 1.3 to 2.5 per 10,00,000 (Mean 1.8). There was an increasing trend of annual incidence but as compared to 2001, significant increase (about 2.5 – 3 folds) was observed from 2005. The cause specific death was slightly on higher side since 2001 to 2003 but 2004 onwards it was almost consistent.

Out of total 141650 cases, 88485 were registered between two quarters i.e. April – June and July – Sept. contributing about 63% of total cases. The highest average annual

incidence (0.74/10000) was observed in July – Sept. quarter while it was lowest (0.40/10000) in Oct – Dec. The average annual incidence of April – June and July – Sept quarter was found consistent (0.70/10000).

Further analysis about geographical distribution of kala-azar cases revealed significantly higher proportion of cases (75589, 53%) in North areas of Bihar followed by East (58283, 41.5%), South (4160, 3%) and West (3618, 2.5%). The average annual incidence was significantly higher (3.9/10000 and 2.8/10000) in the East and North areas respectively as compared to West and South (0.33/10000). The average cause specific death was highest (2.7/10,00,000) in the North areas whereas it was more (1.3/10,00,000) in West areas as compared to East (1.1/10,00,000) in spite of the lower average annual incidence of the disease.

The study is in progress.

5. Parameters associated with progression of asymptomatic to symptomatic VL cases.

R.K. Topno et al.

Objectives:

- To identify early indicators e.g. hematological picture and its relation in progression of asymptomatic to symptomatic kala-azar
- To identify the relation of the immunological marker like IL-10, Interferon-Y in progression of the Kala-azar cases from asymptomatic status.

Progress:

The study was initiated in Narayanpur and Nandan ketuka villages of Parsa PHC, district Saran wherefrom good number of kala-azar cases were reported last year. During door to door visit, demographic profile of the households were taken through structured questionnaire and they were serologically screened for leishmania infection by rK39 strip test. Out of 1200 enumerated population, 1017 (Male 456, Female 561) were subjected to rK39, out of which 61 were found positive. For assessment of haematological and immunological parameters, about 2 ml peripheral blood samples were collected from the rk39

positive subjects who had neither past history nor having any current sign and symptoms for VL/PKDL. The study is in progress.

6. An epidemiological study to assess the prevalence of PKDL in endemic areas of Bihar.

V.N.R. Das et al.

Objectives:

- To determine prevalence of PKDL in an endemic community of Bihar.
- To assess management of PKDL cases in the community
- To establish intra-familiar transmission

Progress:

The study was carried out in Rukhai village of Chandi PHC wherefrom regular occurrences of VL cases have been reported. Head (in absence any adult member) of the households were interviewed during door-to-door survey using structured questionnaire to collect information on socio-demographic characteristics like age, sex, occupation, and past history of VL and treatment. Each suspected PKDL cases were clinically and physically examined for detection of any skin lesions.

The total population in the study area was 223 having 116 male (52%) and 107 females (48%). The female to male ratio was 923 per 1000 males, very close to the reported sex ratio of the district as per the census 2001. Out of 223 individuals, 41 had past history of VL occurred during 2001 to 2007, with maximum number of cases in 2006. Out of 41 cases, 40 cases were treated with recommended dosage of Sodium Antimony Gluconate (SAG), and only one case received recommended dosage of Miltefosine. All of them got cured with no history of further relapse. A total of 11 individuals (male-5, female-6) were clinically and serologically identified as PKDL cases.

The survey was further continued in the case-based selected villages of Forbesganj block, Dist. Araria districts. Out of 23,915 population from 4323 households, 12 clinically diagnosed PKDL cases (Male 5, Female 7) were detected. All 12 subjects were subjected to rK39 test. Out of 12, 9 had past history of VL (all were rK39 positive). The work in under progress.

Diagnostics

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

D. Singh et al

Objectives:

- Development a new gene target (ITS region of rRNA gene) for the diagnosis of visceral leishmaniasis & comparison of results of PCR (Blood) with results of conventional diagnostic methods

Progress:

A total of 102 peripheral blood samples, collected from clinically suspected kala-azar cases, were subjected to DNA isolation by using QIAamp DNA blood mini kit (Qiagen). Nested PCR of blood samples was performed to amplify the ITS region of rRNA gene of *L. donovani* from previously amplified PCR product to increase the sensitivity and specificity. All the positive samples showed 600bp band whereas negative samples showed no band. Out of 102 suspected VL cases, 87 (85.3%) were positive by blood PCR and 68 (66.6%) cases were found positive by microscopy. No amplification was seen with other intracellular organisms such as *Plasmodium vivax*, *M. tuberculosis*, and *M. leprae*.

Sequencing was performed with randomly selected positive amplicons from clinical samples and positive control (culture isolates) using the ABI 3130xL automated Genetic analyzer. The specificity of PCR was further confirmed by the similar BLAST analysis of the forward primer using the gene data bank sequence of NCBI, which showed perfect complementary with ITS region of rRNA gene of *L. donovani* and did not show homology to any organism other than *L. donovani*. This clearly indicates that the gene sequences are specific for *L. donovani* and do not have any structural homology with genomic DNA from other intracellular organisms. Further, large-scale testing using DNA samples from other clinical sources of endemic areas, is required for validation of efficacy of these primers for its diagnostic applicability. The study is in under progress.

8. Study of Imprint smear microscopy and PCR application on biopsy from dermal lesions for diagnosis of Post kala azar dermal leishmaniasis cases in Bihar.

N. Verma et al.

Objectives:

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

Progress:

After clinical examination for lesions' type, site and coalescence, biopsies from different skin lesions were collected aseptically from 60 PKDL cases (50 fresh and 10 after treatments). Imprint smears were prepared for detection of leishmania parasites under microscope. Biopsy samples were collected in Tris buffer solution for PCR study. PCR detection of whole ITS region of the ribosomal RNA (rRNA) gene was done. DNA was isolated by commercially available 'QIAamp DNA tissue mini kit (Qiagen). A nested PCR has been developed from biopsy samples to amplify the ITS region of rRNA gene of *L. donovani* from previously amplified PCR product to increase the sensitivity and specificity. The amplified reactions were visualised on 1.5 % agarose gel using a DNA marker. The positive samples showed positive ~1100 bp band, whereas negative samples showed no band. Parasitological (L.D.) positive sample was used as a positive control. As a negative control, skin biopsies from 6 known patients of fungal diseases or leprosy were collected.

The comparative analysis of both these tools revealed that leishmania parasite positivity by PCR and microscopy were 95.6% and 91.3 % respectively in papulonodular lesions whereas in case of hypopigmented macular lesions PCR was found to be more sensitive (92.5%) than microscopy (44.4%). The sensitivity of the PCR was found 24% higher than microscopy. Among the 10 SAG treated PKDL cases, 2 were found positive by PCR but microscopically they were negative, possibly due to very high sensitivity of PCR to detect even very low amount of parasitic DNA. The study is in progress.

Clinical

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

Nawin Kumar et al.

Objectives:

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

Progress:

Fresh and parasitologically confirmed VL cases from both sexes admitted in the indoor ward were enrolled in this study. The clinical parameters used in this study were demography, duration of illness before the start of anti-leishmanial drugs. Size of spleen was measured 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, serum 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.

After administration of anti-leishmanial drugs 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 increased in few VL patients suggesting involvement of pancreas during VL infection. The entire above mentioned laboratory parameters improved during the course of therapy. It was observed that transferrin, albumin and iron which were 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.

Till now 24 fresh VL cases were included in the study, of which 18 were treated with Ampho B and 6 cases with Miltefosine. So far as assessment of predictive value of clinical and laboratory parameters on other anti-VL drug like SAG is concerned, this drug is not in regular practice for admitted VL patients of the Institute and the retrospective data does not incorporate all the parameters as per the study protocol.

Table: Laboratory parameters at different time points (N=24)

Parameters	0 Day	7 Day	14 Day	21 Day	EOT	1-month Follow up	6 month Follow up	Normal
Albumin	1.0 – 3.4	2.7 – 4.1	3.4 – 4.4	3.4 – 4.6	3.7 – 4.7	3.5 – 4.9	3.8 – 5.0	3.2 – 5.0
Amylase	59.0 – 176.0	58.0 – 189.0	80.0 – 182.0	70.0 – 261.0	63.0 – 169.0	70.0 – 150.0	68 - 120	<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	174.0 - 278	200.0 – 380.0
Iron	42.9 – 64.8	49.0– 68.2	44.9 – 69.0	48.9 – 68.0	51.2 – 70.2	60.5 – 102.0	62.8 – 132.0	65.0 – 175.0 (M) 50.0 – 170.0 (F)
Apo A1	71.0 – 131.9	88.0 – 125.1	92.0 – 128.2	98.8 – 128.9	101.2 – 138.8	110.1 – 140.6	114.2 – 150.6	122.0 – 161.0
Triglyceride	88.0 – 183.0	58.0 – 250.0	40.0 – 168.0	55.0 – 177.0	54.0 – 148.0	72.0 – 146.0	76.0 - 148.8	<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	186.8 – 286.8	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 – 10.5	7.8 – 10.6	8.9 – 10.8	9.0 – 11.6	10.0 – 11.8	11.8 – 13.6	11.9 – 13.8	10.0 – 14.0
TC	1650 – 9980	4260 – 8900	4800 – 10500	5200 – 9600	6300 – 10960	6150 – 10980	6450 - 11200	4000 – 11000
Platelet	76000 – 180000	78000 – 210000	120000 – 250000	200000 – 260000	215000 – 278000	216000 – 292000	238000 – 324000	150000 – 350000

10. Susceptibility to Visceral leishmaniasis in human beings – the role of testosterone.

K. Pandey et al.

Objectives:

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

Progress:

In this study, we examined the influence of testosterone in *L. donovani* infection in altogether 28 clinically and parasitologically confirmed VL cases under different clinical

manifestation and response to treatment. Subsequent experiments were performed to find out the correlation of leishmania load with level of testosterone in VL cases, if any.

The data revealed that testosterone level was decreased in VL cases than normal cases who were asymptomatic (n=2) or recently diagnosed as full blown VL cases (n=2). On the contrary, testosterone level was observed to be 6.75 ng/ml i.e. within the normal range of 1.8 – 9 ng/ml in successfully cured VL cases. In addition, severity of VL infection was observed to play an important role in androgen expression. This was observed through findings that VL cases with 1+ parasitic load had low testosterone level whereas in cases with high parasitic load (2+), low testosterone level than normal range of 1.8 – 9 ng/ml. In patients with 5+ parasitic load, there was further decrease in the testosterone level in 100% VL cases.

Table 1:

Type of Cases	**No.**	**UD**	**Level**	**Remarks**
Asymptomatic (rk39 +ve)	2	0.5	1.55	Abnormal
Fresh VL cases	2	1.498	<0	Abnormal
Cured VL cases	3	0.616	6.75	Normal
Resistant VL cases	1	0.336	4.2	Normal

Table 2: Parasitic load vs Testosterone level

Parasitic Load	**No**	**Testosterone level**	
		Normal	**Less**
1+	3	2	1
2+	7	2	5
3+	6	5	1
4+	3	2	1
5+	1	0	1

Basic aspect

11. Crucial role of plant's extract in propagation of *Leishmania donovani* promastigotes.

A.K. Gupta et al.

Objectives:

To explore the possibilities of some plants' extract

1. as a source for replacement of FCS/serum/ blood/ blood products in routine culture of *L. donovani* promastigotes.
2. as a source of antileishmanial compound, if show lethal effect.

Progress:

Propagative effect: After experimental confirmation of possible application of few plants' extract as replacement of FBS, animal products and peptone in long-term in-vitro propagation, cryo-preservation as well as cloning of leishmania parasite, the study was further extended to know whether a) extract obtained from whole dry leaves powder, or b) dried plants' extract or c) dried medium after admixing all the ingredients (except $CaCl_2$) at a time in plants' extract retain their proliferation-promoting activity sufficient to replace FCS/blood as a supplement or not.

Whole dry leaves powder of 3 plants and dried plants' extract of 3 plants were prepared and supplemented with different concentration (wt. / vol.) of solid mass to check the retention of fertility property in medium during long term continuous successive sub-passaging. In other sets of experiments, solid mass of dried medium after admixing all the ingredients (except $CaCl_2$) was dissolved in appropriate amount of D.W. and added to plants' extract for sub passaging. Animal product free culture medium (LGPY) was used as a basal medium. The medium with heat inactivated FCS (10%, v/v) was taken for positive control and plain medium was taken as negative control. Regular sub-passaging and observation to assess the propagation is under progress.

Anti leishmanial effect: During screening of plants' extract for propagative effect on leishmania promastigotes, 3 plants showed 100% lethal effect on promastigotes at 20% concentration in RPMI-1640, SIM and LGPY medium, even after addition of 10% FCS after

exposure of 72 hours. Out of these three plants, one was non-traditional medicinal plant which leaves are usually consumed as vegetable. This plant was taken for study.

To further explore its lethal effect, fully developed and healthy plant free from recent use of insecticides/pesticides/fertilizers, was harvested from non-polluted place during peak season and processed within 2 - 3 hours for preparation of its aqueous and hydroalcoholic extracts. The extracted material was stored at -20°C after filtration and drying. Different concentrations of extracts were tested for their efficacy against *L. donovani* promastigotes. Preliminary results showed that IC^{50} of crude aqueous extracts was 100 µg/ml. The study is in progress.

12. Association of HLA Class I and Class II Alleles in susceptibility to Visceral leishmaniasis in endemic and non endemic regions of Bihar.

D. Singh et al

Objectives:

- To investigate the genetic diversity of HLA in person having kala-azar from endemic and non endemic regions.
- To determine the HLA class I and class II allelic distribution in the patients and healthy controls.

Progress:

Peripheral blood samples were collected from clinically diagnosed and rK39 positive VL patients (n=5) as well as their healthy contacts (n=5) from endemic region of Bihar. Five unrelated healthy control samples each from endemic and non endemic region were also collected, which had neither previous history of VL in their households nor were they clinically suspected for VL or rK39 positives.

All blood samples were subjected to DNA isolation using Qiagen blood mini kit. PCR was performed using primers of Class I (HLA-A, HLA-B, HLA-C) and Class II (HLA-DRB1, HLA-DQB1, HLA-DPB1) locus. PCR products were analyzed by electrophoresis on agarose gel and were showed 2 Kbp (HLA-A); 1.2 Kbp & 1.5 Kbp (HLA-B); 1.2 Kbp (HLA-C); and 365 bp (HLA-DRB1), 400 bp (HLA-DQB1 & DPB1) band respectively. Amplicons were purified by using ExoSAP-IT and subsequently cycle sequencing reaction was performed using internal primers provided with Abbott, HLA kit. Sequencing was performed

in ABI 3130xL Genetic analyzer and results were analyzed by HLA analysis software (Conexio Analyzer).

The interim result indicates possible association of HLA-A *02010101 and HLA-DRB1 *150201 alleles with protection against visceral leishmaniasis and HLA-A *020601 & *24020101 and HLA-DRB1 *150101 alleles with susceptibility to visceral leishmaniasis.

Further work to include more number of samples is under progress to know the actual frequency of the disease with respect to community.

13. Studies on some nutritional factors in severity of Visceral Leishmaniasis (VL).

C.S. Lal et al.

Objectives:

- To identify and assess the nutritional markers/factors in the malnourished VL patients
- To evaluate the correlation between malnutrition factors and VL
- To assess the nutritional factors predisposing to severity in VL

Progress:

We examined altogether 66 individuals in this year, out of which 44 were malnourished VL patients and 22 healthy people not exposed to leishmania parasite. The nutritional markers studied were albumin, total protein, zinc, copper, iron, total iron binding capacity, transferrin calcium, and magnesium.

Till now, we have identified some nutritional factors i.e.; cholesterol, triglyceride, albumin, total protein, copper and zinc during VL infection and its association with the BMI index in VL infection. The trend of down regulation of zinc was also observed as the severity of malnourishment increases. Albumin was down regulated as the BMI index decreases. The results of calcium did not showed any difference in both the groups (malnourished VL and Control VL). Magnesium was observed to be increased only in severely malnourished VL (BMI<15) while Mg was normal in other malnourished VL as to the healthy individuals. Total protein showed significant lower trend as the BMI index decreases. The detail results are shown in Table 1.These nutritional markers might be playing its role in the severity of the disease also. A correlation with the parasitic load was also studied with few nutritional

markers which is playing their role in VL infection (Table 2).The other nutritional factors did not showed any significant results.

During the investigation, quality control sera were also analyzed. The external quality control measures were also taken into account during the experiment.

Table 1: Parameters evaluated in malnourished and control VL

Category →	VL Control	VL (Malnourished)		
BMI→ Parameters	>20	Mild 18 – 20	Moderate 15 – 18	Severe 10 - 15
Cholesterol (mg/dl)	110.6	56.3	58.74	49.49
Triglyceride (mg/dl)	142.9	90.35	142.16	212.37
High density lipoprotein	23.0	25.4	23.0	22.0
Low density lipoprotein	63.0	56.0	48.4	34.0
Albumin (g/dl)	2.8	2.3	2.3	1.9
Apolipoprotein A1	65.0	59.0	59.0	58.5
Apolipoprotein B	70.0	67.0	72.1	66.7
Zinc (μg/dl)	89.0	72.0	62.8	60.2
Copper (μg/dl)	102.0	90.8	68.4	56.1
Magnesium (mg/dl)	2.4	3.6	3.3	2.8

Table 2: Correlation of significant nutritional markers with parasite load

Parameters	Healthy Control	Parasitic Load in VL cases				
		1+	2+	3+	4+	5+
Cholesterol (mg/dl)	186.4	83.1	93.06	74.1	44.26	46.75
Triglyceride (mg/dl)	166.4	119.4	133.05	215.06	210.65	260.66

14. Biochemical analysis of reticulo-endothelial system and its correlation with the peripheral venous blood in the establishment of visceral leishmania infection.

C.S. Lal et al.

Objectives:

- To identify the biochemical molecules responsible for the growth of the parasite within the RES.
- To assess the biochemical variables in both RES and venous serum.
- To target certain biochemical constituents with view of parasetemia.
- To explore immunological markers in the establishment of infection within RES

Progress:

The mass cultures of promastigotes were prepared to the strength of $1x10^7$ and it was inoculated into 10 Balb/C mice to prepare the animal model. After the lapse of about two months, the infected mice were processed for taking the blood as well as its reticulo-endothelial system (RES) tissues. Till date, 5 mice were sacrificed for this study. The samples (whole blood and RES tissues) were processed for biochemical investigations. The RES tissues were kept in normal saline and homogenized. A panel of ten (10) biochemical variables urea, creatinine, cholesterol, triglyceride, total protein, albumin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and glucose were studied by dilution procedures on the chemistry analyzer. The observed values of different parameters are as below:

Table: Biochemical variables in serum and RES tissues of Mice

Parameters	Mice Serum		Mice RES Tissue (Spleen)		Mice RES Tissue (Liver)	
	N	I	N	I	N	I
Total Cholesterol	64.4	50.4	2.4	4.4	14.4	10.8
Triglyceride	66 .0	97.0	8.4	18.6	127	75.2
Total Protein	5.5	5.3	0.2	0.2	1.8	0.9
Albumin	2.9	2.6	0.1	0.1	1.0	0.6
Glucose	117.8	106.4	1.8	3.8	244.2	220
Urea	69.0	50.0	5.8	5.4	36.6	24.2
Creatinine	0.4	0.4	0.06	0.05	0.08	0.10
ALAT	65.0	81.3	144.5	40.4	10112.9	9423.0
ASAT	206.8	296.1	360.9	267.8	9738.2	13989.5
Alk. Phosp.	106.0	124.6	185.8	19.2	49.6	53.6

Note: The results are shown as mean value; N: Normal mice; I: Infected mice

15. Identification of Anemia as pathogenic factor in Visceral Leishmaniasis.

S. Narayan et al.

Objectives:

Primary objective

- To understand the role of *Leishmania* parasite in causing anemia in visceral leishmaniasis.

Secondary objectives

- To estimate the erythrocyte number, haemoglobin levels in primed and non-primed blood samples.
- To estimate Heme and Non-Heme Iron concentration in parasitic exposed and non-exposed blood samples.
- To estimate attached haemoglobin in the flagellar pocket of parasite.

Progress:

Intracellular amastigotes of *Leishmania donovani* were obtained from spleen aspiration of Kala-azar patients and promastigotes were initially grown in modified Tobie's medium. Mass culture of promastigotes was done in cell free monophasic Schneider's insect tissue culture medium supplemented with 10% FCS. The parasite of 6^{th} and 7^{th} generation, having maximum infective capacity and high tolerance (Resistant 0.44µg/10^7 parasites/ml) and sensitive (0.34µg/10^7 parasite/ml) to Amphotericin B as calculated by HPLC, were procured for all experimental protocol. The peritoneal macrophages of murine derived amastigotes were also obtained.

The clinical blood samples of parasitological confirmed Kala-azar positive cases (n= 6) were subjected for experimentation. The blood sample of the patients having hemaoglobin < 5.0g/dl, complications such as pneumonia, jaundice, malaria, tuberculosis, renal or cardiac disease, diabetes or HIV infection; serum concentration of aspertate and/or alanine aminotransferane ≥ 3 times the upper limit of normal, serum concentration of creatinine more than 1.5 times of upper limit of normal, or had already received any anti-leishmanial drug, were excluded.

The erythrocytes (1x10^6/ml) from the samples were separated on Percoll at 1000 rpm for 10 minutes and washed three times with phosphate buffer saline (PBS) with 1% glucose. Erythrocytes were incubated with equal volume of parasite at 25° C and with equal volume of assay buffer for required period of time. After incubation the analysis of RBC, MCH HCT and Hb were done through cell counter (PoCH-100i sysmex, Transasia) in both the erythrocyte culture with and without parasite and compared.

Heme and non-heme iron content in the unprimed and *L. donovani* primed erythrocytes of VL patient (n= 6) were estimated. Erythrocyte samples with parasite and without parasite were incubated and cultured on 24±1°C for 72 hours in the sample groups of erythrocytes, the rest existing erythrocyte were lysed by adding lysing buffer. The heme

content was estimated spectrophotometrically by reading the absorbance at 398nm. For the estimation of non-heme iron content in both the samples, ferrozine reagent was added and estimated by reading the absorbance at 560 nm. The comparative study between the samples of erythrocytes with parasites and without parasites was also carried out to estimate the consumption of heme by the used parasites. The experiments have been standardized and the study is in progress.

16. Study of Haemoglobinopathies in anaemia of kala-azar cases from Bihar.

N. Verma et al.

Objectives:

- To screen the anaemic cases of kala-azar and others for presence of any disorder of haemoglobin, i.e, β- thalassemia, sickle cell anaemia (HbS) etc.
- To correlate the findings with Hb%, RBC morphology, MCV, MCH, MCHC and reticulocyte count.
- To screen the cases and identify carriers of β-thalassemia and other abnormal hemoglobin for genetic counseling.

Progress:

Blood samples (about 3 ml) from 75 kala-azar patients and 36 other cases from RMRI OPD were collected in EDTA vials and the blood smears were prepared. Properly mixed blood samples were tested in Automatic Blood Cell Counter for complete blood count (CBC). Haemoglobin %, Total RBC, WBC and Platelet counts, differential counts of WBC, PCV, MCH, MCHC MCV were recorded. Stained blood smears were examined microscopically for presence of any morphological changes in RBC i.e. microcytes, macrocytic, hypochromic, hyperchromic or normochromic, target cells, any basophilic stippling, immature RBC or any other abnormality.

Blood samples of both anaemic or nonanaemic cases of kala azar and other cases were run in the 'Variant' machine to detect any abnormal haemoglobin. Percentage of Hb., HbA, HbA_2 and HbF have ben presented in the table with other haematological findings (i.e. Total WBC, RBC, MCV, MCH, MCHC and hematocrit (PCV). Study is in progress.

Table: Value of Hb., HbF, HbA0 & HbA2 with hematological findings in kala azar and other cases. (mean, S.D., range)

Type	Age	HB%	WBC	RBC	MCV	MCH	MCHC	PCV	HBF	HBA0	HBA2
KA Cases	24..4 (5-60)	9.7± 2.50 (4.7-13.1)	5100± 2406 (1000-10300	3.44± 0.84 (1.72-5.27)	70.2 ±9.4 (54-91.5)	28.6± 3.89 (22.5-37.7)	40.3 ± 3.37 (24.5-44.3)	24.3± 6.79 (11.2-40.2)	0.28± 0.37 (0.0-1.4)	87.68± 5.06 (**60.9**-91.3)	3.61± 4.8 (1.1-**29.8)**
Non-KA Control	32.4 (7-59)	11.9± 2.43 (5.6-16.1)	8485± 3151 (2300-16800)	4.05± 0.73 1.64-5.73)	76.5± 10.62 44.2-92.8)	29.9± 3.94 (15.8-36.2)	39.2± 1.79 (35.7-41.6)	30.6± 6.2 (14.4-42.7)	0.12± 0.44 (0.0-2.2)	86.3± 2.16 (82.2-89.4)	3.31± 1.55 (1.4-**6.6**)

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

N. Verma et al.

Objectives:

- 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

Specific Objectives:

- 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-γ, IL-4 and IL-10) in PKDL cases and compare it with VL and control subjects.

Progress:

T-cell subsets and cytokine profile has been studied in 26 PKDL cases and 8 healthy controls. Study conducted on peripheral blood mononuclear cells of PKDL cases reflect that the absolute number of circulating CD4+ and CD8+ T cell subpopulation and surface expression during active infection in PKDL cases was reduced (mean value 655/µl and 499/µl respectively) in compare to those of healthy controls (mean value of CD4+ cells

1064/µl and CD8+ cell 699/µl). The involvement of the CD4 & CD8 T cells in the pathology of PKDL lesions will be studied in the dermal lesions. Lower expression of cytokines IFN-γ (> 1-fold) and IL-4 in CD4 T-cells of peripheral blood from PKDL cases as compared to control indicates immunosuppression in PKDL cases. The study is in progress to find out whether the PKDL Leishmania parasite is causing T-cell anergy or it is due to localized infection.

Table: CD-4, CD-8 cell subpopulation and Cytokine profile of PKDL cases

	CD4 cells/µl	**CD8 cells/µl**	**CD4 : CD8**	**IFN-γ US(%)**	**IL-4 US(%)**
PKDL Cases (21)	655 ± 257.5	499 ± 208	1.35 : 1	1.56 ± 1.04	3.03 ± 1.59
Controls (6)	1099.5 ± 429.05	652.8 ± 287.57	1.6 : 1	3.5 ± 2.3	4.6 ± 3.6

18. Innate Immunity function in Visceral Leishmaniasis and under malnutrition.

Vikash Kumar et al.

Objectives:

Primary objective

- to define the role of innate immune response in non malnourished VL and malnourished VL patients.

Secondary objectives

- To study the expression of surface molecules of monocytes, neutrophils and NK cells.
- To study phagocytotic activity and respiratory burst activity of monocytes and neutrophils.
- To study the production and secretion of chemokines pro and anti- inflammatory cytokines of monocytes, neutrophils and NK cells.

Progress:

The surface expression molecules viz CD62L and CD11b, respiratory burst, phagocytosis activity, chemokine and cytokine secretion was determined by whole blood techniques in malnourished, non-malnourished VL patients after stimulation with SLA, LPS, and K-562 myloma

cells. In un-stimulated innate cells of VL patients, CD62L expression was observed very low (479 mean values) compared to control (1253 mean value).The Mean fluorescence intensity (MFI) for CD11b before and after LPS treated was lower in VL patients (18865, 23619) than control (32473, 58704)). The ROS production and phagocytosis activity (as means result obtained) in the malnourished VL patients did not exhibit significant change after stimuli with LPS and SLA. There was less secretion of pro and anti inflammatory cytokine viz IFN-γ, IL-10, IL-8, produced by innate immune cells after stimulation with LPS, SLA and K-562 myloma of malnourished VL patients, as compared to control. The significant change of CD107a (lamp-1) expression was observed on activated NK cells in patients compared to control. However, the degree of activated NK cells activity after stimulation with K-562 myloma cells in control CD107a (lamp-1) expression on CD56+ CD107a+ NK cells was 23. Activated NK cells CD56+ CD107a+ 5 in VL patients after stimulation with K-562 myloma Cells. The results observed so far indicates that malnutrition status has impact on activation of PMN, monocytes and NK Cells.

19. GPI-anchored membrane proteins of *Leishmania donovani* mediated regulation of Toll-Like Receptors and costimulatory molecules on antigen presenting cells and induction of cytokines.

S. Das et al

Objectives:

- To isolate and characterize GPI-anchored membrane proteins from promastigotes of *L. donovani* .
- To study the cell proliferation and surface expression of Toll-like receptors (TLRs) in GPI anchored proteins stimulated human PBMC derived antigen presenting cells.
- To study the role of GPI anchored proteins in production of pro-inflammatory cytokines of the antigen presenting cells.
- To study the translocation of NF-kB from cytoplasm to nucleus of the APCs.
- To determine the capacity of GPI anchored proteins to induce maturation of DC by up-regulating the expression of co-stimulatory molecules.

Progress:

The GPI-anchored proteins were isolated from promastigote cultures of *Leishmania donovani* by using Triton-X 114 phase separation technique as described by Ko and Thompson. SDS PAGE analysis of both the phases showed maximum membrane bound

hydrophobic proteins in detergent phase. Some high molecular proteins were noticed near the junction of stacking and resolving gel. To characterize the GPI-anchored proteins, the proteins in the detergent phase were treated with PI-PLC. The GPI-anchored proteins became hydrophilic after the PI-PLC treatment and found in the aqueous phase as PI-PLC treatment cleaves off the lipid anchor of the proteins. Role of GPI anchored proteins in proliferative response were studied by cell cycle analysis. It was observed that the GPI-anchored proteins were able to proliferate cells as because 4n stages were noticed.

Role of GPI-anchored proteins in expression of Toll like receptors was studied in human mononuclear cells. Peripheral blood mononuclear cells (PBMCs) isolated from venous blood of healthy and VL patients were stimulated with known ligands of Toll-like receptors (like LPS), SLA(soluble leishmaial antigen) and GPI-anchored proteins to study the expression of TLR2 and TLR4. In healthy subjects, the GPI-anchored proteins were able to upregulate TLR2 and TLR4 from their basal expression levels. The GPI-anchored proteins when used to stimulate the PBMCs of VL patients, demonstrated upregulation of both TLR2 and TLR4 present on neutrophils, macrophages and lymphocytes. This suggests the involvement of GPI-anchored proteins of the parasite in modulating the innate immune response of the host via the TLR signalling pathway. The action of the GPI-anchored proteins was more prominent on the macrophages in respect to the up regulation of TLR expression.

The production of proinflammatory cytokines in response to GPI-anchored proteins was studied in healthy and VL patients. The GPI- anchored proteins up regulated the production of TNF-α and IL-1β. The study of production of other proinflammatory cytokines is in progress.

20. Studies on immunological changes in lymphocytes after leishmania infection.

R. Banerjee et al.

Objectives:

The overall objectives of the proposal are to study the fate of the anergic lymphocytes in infected hamster and also in patients and especially in different cell population in presence / absence of various molecules.

- To find out the mode of cell death and the pathway in the leishmania infected animal model.

- To observe the anergic lymphocyte population in patients during treatment.
- To identify the responsible cell population for the lymphocyte death.
- To identify the specific molecule for the lymphocyte death.
- To study different cell surface marker in the patients and its changes during the treatment.

Progress:

The disease progression leads to gradual impairment of lymphocyte proliferation which can not be restored even in the presence of concanavaline A. Fate of the anergic lymphocytes is intrinsic apoptosis as evident by depolarization of mitochondrial membrane potential, cytosolic release of cytochrome C, caspase activation and DNA fragmentation. More interestingly, TGF-β, secreted by macrophage like adherent cells, was significantly upregulated in lymph node compartment of infected hamsters. Addition of neutralizing TGF-β and recombinant TGF-β showed down-regulation and induction of lymphocyte apoptosis respectively. Furthermore, it has been observed that TGF-β triggers apoptotic death of lymphocytes through up-regulation of tyrosine phosphatase activity, as evident by ELISA and the use of sodium orthovanadate (NaOVa, a tyrosine phosphatase inhibitor) reduces the apoptotic frequency. TGF-β not only induces PTPase but also serine/threonine phosphatase activity, as evident by PP2a activity measurement by ELISA. TGF beta follows smad independent pathway and thus induces PP2a by phosphorylating TAK1 molecule.

21. An analysis of the *Leishmania donovani* parasite and part played by its antigen on immunological imbalances during visceral leishmaniasis.

S. Bimal et al.

Objectives:

- To identify *Leishmania* antigens at different stages of development and their influence on Th-1 and Th-2 effectors.
- To purify the individual components of antigens of *Leishmania* from different developmental stage involved with sudden overexpressed Th-2 effector cells.
- To study the signal transduction machinery utilized by the antigens with Th-2 effector cells involved.

- To evaluate strategy to replace Th-2 signal transduction machinery by a Th-1 signal transduction shift.

Progress:

KMP-11 gene, cloned in pQE-30 plasmid, was purified by Nickel chelate affinity chromatography and its presence was ascertained through immunoblotting with mAb specific to KMP-11 protein. The *infected* macrophages from VL patients (n=5) after priming with KMP-11 antigen of *Leishmania donovani* were co-cultured in the presence of uninfected CD4 cells of healthy control subjects. The T-cell recognition and function ability as well as alteration in the oxidative burst release by infected macrophage of VL patients was taken as a parameter to show the significance of the KMP-11 molecule *in vitro* in protection from VL. Role of KMP-11 antigen was first explored in the inductive phase of T-cell activation by cell cycle analysis after stimulation with 50 µg/ml of KMP-11 antigen or 10 µg/ml of SLA at 2 hrs and 16hrs. The mean percentage in s-phase of healthy T-cells induced by KMP-11 was found increased during 16hrs incubation. The primed T-lymphocytes responded to KMP-11 in vitro, resulting in the release of IFN-gamma but not IL-4 in healthy donors *together* with the induction of super-oxide radicals in macrophages of the patients. This suggests that the KMP-11 molecule can be examined further as vaccine candidate for Kala-azar.

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

S. Bimal et al.

Objectives:

- To understand the CD2 deficiency in Kala-azar 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:

BALB/c mice in 5 cohort (5 in each cohort) were infected by injecting 0.1 per animal of $3x10^7$ stationary phase promastigotes of either sensitive (Ld strain 143) or resistant (Ld strain 153) via the intra-cardiac route. Mice infected for 55days were used for experiments. One group was left untreated. One group of infected mice did not receive any treatment. Animals in remaining 3 cohorts were treated differentially (SAG, CAG+CD2, CD2) day 10,

day22 and day 34 post establishment of infection. Animals in batches were sacrificed day 10, 22 and 34 for measurement of weight spleen size, anti-leishmanial antibodies titre, T cell function (IFN-y) and anti-leishmanial macrophage function (TNF-α, oxidative burst measurement and nitric oxide production).

Mice treated with SAG along with CD2, irrespective of type of parasite, used to establish infection (Ld. S and Ld. R) induced T-cell to produce IFN-γ. The effect of immunochemotherapy on microphage function on mice infected with sensitive and resistant strain was not same for all parameters. Neither SAG therapy nor a treatment of SAG with CD2 had any positive impact on release of nitrite till day 36 by macrophage infected with either sensitive/ resistant strain of *L. donovani*. The analysis of super-oxide generation by differentially treated macrophages in response to sensitive and resistant strain of *Leishmania* parasite revealed that macrophages of non-infected mice showed an intrinsic super-oxide generation. This ability was lower when infected with resistant parasite compared to sensitive strain. The super oxide generation after SAG treatment with CD2 began enhancing very early on day 24 post infection during which SAG action was at minimum in mice infected with sensitive strain. The ROS generation ability was found high with SAG/SAG+CD2 on day 36. Despite observing a lower range, the O_2 generation ability was not found to be inhibited.

After either SAG/SAG+CD2 therapy in macrophage infected with resistant strain of the parasite. Similarly, level of TNF-α was higher after combination therapy than SAG therapy in mice infected with sensitive parasite strain. Addition of CD2 in injection of SAG, however, failed to induce more TNF-α when mice were infected with resistant strain. Along with these findings, we observed a regression in spleen mass from 3.97gm on day 24 to 1.07gm in combination therapy group on day 48 after initiation of infection. Addition of CD2 in SAG regulates outcome and response to treatment of Kala-azar, which may be caused either by sensitive/resistant strain by promoting IFN-γ, TNF-α and oxidative burst production but not nitric oxide.

23. *Leishmania donovani* antigen and their influence on Natural T-regulatory Cells in immuno-suppressed VL patients.

S. K. Singh et al.

Objectives:

1. To explore the possible existence of Natural T-regulatory cells in human VL subjects.
2. To identify the possible correlation if any between *Leishmania* parasite and increased accumulation and proliferation of Natural T-regulatory cells.
3. To investigate the possibility of inactivation of *Leishmania* antigen to prevent accumulation of NTRs and hence to activate immune system to control leishmaniasis.

Progress:

Presence of natural T-regulatory cells were examined in peripheral blood and bone marrow of VL cases during different stages of treatment by Flow cytometry using florescence labeled monoclonal antibodies to CD4, CD25, Foxp3 and CD127. An up-regulated expression of natural and adaptive T regulatory cells was observed in VL patients before start of treatment. A marked difference was observed in untreated and treated VL patients. Further, samples with high frequency of natural T regulatory cells showed increased expression of IL-10 and TGF-β. An increasing trend in natural T-regulatory cells was also observed in response to *in vitro* stimulation with *Leishmania*. Natural T-regulatory cells were found significantly high in mice infected with *Leishmania* having major population of phosphotidyl serine positive cells. It indicates positive correlation between pathogenicity of *Leishmania and* expression of natural T regulatory cells. Similar finding was observed *in vitro* experiments on peripheral blood of human VL subject. In adaptive transfer experiments, natural CD4 T-regulatory cells from VL subject were incubated with macrophage from healthy subject and natural CD4 T-regulatory cells from healthy subject were incubated with macrophage from VL subject in presence or absence of *Leishmania* parasite. It was observed that the natural CD4 T-regulatory cells proliferated on presence of *Leishmania.* The mechanism by which natural CD4 T-regulatory cells proliferate in response to antigen is in progress.

24. Protective efficacy of purified membrane antigens (Phospholipids vs Lipophosphoproteins) isolated from *Leishmania donovani* metacyclic promastigotes.

S. Bimal et al

Objectives:

- Detection of protein antigen with glycophospholipids anchors in *L. donovani* promastigotes.
- Biochemical characterization of antigen proteins lipids and phosphate.
- Assessment of protective immunity

Progress:

Immunoprotection of phospho-protein of L. donovani*:*

Mice of 4 different experimental group formulations are monitored for obtaining survival rate in %. No death is reported till date in any of the experimental groups.

Anti-leishmania antibody response at different time points after immunization:

The group immunized with soluble leishmania antigen and phosphoprotein with 22 KDa protein induced a high antibody response in all 5 (100%) mice on day 7 compared to their counterparts immunized with phospho-proteins where only 2 of 5 mice were observed with antibody titre.

Cytokine response to SLA or phosphoprotein antigen:

Increased Th-2 cells associated function in phosphoprotein immunized hosts was later observed with raised IL-10 (6.4 fold) and IL-4 (about 2.7fold) production in correspondence to about 8.57 fold more production of IFN-γ by T-cells compared to values obtained in infected unimmunised host. More enhanced values for IL-10 (24.1fold) and IL-4 (11.70 fold) were observed in lymph node. When 22 kDa protein antigen was added in the vaccine construct of phosphoprotein, notably IFN-γ was produced after 22kda addition to phosphoprotein but it was only 3.8%. Th-2 cells function was observed still promoted greatly in spleen by phosphoprotein but in spleen, IL-10 and IL-4 increased up to 1.8 fold and 2.0 fold but at this stage, IFN-γ release was observed almost undetectable. An almost similar result was observed when phosphor-protein was given along with 22kDa antigen in spleen.

It appeared that phosphoproteins studied were more related to increased pathogenesis of VL with little impact on the protective immune response.

Table: % of IFN-γ, IL-4 and IL-10 produced *ex-vivo* by lymph node cells and splenocytes from *L. donovani* infected mice before and after immunization.

	% of cytokine produced ex-vivo by lymph node cells				% of cytokine produced ex-vivo by splenocytes			
Cytokine	**G-1 (Inf)**	**G-2 (SLA)**	**G-3 (PP)**	**G-4 (PP+ 22KDA)**	**G-1 (Inf)**	**G-2 (SLA)**	**G-3 (PP)**	**G-4 (PP+ 22KDA)**
IL-4	0.8	2.70	21.60	8.2	9.4	11.40	21.6	28.35
IL-10	0.34	0.70	2.18	8.2	1.52	0.67	2.76	1.6
IFN-γ	1.00	0.60	8.57	2.2	0.6	6.90	0.6	4.5

Inf: Infected, PP: Phosphoprotein

25. Studies on genomic diversity in *Leishmania donovani* using genomic DNA microarray.

Abhik Sen et al.

Objectives:

Primary objective

- To work on the analysis of the genome wide diversity of the causative agent, *Leishmania donovani*, with a view to understand the genetic basis of the phenomenon such as drug resistance and infectivity that are of importance in the management of the disease.

Secondary objectives

- To construct the shot gun genomic DNA library of *L. donovani*
- To sequence the gene(s) that are differentially expressed and responsible for drug resistance and pathogenicity in leishmaniasis
- To study the functions of the important genes involved in drug resistance and pathogenesis

Progress:

Semiquantitative PCR.

The differential expression of amastin, calpain like cysteine protease and 20 S proteasome β subunit analyzed by semi-quantitative RT-PCR showed 4.2 fold, 5.1 fold and 4.1 fold up-regulation respectively in lesion derived amastigotes compared to promastigotes confirming, the microarray data. In control experiments, the parasites were induced with charcoal and no induction of amastin was found.

Studies on 20S Proteasome beta subunit protein.

The 20 S *Proteasome beta 6-subunit fusion protein* was found to be 31kDa size, which included 27.9kDa of the native protein and 3kDa was due to the V5 epitope and 6-His residues. The purified recombinant protein was used to raise antibody in BALB/C mice and the hyper immune sera has been used to study the immunenoreactivity with the whole cell lysate of *L. donovani* by western blot. The anti Proteasome antibody highlighted 8 immunoreactive bands against *L. donovani* whole cell lysate ranging between 20–35kDa.

The protein was found to be localized in the cytoplasm of both promastigotes and amastigotes forms of *L. donovani* visualized under confocal microscope but in amastigotes, the abundance was more. In characterization, the recombinant purified 20 S *Proteasome beta 6-subunit fusion protein* showed protease activity in the gelatin containing zymography gel. The activity of the protein was found maximum at pH 7.2.

It was also observed that lactacystin inhibited cell division in a dose-dependent manner. Lactacystin markedly interfered with parasite survival inside the host cell over a period of 96 h, when the percentage of infected macrophages decreased from around 65–8% with the lactacystin-treated parasites.

Studies on Amastin protein

To characterize the Amastin like protein, which is predicted to be a surface glycoprotein, the gene was amplified and cloned for recombinant protein expressing in pBAD-TOPO® expression vector as described earlier. The recombinant fusion protein was found to be of 27kDa. The anti-amastin antibody raised against the purified recombinant protein was used for immunoblotting against the whole cell lysate of both promastigote and amastigotes. In the immnunoblot the antibody highlighted immunoreactive bands at 23 kDa, 27kDa and 30 kDa region. The different bands may be due to the presence of large number of homologues amastin gene families.

In the amastigotes, the protein abundance was higher than promastigotes. During immunolocalization studies, the protein was found localized on the surface of the amastigotes while, in promastigotes the presence of the protein was found negligible. The recombinant Amastin protein induced both TH1 and TH2 type of cytokine expression in CD4+ population. The range of Amastin concentration used was 2-20 µg/ml. The percentage of IFN-γ secreting cell remains nearly constant throughout the range, while the IL-10 positive cell achieved the maximum value at 5 and 10 µg/ml and sharply got reduced at 20 µg/ml. At 5 and 10 µg/ml concentration both type cytokine positive cells are comparable but at 20 µg/ml the IFN-g producing cell number is significantly high than IL-10.

Studies related to identification of genes responsible for drug resistance and their functional characterization is under progress.

26. Mechanism of Amphotericin B Resistance in *Leishmania donovani* parasites

N. Nandi et al.

Aims and Objectives:

The overall aim of the proposed project is to study the resistant mechanism conferred by the drug resistant *Leishmania* parasites against Amphotericin B and probable difference in the resistant and sensitive strains in sterol composition and content, drug efflux mechanisms and ROS mediated cell signaling pathways in conferring resistance. This study will help in identifying the key factors associated in mediating drug resistance which may eventually lead towards the finding of newer targets for future rational chemotherapeutic drug designing.

Specific Objectives:

- To select AmB resistant and sensitive parasites.
- To analyse the difference in the content and composition of the free sterols and fatty acids in resistant and sensitive strains
- To study the uptake of AmB in sensitive and resistant Parasites with time
- To study the efflux of AmB from sensitive and resistant Parasites with time and probable involvement of ABC Transporters in drug efflux if any
- To study the difference in thiol levels in drug sensitive and resistant parasites

- To study the difference in the expression level of Trypanothione dependent peroxidase, Na^+-K^+ ATPase, Trypanothione reductase, γ-glutamyl cysteine synthetase and Ornithine Decarboxylase in the resistant and sensitive parasites with time
- To study the generation of ROS in both drug resistant and sensitive parasite with time and to find out the possible difference in ROS mediated pathway in both resistant and sensitive parasite if any

Progress:

Selection of Amphotericin B Resistant and Sensitive Strain and in vitro and ex vivo Drug Sensitivity Assay:

To select true AmB (AmphotericinB) resistant and sensitive strain of *Leishmania donovani* and to determine their LD_{50} (Lethal dose 50), clinical isolates were obtained from AmB responder and non responder VL patients treated in the indoor ward facility of the Institute and their growth kinetics as well as in-vitro and ex-vivo drug sensitivity assay was performed. The LD_{50} for the resistant and sensitive promastigote is 0.375µg/ml and 0.125µg/ml and that for the axenic amastigote and intracellular amastigote is 0.750µg/ml and 0.225µg/ml respectively. It was observed that LD_{50} of AmB for the resistant parasite is almost 3 folds higher in comparison to the sensitive parasite. The drug dose, showing LD_{50} for the sensitive strain, showed only 10-20% inhibition in resistant strain at late log phase. These results confirmed the resistant and sensitive clinical isolates obtained from the clinically diagnosed VL cases.

Uptake and efflux of AmB in sensitive and resistant promastigotes.

The intracellular AmB content in the *L. donovani* sensitive strain increased with the incubation time, whereas that of the resistant cells increased slowly up to 8 h and decreased afterward. As a function of extracellular AmB concentration, uptake was linear for sensitive promastigotes whereas for resistant promastigotes, the saturation reached at 0.3 µM.

Relative expression level of ABC Transporters involved in Drug Efflux and Sequestration:

Studies on the expression level of important ABC Transporter genes in Amphotericin B resistant mechanism showed ~2 fold increase in the expression level of mdr for the resistant strain, but the expression level of pgpA was almost similar in both the strains.

Therefore, there may be a probable involvement of drug efflux rather than drug sequestration for the AmB resistance mechanism.

Accumulation of ROS in resistant and sensitive strain

The accumulation of ROS with exposure to drug was determined. It was found that ROS generated was almost 2 fold higher for the resistant strain in comparison to the sensitive.

Relative expression level of Thiol Metabolic Pathway genes involved in ROS detoxification:

Studies on the expression level of important Thiol metabolic pathway genes in Amphotericin B resistant mechanism demonstrated that the relative expression level of genes viz. odc (Ornithine decarboxylase), sps (Spermidine synthetase), trys (Trypanothione synthetase) involved in trypanothione biosynthesis were approximately same in both the strains, whereas the expression level of tr (Trypanothione reductase) was almost two fold higher in the resistant strain. However, the expression level of the genes involved in the ROS detoxification mechanism was higher in the resistant strain in comparison to the sensitive. ctp (Cytoplasmic tryparedoxin) was almost 1.8 fold upregulated whereas the pxn (peroxiredoxin) was almost 1.5 fold upregulated in the resistant strain. Detoxification of ROS was accomplished by peroxiredoxin (pxn) which derived their reducing equivalents from a cascade composed of tryparedoxin, trypanothione and trypanothione reductase with NADPH as the primary electron source. Due to presence of an upregulated ROS detoxification machinery the amount of ROS accumulated in the resistant strain was less as compared to sensitive strain.

Studies related to difference in the content and composition of the free sterols and fatty acids in resistant and sensitive strains and comparative analysis of the membrane fluidity of both the strains are under progress.

27. Biochemical and functional characterization of Iron-sulfur cluster (ISC) assembly and cellular localization of LdIscS, LdIsU proteins in *L. donovani*.

V. Ali et al.

Objectives:

- Characterization of Iron-sulfur (Fe – S) clusters assembly biosynthesis and Fe – S proteins to understand parasite survival and multi-drug resistance mechanism.

Progress:

Leishmania genome search using NCBI/TIGR/Sanger/*Leishmania* database showed the presence of ISC system including CIA and export machinery in *L. major* and *L. infantum*, suggesting the presence of complete ISC system in the *Leishmania* parasites. Homologues of IscS, IscU and Isd11 are present on chromosome 27, 35 and 15, respectively, in both *L. infantum* and *L. major*. Surprisingly, HscB and IscA homologs are found to be present in former but absent in later. We have found all the components of CIA machinery in both *L. major* and *L. infantum*. Homologues of Cfd1, Nbp35, Nar1p, and Cia1 are present on chromosome 26, 21, 5 and 10, respectively, in both *L. major* and *L. infantum*. *Leishmania* genome search shows the presence of Atm1, Erv1p and Grx homolog on chromosome 32, 15 and 33, respectively in *L. major, L. infantum and L. braziliensis*. Thus, genome wide analysis of Fe-S clusters machinery components is clearly showed the presence of complete ISC system which can produce Fe-S clusters in the mitochondria, cytoplasm, nucleus and their maturation as well as export. Since, genome sequence of *L. donovani* is not yet complete so the presence or absence of these genes in *L. donovani* can't be concluded at this stage. This suggested that *Leishmania donovani* parasite to be an interesting model for investigation of Fe-S cluster assembly pathway as such significant differences could help in understanding the pathological significance of this pathway. *Leishmania* parasites lack NIF and SUF systems those present in other organisms. NIF system is present in limited organisms especially in microaerophilic or anaerobic organisms. However, SUF system is present in archea bacteria, plants, and few parasites play a role in oxidative stress conditions and Fe-deficient conditions. The sequence of IscS (Genome id LinJ27.0650) and IscU (Genome id LinJ35.3720) of *Leishmania infantum* strain JPCM5 was selected as our working sequence

because we could not find these sequences in *L. donovani* due to incomplete *L. donovani* database.

The IscS and IscU genes were amplified using the genomic DNA isolated from the Ag83 strain of *L. donovani* using Pfu DNA polymerase. Althogh, PCR product of these genes were also amplified with other reference strains and unresponsive strains reported another place. The product was digested using restriction digestion enzymes BamH1 and SalI (IscU), & BamHI (IscS). Similarly the vectors were also digested as above mentioned restriction enzymes and both were purified and ligated using the Qiagen PCR cloning kit. We have chosed cohesive end cloning of both genes which reduced lots of method for cloning. Cohesive end cloning is much easier as compared to blund end or TA cloning methods.

The IscU is cloned in pET-22 vector possesses C-terminal histidine. The histidine tag is used to purify the over expressed protein using Nickel affinity chromatography. However, IscS gene cloned in pGEX-4T1 containing GST tag which can help to express protein in soluble form. The two different tag used in cloning is useful for future study to analyse IscS and IscU interaction.

The positive clones were transformed in the competent *E. coli* host cells and the expression of the recombinant proteins were achieved in soluble and insoluble form of IscU and IscS, respecytively. We have titrated the IPTG concentration and temperature for both of proteins. We have found that lower temperature and IPTG concentration helps to increase expression of fusion protein in soluble form. We have also purified IscU protein by batch purification using affinity chromatography showed about 80-90% purity of protein. We will improve the expression and purification of these proteins in future. Our project work is continue and might be provide fundamental knowledge of Fe-S clusters machinery in *Leishmania* parasites.

28. Study of Trypanothione metabolism and associated pathways in *Leishmania donovani*: cloning, biochemical characterization and physiological significance of trypanothione synthetase and trypanothione reductase.

V. Ali et al.

Objectives:

- Characterization of Trypanothione metabolism of *L. donovani* to understand its role in multi-drug resistance mechanism.

Progress:

The ability of *Leishmania* amastigotes to survive within the drastic environment conditions and the oxidative stress in the phagolysosomes of mammalian macrophages and resistance to drugs like antimonials (SAG) is thought to be due to the molecule (Trypanothione) which is central molecule of the oxidative stress relieving redox system in the parasites, so the detailed study of the Trypanothione system will increase our understanding of the intracellular survival of the parasites under stressed conditions and in presence of drug i.e. drug unresponsiveness. If we can block this system at a critical point then we will be able to combat the problem of drug resistance. We can also design new inhibitors targeting TSA enzyme which will be a breakthrough in removing the disease Leishmaniasis.

Our objective was to clone and characterize the gene TSA, which was found to be playing a major role in parasite survival and drug unresponsiveness. To achieve our objective, primers were designed against the *L. donovani*. TSA gene NCBI database Acc. No. AJ 430863 so that Open Reading Frame (ORF) of the gene be maintained when it is cloned in PET-15b vector.

The gene was amplified using the genomic DNA isolated from the Ag83 strain of *L. donovani* using Taq DNA polymerase and Pfu DNA polymerase. The product was digested using restriction digestion enzymes BamH1 and NdeI, similarly the vector was also digested and both were purified and ligated using the Qiagen PCR cloning kit. We have chosed cohesive end cloning of TSA gene which reduced lots of method for cloning generally used in some laboratories. Cohesive end cloning is much easier as compared to blund end or TA

cloning methods. We have got many positive clones which possess TSA gene in correct orientation as T7 promotor of expression vector.

The regulatory element in the vector is lac operon and in the presence of IPTG the protein can be over expressed for the isolation purpose, the vector also provides a Histidine tag to the recombinant protein which can be later removed using a specific enzyme acting at intervening site. The histidine tag can be used to purify the over expressed protein using Nickel affinity chromatography.

The clone was transformed in the expression vector and the expression of the protein was achived in such conditions for expression at optimum levels were optimized by varying temperature, time amd concentration of inducer. We have found that the protein was expressed in soluble and insoluble fraction at 25 °C, with 1.0 mM IPTG for 5-6 hrs. It would be better to titrate the IPTG concentration in future to get more protein in soluble form. Although, we will get sufficient amount of soluble protein for biochemical analysis in above mentioned conditions but better to confirm optimum conditions for protein expression. The purified protein can be used for the characterization purpose, for inhibition studies, enzyme activity studies, screening for ligands which can be used against the target as potential drugs and for raising the antibodies against the protein for localization studies and further purpose.

Bioinformatics

29. Function Prediction and Database Design of the Genome of *Leishmania donovani* prevalent in Bihar and adjacent regions.

G.C. Sahoo et al.

Objectives:

- *In silico* analysis of functional properties of different important proteins of *Leishmania donovani* using different algorithms e.g. SVMProt of BIDD (bioinformatics & drug design), Singapore.
- Design a database for *Leishmania* strain bank repositories which are endemic to Bihar and adjacent areas according to different cell culture strategies, on the basis of clinical symptoms in patients, drugs under trial, and functions of the protein.

Progress:

Our study from SVMProt and available *L. donovani* sequences suggests that various proteins of *L. donovani* genome possibly belong to diverse protein functions which are expected to occur in the life cycle of *L. donovani*.

Considering the biological significance of *Leishmania* protein and with the aim of providing easy access to large and growing volume of data, LEISHPROT, a web based repository database for all known *Leishmania donovani* proteins was developed to provide sequences as well as annotation information. The *Leishmania* proteins have been analyzed, organized and integrated to develop a high user friendly database and analysis system. The database can be queried comprehensively through argument such as UniprotID, Uniprot accession No., gene name, different protein name, different predicted functional family and stability data through global search parameter all achieved through same search form.

A relational database was constructed in MySQL to facilitate storage, query and visualization of annotation information. It includes: 'functional analyses, 'molecular analyses, 'cleavage sites' and '3-D structure' for proteins. The *Leishmania* protein data and related information are stored in MySQL relational database tables. The application layer between the web interface and the backend relational tables has been implemented using PHP.

In an effort to improve access to diverse *Leishmania* protein data, The LEISHPROT has been modified to include an abundance of linkage to other database like PIR, PROSITE, PUBMED etc. The database is unique, since no other such database already exists, and will be useful for both molecular biologists conducting experiments, but also for bioinformaticians that manage large amount of data, building algorithms and performing functional classification and comparative analysis of proteins.

30. Comparative molecular modeling of various important proteins of different *Leishmania* strains and ligand-protein interaction study of different anti-leishmanial drugs.

G.C. Sahoo et al.

Objectives:

- Molecular modeling of different important proteins of *Leishmania donovani* using different algorithms i.e. homology modeling, ab initio prediction and threading.

- Compare the structures of important proteins of *L. donovani* with those of other *Leishmania* strains.
- Ligand-protein interaction study of different anti-leishmanial drugs e.g. miltefosine, paromomycin.

Progress:

The structure of various proteins of diverse strains of *Leishmania* have been elucidated and compared. In some cases, sequence of the protein is not available from public domain database (NCBI); hence the corresponding sequence was obtained following culture of *Leishmania donovani*, DNA extraction, PCR of the corresponding gene and automated sequencing of the gene with specific primers (tubulin). Structural models of twelve different proteins of various *Leishmania* strains have been developed. Few proteins (e.g. sodium stibogluconate resistance protein) were fully novel protein which didn't show any matching with template protein in protein databank (PDB). Each protein has been tested for its ligand protein interaction with diverse sets of ligands (antileishmanial drugs, drugs / ligands acting on template protein, other ligands which may have interaction from literature) by computational methods using ligand protein interaction tools of discovery studio, GOLD software. Some of other proteins on which structural model has been constructed are Cathepsin B like cysteine proteases, LPG2, KMP11. Near about one thousand compounds or their analogues have been studied to know their ligand protein interactions with seven different important proteins (cysteine protease, topoisomerase II) of *Leishmania*. Anticancer drug sulforaphane has been indicated for leishmaniasis by in silico ligand protein interaction studies of a surface protein of *Leishmania* i.e. KMP11.

From structural analysis of DNA topoisomerase II protein of *L. donovani* and ligand protein interaction study of various DNA topoisomerase inhibitors and their analogs with three binding sites, few ligands i.e. Teniposide, Irinotecan, Quinacrine, Moxifloxacin, Pentamidine, Temafloxacin and Moxifloxacin have shown highest binding affinity and hydrogen bond formation with different amino acid residues of LdTOP2 in both GOLD and AutoDock programs.

A 3-D structure of P-glycoprotein and trypanothione reductase was build through homology modeling using respective templates by Modeller software. After validation of the final model through PROCHEK & VERIFY 3-D graph, it was used for the virtual high throughput screening of around 150 compounds using AutoDock4.1, GOLD2.1 and Ligand

Fit. Out of these, two compounds Conivapton and Nizatidine showed highest ligand fit score while Imatinib, Telmisarton, Dasatinib showed better fitness score(GOLD) and formation of hydrogen bonds. These compounds may act as potential drug candidates against P-glycoprotein of *Leishmania donovani.*

On the basis of template structure of maltose binding protein fusion with RACK1 from *A. thaliana* (pdb id: 3dm0), LACK protein showed β-propeller like structural folding. The ligand protein interactions of the modeled LACK protein were carried out with several anti-leishmanial drugs and some other drugs through Discovery studio2.1 (ligandfit) and GOLD4.1. From structural and docking analyses, pentamidine & posaconazole were found to have comparable docking scores and formation of H-bonds with vital amino acids of LACK protein of *L. donovani*.

The modeled protein structure of sodium stibogluconate resistance protein (SSRP) was verified using discovery studio (profiles-3D), Procheck, WHAT-IF and Swiss Model Server. Ligand protein interaction study of near about 200 hundred compounds was screened out in various programs like GOLD, AutoDock, and ligand fit tool of discovery studio. Among different ligands Indoglycerol phosphate and derivatives of Malathion have shown lowest energy conformation and having potential binding to SSGRP active site amino acids (His404, Meth405, Arg445, Glu450 and Arg550) which have shown higher number of H-bonds.

Modeling of other important proteins of *Leishmania donovani* and their interaction with various ligands are under progress.

31. Development of novel algorithm to find microsatellites in *Leishmania* genome and its database.

M.R. Dikhit et al.

Objectives:

- To develop an algorithm to find out microsatellites in *Leishmania* genome which may be used as molecular marker in different fields like genome characterization, Mapping, phylogeny and evolutionary biology.
- To develop a curated and integrated web-based relational database providing centralized access to publicly available *Leishmania* microsatellites.

Progress:

LeishMICROSATdb, a database of microsatellite repeats of *Leishmania* species, has been developed using genome sequence data available in NCBI. The genome sequences were assembled in accordance with chromosomes. *LeishMICROSATdb* contains di to hexa nucleotide repeats of three species of *Leishmania* i.e. *L. major*, *L. infantum* and *L. braziliensis.* Precise need based microsatellites data may be retrieved from this database using different input parameters like microsatellite type (simple perfect), repeat unit length (mono to hexa nucleotide), repeat number, microsatellite length and chromosomal location in the genome. Furthermore, clustering information of different microsatellites in the genome can also be retrieved. Finally, to facilitate primer designing for PCR amplification of any desired microsatellite locus, 200 bp upstream and downstream sequences are provided.

Vector Biology & Control

32. Evaluation of pathogenesis in visceral leishmaniasis, part played by *Leishmania donovani* Vs vector salivary gland homogenate (SGH).

S. Narayan et al.

Objectives:

- To evaluate *in vitro* infection and immunity in mononuclear cells stimulated by SGH primed killed parasites antigen, SGH alone and killed parasite antigen alone
- To evaluate *in vitro* infection and immunity in non-stimulated mononuclear cells and parasitized by SGH primed live parasites and live parasites alone

Progress:

The late log phase of newly isolated promastigotes of Visceral leishmaniasis (10^8/ml), maintained in Schneider's insect tissue culture medium supplemented with 20% FCS, was used for preparation of whole antigen stock. The salivary gland (20 pairs/ml) of *P. argentipes* was used for preparation of vector salivary gland homogenate (SGH) by six cycle of freezing thawing technique. The stimulation dose/10^6 mononuclear cells of host was prepared by adding *Leishmania* antigen of about 2000 killed promastigotes with the SGH of two pairs of glands.

The pooled blood of murine was drawn and layered on Ficoll-Hypaque (Pharmacia, Uppasala, Sweden) and after centrifugation, the buffy coat of mononuclear cells was separated and washed three times.

In one set (A) of experiment, harvested mononuclear cells were stimulated with prepared SGH primed killed parasites, SGH alone and killed parasites alone as test and no stimulation as control. In one subset (A1) of experiment, prior to completion of incubation, PMA (1ng/ml), Ionomycin (2μM/ml) and golgi stop (0.7μg/ml) were added to culture. The CD_4^+ cells were stained with Per cP labeled anti-CD_4 monoclonal antibodies. The intracellular cytokines IFN-γ and IL-4 of CD_4 cells were stained by PE and APC labeled anti-mouse monoclonal IFN-γ and APC antibodies respectively. Iso-type control and compensation were included in all the assays. Data were acquired by using FACS calibur and analyzed by using cell quest software (Becton Dickinson, SanJose). In other subset (A2), the stimulated mononuclear cells were challenged with infective stage of promastigotes and the infection pattern was observed.

In the another set (B), the non stimulated mononuclear cells were incubated and parasitized by live parasites primed with SGH as test and by normal live parasite as control for observation of infection pattern.

The interim observation reveals that vector salivary gland homogenate has dual mode of action as it along with killed parasites gives protection to the host and on the other hand along with live parasites as inoculums it provides infection to the host. The study is in progress.

33. Identification of sibling species of *Phlebotomies argentipes* population in Bihar.

D.S. Dinesh et al.

Objectives:

- To find out variations among population of *P. argentipes* responsible for transmission of the disease.

Progress:

To differentiate the morphotype and geographical different species of *P. argentipes* on molecular basis, DNA has been extracted using Qiagen kit from such specimens. The

different forms of *P.argentipes* were scrutinized based on the relative length of ascoid and antennal segment 4th (type-I, II & III). *P. argentipes* of two geographical regions one from plains of Bihar and hilly region of Ranchi were considered for this study. The strains from Bihar represent the endemic zone and that from Ranchi represents the non-endemic zone. The DNA was amplified using universal primer developed by Depaquit (2000). Primers from 18S were designed and being tested. The study is in progress.

34. Determination of infection of *Leishmania donovani* among *Phlebotomies argentipes* populations in different endemic areas of Bihar.

D.S. Dinesh et al.

Objectives:

- To determine the relative abundance of *P. argentipes* and infectivity rate in areas of low and high transmission zone of Kala-azar.

Progress:

Based on State Govt. data, three villages were selected namely; Harpur Singhara from Vaishali district, Rookhai from Nalanda district and Bhawanibigha from Nawada district. The village of Vaishali district was selected as active zone for Kala-azar transmission, Nalanda as continuous but very low enedemic zone and Nawada as non-endemic zone based on the available Govt. data. Sandflies were collected early in the morning with the help of aspirator and flash light from all the selected villages. *P. argentipes* flies were dissected for natural infection of *L. donovani* by microscopic examination. The contents were transferred into microcentrifuge tube and DNA was extracted using Qiagen kit. The PCR was conducted using primers of ITS region. The man hour density of sandflies and the number of reported kala-azar cases for all the three regions were also recorded. So far, about 200 sandflies were dissected and all were found negative for presence of *L. donovani* after microscopic examination. PCR result is awaited.

35. Study of host preference and behavioural changes in *Phlebotomus argentipes* in DDT sprayed and unsprayed areas of Bihar.

V. Kumar et al.

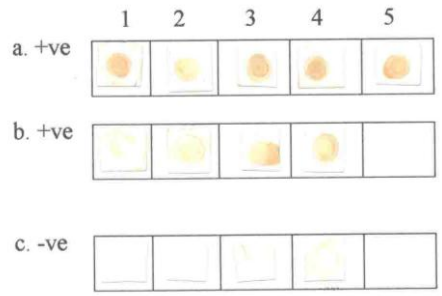
Objectives:

- To find out the effect of spraying on the behavior of sandfly.
- To find out the blood meal index in the sprayed and unsprayed areas.

Progress:

Three districts namely Vaishali, Muzaffarpur and Samastipur were selected as DDT exposed area and Gaya district was selected as DDT-non-exposed areas. To study the behavioural changes viz. endophilic and exophilic nature in response to DDT spray, indoor and outdoor collection of sandflies were made in endemic as well as non-endemic areas with the help of CDC light trap. During outdoor collection made so far, not a single fly could be collected.

To study the host preference through blood meal analysis, freshly fed *P.argentipes* were collected with the help of aspirator and the female flies were immobilized by keeping them in 4 degree centigrade. Then the flies were crushed in the filter paper and the head were removed for identification of the fly. Dot-ELISA for blood meal analysis has been standardized. Freshly fed female *P.argentipes*, 34 from DDT sprayed and 29 from non-sprayed areas were collected for blood meal analysis.



(a: +ve for Rabbit, b: +ve for Chicken, c: -ve control)

The study is in progress.

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

D.S. Dinesh et al.

Progress:

The study has been extended in collaboration of New Mexico A School of Medicine, USA.

37. Remote Sensing and GIS: Tools for the prediction of epidemic for the intervention measures.

G.S. Bhunia et al.

Objectives:

- To identify favourable areas for breeding of vector through Remote sensing and GIS
- To identify appropriate environmental health control measures
- To demonstrate how remote sensing data acquired at various scales and spectral resolutions can be used to study spatial distribution of infectious disease pattern.

Progress:

To identify the suitable breeding sites, 280 study areas (20 sites in each PHCs) were selected in 2x2 sq. km considering different environmental parameters i.e. urban, peri urban region, water logging condition (within 5 mt., 10- 15mt. and >20 mt. distance from the habitation), presence of garbage (i.e. <5mt., 5-10mt., >15mt. from the settlement), Presence of river/canal/nala (i.e. <15mt., 15-30mt., >30 mt. distance from the river), vegetation density and demographic characteristics.

Village level information about case density, pattern of disease spread, land cover variables, base map, census etc. was collected. The pre-field laboratory work has been started on digital image processing i.e. enhancement, rectification, subsetting. The vector layers were generated on district, PHCs, and village level. The work is in progress.

EXTRAMURAL STUDIES

Epidemiology

1. Implementation Strategies for Visceral Leishmaniasis Treatment in India (PHASE-II).

(Sponsor: WHO/ TDR)

P. Das et al.

Objectives:

Primary objective

- to test the effectiveness of an improved strategy for VL diagnosis and management in terms of better identification of VL cases and greater compliance with treatment.

Secondary objectives

- To test the effectiveness of an improved strategy for VL diagnosis and management in terms of better identification of VL cases and greater compliance with treatment
    - To test the effectiveness and operational feasibility of active case finding (earlier diagnosis) and community based case management of VL and PKDL
    - To determine the effect of improved case management including the use of new VL drugs (miltefosine) in improving treatment adherence and improving cure
- To analyze patient and provider costs of active and passive case detection and management
- To identify country, program and context specific factors that influence the cost-effectiveness of strategies for improved diagnosis and management of VL

Progress:

The study was conducted in two blocks of Saran district namely Parsa and Amnaur, the first one was marked as the 'intervention block (Test area)' where the new strategies were tested and another one was used as control block with the existing conventional approach for

comparison. Test area comprised of 11 villages having altogether 5776 households and the enumerated population was 33928 (M=18208; F=15720) whereas the control area comprised of 11 villages having around 33851 populations. The mean family size was 5.78 and mean age of the population was found to be 24.84 in test area. The age distribution revealed that maximum proportion (15.58%) was in the 6-10 years followed by 15.20% in 21-30 years age group.

Altogether 47 cases of current fever of more than 2 weeks duration was encountered, all were tested for rk-39 strip test. Out of 47, 19 cases were found positive and treated for Kala-azar, while in the same period 14 VL cases were found in the control area. On the other hand in total Kala-azar cases (current & past in last one year) were 111 out of which 39 were female and 72 were male. No case suspected for PKDL like lesion was found in the test as well as in the control area. The estimated annual VL incidence/ 10 000 population was 38.3 and 34.5 in test and control area respectively. Incremental increase in annual VL incidence through active surveillance in test area was found to be 1.5 per 10 000 population. On an average, one active case was detected from about 304 households.

It was observed that the major diagnostic tool for Kala-azar in practice in both test and control area was rk-39 as out of 33 cases confirmation by bone marrow aspiration was done in only one case. Place of the diagnosis in all cases was public set up (PHC/ Dist. Hospital) without any complication during the diagnosis reported by the responders. Out of 33 patients, 32 were treated with miltefosine in both test and control areas, only one was treated with sodium antimony (SAG). Till end of survey, 27 cases completed the full course of treatment (26 with miltefosine and 1 with SAG) without any major side effect, interruption or switch over to other regimen. In test area, the average days gap between onset of fever and seeking health care facility (HCF); between going to HCF to confirmation of disease; gap between confirmation and start of treatment were 3.8 days, 3.8 days and 1.3 days respectively as compared to 10.6 days, 8.5 days and 0.2 days respectively in the control village.

The estimated per patient cost of actively identified cases comes to USD 47 which includes the training and surveillance cost. A total of two public providers and 10 private providers were interviewed in respect of diagnosis and treatment of VL cases. There was no NGO found to be involved in the study area. The study is completed.

2. Towards more cost effective Visceral leishmaniasis (VL) case detection and case management in endemic districts – Implementation strategies - PHASE-III

(Sponsor: WHO/ TDR)

P. Das et al.

Objectives:

- To test alternative strategies for active case finding for VL and PKDL and compare costs, yield of cases, feasibility and other factors across varying contexts. The strategies to be tested are:
    - Camp approach
    - Focal approach (neighborhood search around house of index case)
    - Blanket approach (house to house search)
    - Incentive based approach
- To analyze in real life settings the prospects and constraints of VL management, including drug availability, compliance, options for DOT (direct observed treatment), provider's treatment skills, clinical and biochemical monitoring and identification /management of adverse and serious adverse events, early referrals mechanisms, patient satisfaction and costs.
- To develop policy recommendations for improving early VL case detection and case management.

Progress:

After completion of Phase II study, Phase III was initiated in the same study area; Parsa and Amnaur block of Saran district as intervention and control area respectively. The various case detection approaches investigated were camp, Index and Incentive approach as compared to the blanket approach as gold standard. In intervention block, a group of villages from 4 highly endemic sub-centres i.e. Maker, Jagdishpur, Pachrukhi and Bheldi were selected to cover near about 10 000 population in each sub-centre area. The approaches to be applied in intervention area were Camp, Index and Blanket whereas in the control area it is incentive and blanket only.

Camp approach:

The place for camps were selected with a view to cover entire population, some of the villages were adjoining and hence catered by the same camp and some villages were large that required camps at multiple place. Prior to camp, poster display, pamphlets distribution and audio canvassing was arranged. Altogether 19 days camps were organized to cover 20 villages where 301 individuals (51.2% male and 48.8% female) make there presence for health check up. In camp, 5 individuals having past history of VL and one having past history of PKDL came for their checkup. KA cases were found who were either treated or under treatment. A total 45 fever cases were screened through rK39, out of which 6 was found positive (13.3%) and referred to PHC. No suspected case of PKDL was seen during the camp.

Blanket approach (Household survey):

Immediately followed by the camp approach, household survey was conducted in the respective villages through the trained ASHA Health workers. A total of 47 862 population were enumerated in the intervention area [M 25864 (54%), F 21998 (46%)] from 7927 households with an average family size of 6.04. Mean age of the population was 24.7 yrs. During the screening, 127 known case of VL within last 1 yr were encountered out of which 72 (56.7%) were male and 55 (43.3%) were female. Out of 15 fever cases, found during the survey, only one was found rk39 positive and referred to PHC for treatment. Eight cases with past history of PKDL and 4 individuals having PKDL like skin lesion were reported during the survey. Out of 4 suspected PKDL cases, one was confirmed and treated at this Institute.

Index case approach:

A list of PHC reported cases of VL (n=79) from the study area during the specified period of one year was prepared and their respective household numbers were assigned through database search. These households were marked as Index households and through field visits list of neighbouring households in the perimeter of 50 meter of each index households were prepared to find out focal cases against each index household. Data matching and analysis work is under progress.

Physician Interview:

Six physicians, treating Kala-azar cases, were interviewed, out of them 4 were from public and 2 from Pvt. Sector. Mean years of work experience and VL experience was 16 and 9 yrs respectively. In last one year, the mean number of VL cases treated by them was 23.3. So far

as diagnostic tool is concerned, all were stressed for rK39, besides parsitological confirmation by 33%. All advocated for Miltefsoine, but the physician from Pvt. Sector also stressed for Amphotericin B. The main side effects of miltefosine, uttered by the teating physicians, includes diarrhea, vomiting, renal toxicity and teratogenicity. The main problem regarding home management of VL came up was irregular treatment and difficult management of AE. One respondent responded that DOTs can be effective for Home management.

After selection of study village units in the control area covering around 40 000 population, village wise health workers have been identified and trained. The incentive approach has been recently started. Work is in progress.

3. Sentinel surveillance of Visceral leishmaniasis in endemic areas of Bihar.

(Sponsor: World Bank)

P. Das et al.

Objectives

The main objectives of setting up sentinel surveillance in the endemic regions of Kala-azar in Bihar are as follows:

1. To develop a system for the generation and sharing of reliable information on kala-azar from each surveillance site established
2. To make recommendations on expansion of sentinel surveillance sites in the districts and the state.

Progress:

After several rounds of discussions, the protocol has been finalized. Its approval and funding is awaited.

4. Pharmaco-vigilance and therapeutic effectiveness of Miltefosine for the treatment of Kala-azar in endemic areas of Bihar.

(Sponsor: World Bank)

P. Das et al.

Objectives

- The main objectives of this study in the endemic regions of Kala-azar in Bihar are as follows:
- To improve patient care and safety in relation to the use of Miltefosine
- To improve public health and safety in relation to use of miltefosine
- To contribute to the assessment of benefit, harm, effectiveness and risk of miltefosine, encouraging their safe, rational and more effective use
- To promote understanding, education and clinical training in pharmaco-vigilance and its effective communication to health professionals and the public.
- To make appropriate recommendations for administration of Miltefosine as first line of medicine for the treatment of kala-azar in the districts and the state.

Progress:

After several rounds of discussions, the protocol has been finalized. Its approval and funding is awaited.

5. Rapid cross-sectional survey for prevalence of Kala-zar in endemic states of India.

(Sponsor: World Bank)

P. Das et al.

General objectives:

To obtain detailed baseline information of the programme implementation indicators in terms of

1. Disease prevalence
2. Early diagnosis and treatment and
3. Vector control.

Specific objective:

Baseline survey

To estimate the proportion of

1. diagnosed kala-azar cases completing the standard treatment as per the national guidelines
2. suspected cases of kala-azar diagnosed by rK-39
3. houses in targeted kala-azar endemic areas covered with effective insecticide spray
4. blocks that do not have rK-39 test for kala-azar and/or first line medicine stock out during the last three months

End line survey

1. To evaluate whether at least 50% of the sample blocks achieved the goal of elimination programme i.e. 1 case per 10 000 population.

Progress:

After several rounds of discussions, the protocol has been finalized. Its approval and funding is awaited.

Clinical Studies

6. Treatment Response of Kala-azar/ HIV co-infected patients with Ambisome and Anti Retroviral Therapy. (Sponsor: MSF, Spain)

K. Pandey et al.

Objectives:

***Primary Objective*:**

- To determine the efficacy and safety of Ambisome and ART in the treatment of Kala-azar/ HIV co-infected patients

***Secondary Objective*:**

- To assess initial cure or clinical response for VL at 30 days after end of treatment

Progress:

The clinical study was aimed to determine the efficacy and safety of Ambisome and Anti-retroviral therapy (ART) in the treatment of Kala-azar/ HIV co-infected patients. Altogether 46 patients were enrolled in the age group of 10-42 years (mean age 26) and treated with ambisome in the dose of 5 mg/kg body weight for four days. After end of treatment, spleen size regressed and hemoglobin count increased; other biochemical parameters like SGPT, SGOT, serum creatinine were found within the normal range during the treatment and follow up. All the patients were found initially cured after 1-month assessment. Six-month follow up was completed in five patients and all were found finally cured. After completion of VL treatment, they were put on ART. Two patients had oral thrush and were subsequently treated with oral fluconazole. One of the patients had pulmonary tuberculosis and was subsequently started with anti-tuberculous drug. One patient had headache and vomiting along with abnormal behaviour; and the other one had decreased eye vision.

7. Management of Visceral leishmaniasis cases co-infected with tuberculosis with AmBisome and anti-tuberculous drugs. **(Sponsor: MSF, Spain)**

Nawin Kumar et al.

Objectives:

- To confirm the diagnosis of VL and tuberculosis in cases of MSF referred such suspected co-infected cases.
- To treat the confirmed VL/ TB co-infected cases with liposomal amphotericin B in the dose of 5 mg/Kg body weight for 4 consecutive days for VL and standard dose of Anti-tuberculous Drugs (ATT) for TB.
- To assess the initial cure and follow up for assessment of final cure.

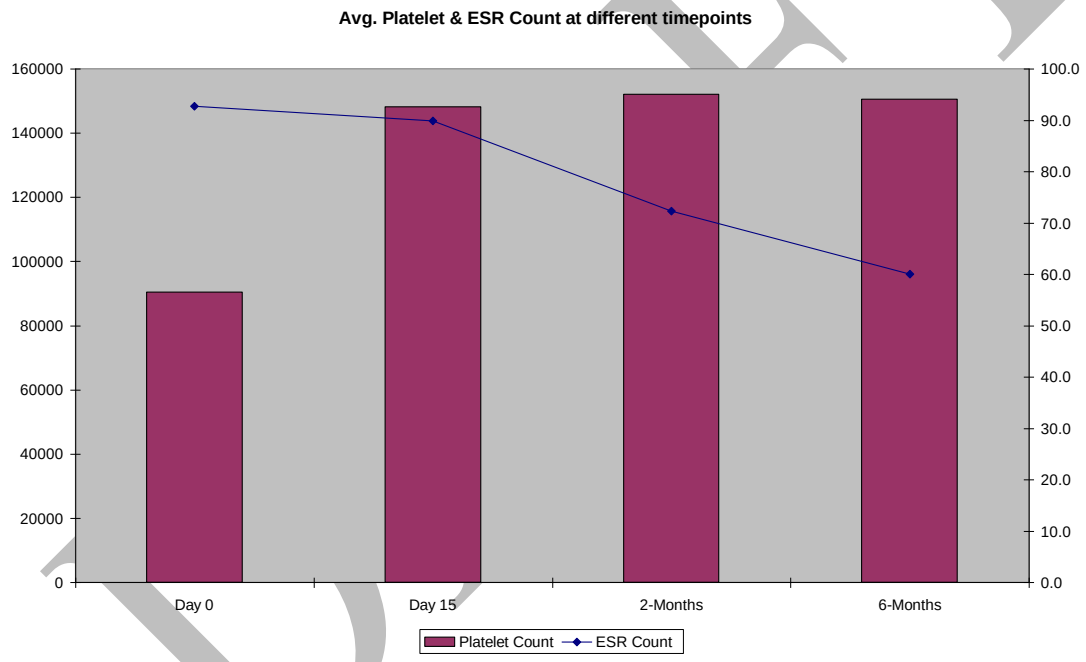
Progress:

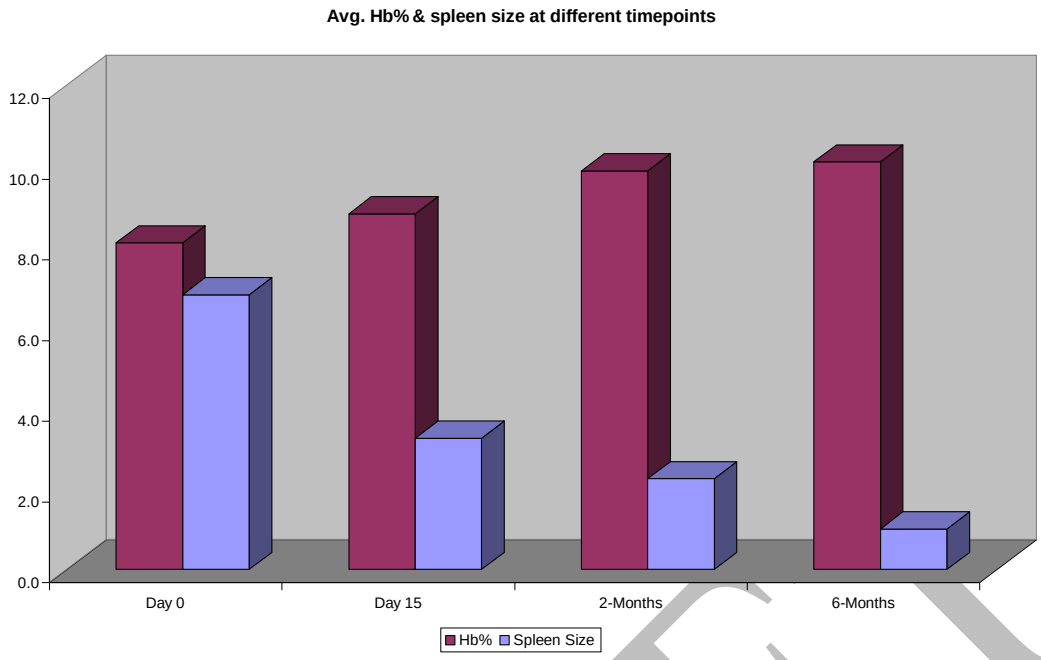
A total of 16 confirmed cases (Male 12, Female 4) of Visceral leishmaniasis (VL) co infected with tuberculosis were enrolled in the study after assessment of inclusion and exclusion criteria. All patients were parasitologically confirmed for VL through splenic

aspiration. Diagnosis of tuberculosis was confirmed by sputum examination in 5 patients and chest x-ray in 11 patients (pleural fluid aspiration in 5 patients).

All the enrolled subjects were treated with ambisome in the dose of 5 mg/kg body weight per day for 4 consecutive days, followed by ATT from day 5. All 16 patients completed full course of ambisome and all were found initially cured for VL. Out of 16 patients, ATT treatment of 6-months duration, were completed in 4 patients and found initially cured for TB and finally cured of VL. Rests are under treatment for TB and simultaneously 6-months follow up for VL.

Assessment of spleen size regression and pathological parameters viz. Total WBC count, Hb%, Platelet and ESR showed suggestive improvement. All the hepatic and renal function tests were found within normal range during treatment and follow up period indicating lack of any adverse effect of ambisome and ATT.





The study is in progress.

8. A comparative evaluation of efficacy and safety of single low dose of Ambisome (7.5 mg/kg/ bw and 10 mg/kg/bw) vs 5 mg/kg/bw for four days (Total 20 mg/kg/bw) for treatment of visceral Leishmaniasis: a randomized clinical trial.

(Sponsor: MSF, Spain)

VNR Das et al.

Objectives:

- To evaluate the efficacy and safety of a single low dose of AmBisome (7.5 mg /kg body weight and 10mg/kg body weight) in comparison to 5 mg/kg body weight for four days (Total 20mg/kg body weight).

Primary objective:

- To determine the efficacy and safety of an optimum single dose of AmBisome.

Secondary objective:

- To assess initial cure or clinical response at 15 days after end of treatment.

Progress:

The parasitologically confirmed VL cases after fulfillment of all the inclusion and exclusion criteria and written Informed consent were randomized in three different arms. Till date, out of 26 screened patients, 20 were randomized (Arm 1- 7; Arm 2- 7 and comparator Arm 3 – 6). Ambisome was tried as a single bolus in the dosage of 7.5 mg/kg body weight in Arm 1 patients, 10 mg/kg body weight in Arm 2 patients and the patients under comparator arm were treated with 20 mg/kg body weight in 4 divided dosage. All the treated patients were found initially cured. No side effects of the drug have been reported. The study is under progress.

9. Safety and efficacy of oral miltefosine in patients with post kala-azar dermal leishmaniasis (PKDL) – dose-finding study comparing 8 and 12 weeks of treatment (D-18506-Z015). (*Sponsor: WHO/TDR*)

P.K. Sinha et al.

Objectives:

- To establish the dosage of Miltefosine for the treatment of PKDL cases in India
- To assess safety and efficacy of different dosage of miltefosine in treatment of PKDL

Progress:

Altogether 17 subjects having nodular and/or papular lesions, 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 12 weeks) and treatment with miltefosine was given. In both arm initial cure was 100% but in arm -1 two patients developed further skin lesion. One patient was dropped due to abstinence of the patient from receiving the medicine. One patient developed eye problem which was referred to AIIMS for further treatment. Patients recruitment target has been covered, Follow up of the treated patients and data analysis is in progress.

10. 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. (VLMP03 – Module II). (*Sponsor:* iOWH)

P.K. Sinha et al.

Objectives:

Primary objective

- To evaluate the effectiveness of access program to provide outpatient VL treatment with Paromomycin IM Inj in progressively more resource constrained, VL- endemic region of Bihar.

Secondary objectives

- To evaluate the safety of Paromomycin IM Inj in an outpatient setting in progressively more resource constrained VL-endemic region of Bihar.

Progress:

In module 2 of multicentric Phase IV study on paromomycin, altogether 500 VL patients were enrolled. All were treated with Paromomycin IM Inj in the dose of 11 mg/kg intramuscularly (IM), once a day, for 21 consecutive days (or over 22 days if one day is missed) in out-patient setting. Patients enrollment and final follow up (6-months after EOT) has been completed. During follow up, no relapse was found but 2 SAE occurred in module 2, one was possibly related and other was not related to the study drug.

11. A randomized, open-label, parallel-group, safety and efficacy study to evaluate different combination treatment regimens (co-administration), of either ambisome and paromomycin, ambisome and miltefosine, or paromomycin and miltesoine for the treatment of acute, symptomatic Visceral Leishmaniasis (VLCOMBO 07).

(Sponsor: DNDi)

P.K. Sinha et al.

Objectives:

Primary objective

- To identify a short course combination treatment regimen that is no less than 7% as effective as the standard amphotericin B therapy.

Secondary objectives

- To compare safety and tolerability of the various treatments measured by vital signs, blood biochemistry, (renal and liver function tests) haematology, spontaneous and elicited adverse.

Progress:

Under this multicentric study, a total of 299 patients were screened at RMRIMS, Patna, out of which 156 were randomized in 4 arms as per the study protocol. On an average, a total of 20 patients have been screened per month and 11 randomized per month. The major reasons observed for the high screen failures were negative parasitological load and/or abnormal laboratory values. Patients randomized in Arm 1 were treated with AmBisome 5mg/kg IV (single dose, Day 1) followed by Miltefosine 50mg twice daily for 7 days (Day 2-8), in Arm 2: AmBisome 5mg/kg IV (single dose, Day 1) followed by Paromomycin sulfate 11mg/kg/day IM for 10 days (Day 2-11) and in Arm 3: Miltefosine 50mg twice daily with Paromomycin sulfate 11mg/kg/day IM for10 days (Day 1-10). Arm 4 was the comparator arm in which the patients were treated with Amphotericin B deoxycholate 1mg/kg IV every alternate day for 30 days (total of 15 mg/kg).

The enrollment target was achieved in July 09 and further screening was stopped at the site. Out of 156 enrolled subjects, altogether 6 were withdrawn; 2 due to SAE, 1 AE leading to withdrawal and 3 treatment failure. Rest all the patients (150) have completed Day

45 visits. Six-months follow up with final cure has been achieved in 90 patients. Further follow up is in progress.

12. The efficacy and safety of a short course of miltefosine and liposomal amphotericin B for visceral leishmaniasis in the Indian subcontinent (LEI PDE 0603). (Sponsor: WHO/TDR)

P.K. Sinha et al.

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 92 weeks) for the treatment of visceral leishmaniasis (Kala-azar) in the India.

Primary objective

- To determine the efficacy of the combined regimen.
- To determine the safety of the combined regimen.

Secondary objectives

- To assess initial cure or clinical response at two weeks after end of treatment.

Progress:

Screening and recruitment under this multi-centric study were designed with three pauses depending upon the prefixed age-group of the subjects and clearance by DSMB to move ahead in the next age group. Altogether 35 VL patinets were enrolled in this study; 20 in the age group of >18-65 years, 5 in the age group of >12-18 yrs and 10 in > 2-11 years age groups.

The patients were treated with co-administration of a single dose of AmBisome at a dose of 5 mg/kg followed by miltefosine for 14 days in the doses of (2.5 mg per kg body weight daily in patients weighing <25 mg/kg; and 50 mg twice daily in those weighing > 25 kg). One patient was withdrawn after 2-days of treatment from the study as the patient withdreew the consent to continue the treatment. The patients tolerated the AmBisome infusion and miltefosine as per the schedule very well with hardly any adverse event. All the treated cases showed initial clinical response at EOT and initial cure at 2 weeks after EOT. All the patients have complted 2-months follow up while 6-months follow up has been

covered for 17 cases. No relapse was found during the follow up and none of the patients showed any sort of SAE.

13. A Prospective, Randomized, Open-Label, Parallel Group, Phase II Study to assess efficacy and safety of three different dosing regimens of Amphomul® (Amphotericin B Emulsion) in patients of Visceral leishmaniasis (Kala-Azar) (BSV-AMBE II-KA-0208)

(Sponsor: Bharat Serums and Vaccines Ltd)

K. Pandey et al.

Progress:

The Phase II study could not be initiated by the sponsor at this Institute. Now the sponsor has approached for its Phase III study which is being placed in the SAC meeting 2009 for suggestion and approval.

14. Safety and efficacy of Liposomal Amphotericin B (Ambisome) in patients with Post Kala-azar Dermal leishmaniasis (PKDL)

(Sponsor: MSF, Spain)

P.K. Sinha et al.

Objectives:

Primary objective

- To assess liposomal amphotericin B regimens 2.5 mg/kg/bw for 20 consecutive days duration for their curative potential in PKDL (parameter: rate of patients with macular/nodular and papular lesions who achieve negative parasitological and clinical severity score of zero 12 months after end of treatment).

Secondary objectives

- To characterize the safety of liposomal amphotericin B when used for periods up to 20 days in three courses at the interval of 15 to 30 days.

- To assess the rates of initial response in relation to duration of treatment.
- To assess the rates of relapse after initial response
- To assess the clinical response of facial erythema and mucosal lesions

Progress:

Altogether 20 parasitologically confirmed PKDL cases (Male 12, Female 8), meeting all the inclusion and exclusion criteria as per the study protocol, were enrolled in the study. All the patients were admitted and treated with liposomal Amphotericin B (Ambisome) in the dose of 2.5 mg/kg body weight for 20 consecutive days as a first course. No adverse side effect was observed, except in one case who developed Gullian-Barrie syndrome after 1st course of treatment. Clinically the lesion appeared to be diminished to some extent in almost all the patients suggesting clinical improvement. Repeat course of the same medication in the same dose and further recruitment of patients in the study to achieve the target of 45 subjects is in process.

Basic aspect

15. Development of a DNA vaccine for Visceral leishmaniasis. (Leish DNA VAX)

(Sponsor: European Union)

P. Das et al.

Objectives:

- To identify sites for carrying out clinical trials.
- Preparation and training for monitoring the trials of Leish DNAVax.
- To carry out the immunological studies on animals with IICB.
- To participate in immunological studies on human cells.

Progress:

The peripheral blood monocytes were isolated from patients with active disease and after cure from disease and also from the sero-positive and sero-negative healthy individuals by histopaque method. The PBMC were re-suspended in cryo-medium and preserved in

-80° C for further use. The further studies will be done with those samples after getting the constructs from the other partners.

16. Laboratory based evaluation of rapid diagnostic tests for Visceral leishmaniasis.

(Sponsor: WHO/ TDR)

P. Das et al.

Objectives:

- To evaluate performance of VL diagnostics.
- To facilitate R & D of new VL diagnostics.
- To facilitate QA/QC in VL diagnosis in all endemic regions.

Progress:

Details have been entered in computerized formats. SOP has been prepared and distributed. Laboratory staffs have been trained for participation in the work. DAT has been standardized. Sera samples have been tested under quality control programme and report has been sent to concern authority. Forty five sera sample of confirmed VL have been tested and report has been sent to concern authority.

Vector Biology & Control

17. Cost effective integrated vector management as a contribution to the visceral leishmaniasis elimination initiative on the Indian sub-continent: a multi-centre study.

(Sponsor: WHO/TDR)

V. Kumar et al.

Progress:

The study has been completed and presented during the previous SAC meeting. The second phase is at the final stage of completion which has been mentioned as separate project entitled "Validation of conventional and new indicators and procedures for monitoring vector control in the VL elimination programme".

18. Validation of conventional and new indicators and procedures for monitoring vector control in the VL elimination programme - Phase II Study **(Sponsor: WHO/TDR)**

V. Kumar et al.

Objectives:

General objective

- To test and further develop indicators to be used for monitoring and evaluating progress and impact of the Vector Control Programme towards VL elimination

Specific objectives

- To assess a) policy and programme guidelines/ indicators on monitoring and evaluation currently used for monitoring different aspects of the Vector Control program in India and b) to critically analyse gaps in M&E policy guidelines and their implementation.
- To evaluate the quality, coverage and effectiveness of the Vector Control Program using IRS.
- To a) validate programme quality (residual effect) of IRS and b) Identify core indicators for monitoring and evaluation

Progress:

In continuation with the earlier work, data on sand fly density in experimental and sentinel houses after 5 months of DDT spray were collected. Bio-assay was conducted in experimental houses. A total of 12 sandflies (Male 8, Female 4) could be collected from experimental houses, whereas from 7 sandflies (Male 4, Female 3) female were collected from sentinel houses. The Bio-assay result varied in between 15.78-26.86%.

Forty-two filter papers with DDT from experimental households were sent to IICT, Hyderabad for chemical analysis and the observed DDT concentration was between 2.389-0.003gm/m2. The result of the filter paper with DDT spraying carried out in ideal condition varied between 2.77-0.69gm/m2. The same test was also conducted at Walloon Agricultural Research Centre, Belgium and the result obtained was in between 1.0-2.0gm/meter sq. for experimental villages and 1.0 gm/meter sq. in ideal condition. In another set of experiment the filter paper was dipped in 5% DDT solution and the DDT concentration was in between 12.93-6.98gm/meter sq (at IICT) and it was 3.0 gm/meter sq. The analysis of randomly collected samples of DDT powder formulation (50%) used in the spraying revealed approximately 48% of DDT concentration indicating almost matching concentration.

19. Usefulness, Feasibility and Cost of Vector Control Monitoring in Kala-azar Endemic District of Bihar, India – Phase III Study.

(Sponsor: WHO/TDR)

V. Kumar et al.

Objectives:

- To assess the usefulness, feasibility and costs of the Monitoring Toolkit developed in Phase II for quality control of IRS, including real coverage, spraying performance.
- To evaluate some specific indicators in the toolkit that require further evidence before large –scale application, its output and impact of IRS.
    - To establish the **norm** for efficient use/application of insecticides (in terms of gm per household and eventually per population) for IRS.
    - To select the most appropriate tool (or tool mix) amongst three different IRS **entomological** monitoring indicators: i. the filter paper method (chemical residue on filter papers as tracers in houses), ii. The bio-assay method and iii. Sandfly captures by CDC light traps for reduction of sand fly density.

- To evaluate the **outcome of IRS** through assessing the insecticide residual action of IRS based on repeated sand fly collections after IRS compared to control.

- To compare the user friendliness and efficiency of two pumps (Hand compression and Stirrup) in terms of number of houses sprayed per day, volume of insecticides used per pump and the operational feasibility of each pump.

Progress:

Based on the Govt. data on reported cases of VL during the period 2006-2008, two PHCs from each of the three districts of North Bihar viz. Muzaffarpur, Vaishali and Samastipur were selected. From each PHC, 2 villages sprayed with DDT were selected, one near to the PHC and the other approx 6-7 Km away (total number of villages 12). From each village, 6 experimental and 6 sentinel households were randomly selected. Similarly, 12 villages, not exposed to DDT spray, were selected from the same PHCs as control.

For assessment of 'toolkit', review of existing documents of the VL elimination programme was done. Interview with key informants at district and PHC level as well as Vector Control Staff at different levels were conducted to assess their knowledge in relation to vector control. It was found that all had acquired IRS training but newly recruited spray men had only one day training at Muzaffarpur. All of them had the guidelines both at district and state level. They responded that village selection for IRS is decided as per the case load and the concerned central and state authorities. The usual period of IRS reported two round i.e. Feb. – March and May – June every year that may differ subjected to various reasons like availability of funds, elections etc. It was observed that storage and the distribution of DDT is monitored by a programme supervisor; several temporary stores were established in Govt. buildings and Health Sub Centres to store DDT about three days prior to spray until use; empty insecticide containers (Sachets) are buried under ground; and no protection devices were available for the spray men.

IRS output assessment was conducted in two study villages after IRS operation where IRS was carried out by one squad/day in the presence of the research team by filling observational checklist. The surface areas covered by IRS in each houses were measured. Villages, where IRS was done in the absence of the research team, was taken as control. It was observed that some of the spray men were inexperienced and they were lacking technical skill of spraying as they were using more than 45-60 strokes/minutes. At the same time, the

experienced ones were quite aware of all the technical skills like DDT suspension preparation, rectification of minor defects of the pumps etc. It was noticed that though the DDT is available as per requirement of the district but there was lacking of sufficient number of pumps and squads, no IEC materials available about IRS, no prior information at all to the villagers about the IRS spraying. In two observations, one squad covered 66 HHs and total sprayed area measured 5999.66 Sq. meter. In another observation, one squad covered 72 HHs and total sprayed area measured 6230.9 Sq. meter.

For assessment of 'entomological indicators', sandflies collection using CDC light trap was conducted in 6 sprayed and 6 sentinel households of each experimental villages; and 6 unsprayed households from each control villages. The average corrected mortality of sandfly, observed through Bioassay test after 2 and 4 weeks of spray, was 47.96% and 32.28% respectively. Heads/spouses of 35 households from each village were interviewed after their consent to assess acceptance indicators. The work is in progress.

20. 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.

(Sponsor: DBT)

V. Kumar et al.

Objectives:

- Establishment of critical parameters to improve the laboratory breeding of *Phlebotomus argentipes* for larger sand fly output.
- Preparation of extracts from virgin male *P. argentipes* of 2-3 days old to see the chemical communication between male and female fly.
- Study of male and female *P. argentipes* behavioral responses to the natural extracts in the laboratory to assess the presence or absence of chemical communication between male and female *P. argentipes,* for mate and host location.

Progress:

It has been demonstrated that sitting frequency of female sand flies on male extract is significantly higher in comparison to control and also the frequency and time spent on male extract is much higher than on bait. Further, experiment on behavioral bioassay test was conducted to explore the role of male sand fly for host location by observing change in behavior (if any) of female *P.argentipes* in presence and absence of male ones. Earlier it was proposed to use Y-Tube Olfactometer as bioassay equipment, but based on the experiments conducted by the collaborating Institute it was decided to continue the bioassay experiment in cage only.

Under this behaviour bioassay test, 12 set of experiments were conducted. In each set of experiment, 30 newly emerged female sand flies (virgin) were released in the specially designed cage with animal bait (rabbit). After half an hour observation, it was noticed that none of the sand flies took feed and they sat on the walls of the cage. But after releasing 30 newly emerged male sand flies in the cage, it was observed that male sand flies sat on the bait in the form of ring and the female sand flies started to come near the ring of male flies and feeding process began. The observed aggregation ratio of male and female (lek formation ratio) as well as feeding percentage was as below:

Table: Male –female ratio on bait (Lek formation) and feeding %

Set No.	Male sandflies released	Female sand flies released	Aggregation ratio of male and female (Lekking)	Feeding %
1	30	30	7:1	68%
2	30	30	21:3	72%
3	30	30	19:3	64%
4	30	30	15:2	60%
5	30	30	9:2	76%
6	30	30	9:1	68%
7	30	30	18:3	76%
8	30	30	19:2	80%
9	30	30	9:1	68%
10	30	30	5:1	80%
11	30	30	7:1	72%
12	30	30	13:2	64%

21. Monitoring insecticide resistance to *Phlebotomus argentipes*.

(Sponsor: European Union Commission)

D.S. Dinesh et al.

Objectives:

- To assess susceptibility status of the vector *Phlebotomus argentipes* against deltamethrin and DDT through WHO kit.

Progress:

The study was conducted with the standardization of exposure period (15', 30' and 60') of *P.argentipes* to 0.05% Deltamethrin to achieve approximately 100% mortality under laboratory conditions maintaining temperature 25-27^{0}C and relative humidity 72-80%. The exposure period followed with 24 hours of recovery period. Five replicates (n=20 each) of both fed and unfed *P. argentipes* were taken in experiment. The observed mortality was 98-100 % with 60' exposure. Unfed *P. argentipes* showed 72%, 96% and 99% mortality with the exposure period of 15', 30' and 60' respectively whereas fed sandflies showed 55%, 86% and 100% mortality respectively under the similar exposure period. The observation suggests exposure period of 60' for the tube bioassay test with 0.05% Deltamethrin impregnated WHO kit paper against *P. argentipes* in the field condition. In the field condition the same experiment was repeated and similar mortality was observed with 0.05% deltamethrine.

However, with 4% DDT mortality in fed and unfed *P. argentipes* was recorded 36-37% in laboratory condition whereas in the field condition (in the intervention village of Muzaffarpur district) it was 46%. The study is under the progress.

22. 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.

(Sponsor: ICMR Task Force)

S. Kesari et al.

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:

During February 2008 to May 2009, total numbers of sandflies collected from the endemic and non-endemic sites were 538 (Male 53.90%, female 46.10%) and 270 (Male 57.41%, female 42.59%) respectively. The major percentage of Phlebotomous species collected from endemic and non-endemic sites belong to *P.argentipes* (65.98% from endemic and 61.48% from non-endemic sites) followed by *Sergentomiya* (32.53% from endemic and 38.52% from non-endemic sites) and very small percentage of *P. papatasi* (1.49% from endemic sites).

A total of 468 houses were surveyed within the two study sites (endemic and non-endemic) to obtain information on socio-cultural practices. Housing pattern, presence or absence of cattle shed, cattle population and area were considered as independent variables; whereas presence or absence of vector in the endemic and non-endemic region considered as a dependent variable. From the "Backward Stepwise (Likelihood Ratio)" logistic regression analysis, it was found that brick and brick with mud wall (*P value = <0.001)*, thatched roof (*P value = 0.059*), and mixed dwelling (*P value = 0.010*), area (*P value=0.001*) were strongly correlated with vector abundance of the study area. The result also pointed out that housing structures like thatched wall (*P value = 0.009*), brick wall (*P value= 0.001*), tiles and cuprile roof (*P value = 0.018*), and separate cattle shed (*P value = 0.*861) have a little bit influence on vector abundance in the study sites.

Pearson's correlation coefficient test showed that there was a strong relationship with minimum NDVI and sandfly density i.e. r= 0.54 and 0.64 in an endemic site and r=0.42 and 0.46 in non-endemic site for *P.aregentipes* and total number of sandfly respectively. There is no significant relationship with maximum NDVI (r = 0.04 and -0.03 in an endemic site; r = -0.08 and -1.03 in a non-endemic site).

The main landscape features observed in the region were permanent water body, temporary water body, marshy/swampy land, wet fallow, dry fallow, crop land, agricultural fallow, plantation with settlement etc. The land use/land cover classifications showed that maximum portion of the area covered by wet fallow land and water body in the endemic region whereas in non-endemic area, it was covered by forest and agricultural fallow land. An overall classification accuracy of the land cover classification showed more than 80% of each PHCs in an endemic and non-endemic site and Kappa statistics is also intended for each land cover classes more than 0.85.

The chemical properties of soil shown that the Silicon-di-oxide (SiO_2) content ranged from 41.04-41.90% and 61.22-62.323%; Aluminum oxide (Al_2O_3), 10.76-14.75 and 9.02-9.43; Sodium oxide (NaO_2), 8.76-12.76 meq/L and 2.41-2.45 meq/L; Potassium oxide (K_2O), 1.76-2.14 meq/L and 1.79-1.83 meq/L; Phosphorous oxide (P_2O_5) ranged from 0.08-0.02 and 2.58-2.76; Titanium-dioxide (TiO_2), 0.00-0.03 and 1.46-1.68 in endemic and non-endemic site respectively. The results also revealed that magnesium and calcium ranged from 2.42-4.61 meq/L and 4.76-8.12 meq/L in endemic site and 0.53-0.58 meq/L and 1.22-1.32 meq/L in non-endemic site. Soil pH in an endemic district is alkaline nature (7.84) where as in non-endemic district it was (6.12) and moisture content is high (12.21%) in an endemic district and very low (3.75%) non endemic district.

D. Others

Meetings/ Trainings/ Workshop/ Symposium held at the Institute

- Dr. Peter Walden, Biochemist & Head, Clinical Research Division, Humboldt University, Berlin, Germany delivered a talk on "Antigens for Leishmania vaccine" on 30th March 2009.
- Dr. D.J. Perkins, Assistant Professor, University of Pittsburg, USA delivered a talk on "Mechanism of anemia in severe malaria" on 06th April 2009.
- Workshop on "Application of Bio-informatics in Medical Sciences" was organized from 21st – 22nd June 2009
- Dr. G.S. Bhattacharya, HOD & In-charge, Advanced Medical Research Centre, Kolkata delivered a talk on "Biomedical Research Ethics" on 23rd June 2009.
- Two-days meeting for discussion on "Snowballing technique as a surveillance tool" was held under expertise of Dr. Arvind Pandey, Director, NIMS (ICMR)

Meetings/ Trainings/ Workshop/ Symposium attended

- Mr. R.B. Verma, Technical Officer attended WHO In-Country Fellowship Training Programme at CMC, Vellore from 2nd Feb – 30th Apr 2009.
- Dr. Vahab Ali, Scientist 'C' participated in the Poster presentation session of "The 9th Awaji International Forum on Infection and immunity", held at Awaji Yumebutai Internation Conference Centre, Japan from 8th – 11th Sept. 2009.

Distinguished visitors

- Dr. Peter Walden — 30 Mar 2009
  Biochemist & Head, Clinical Research Division
  Humboldt University, Berlin, Germany

- Dr. D.J. Perkins — 06 Apr 2009
  Assistant Professor, University of Pittsburg
  USA

- Dr. A.I. Ashan — 21 June 2009
  Pro-Vc, Patna University &
  Former Prof. & Head, Bio-infomatics,
  Jamia Milia Islamaia, Patna

- Dr. G.S. Bhattacharya — 23 June 2009
  HOD & In-charge,
  Advanced Medical Research Centre, Kolkata

- Dr. Arvind Pandey — 29 – 30 June 2009
  Director, National Institute of Medical Statistics
  New Delhi

Publication

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13. Kumar N, Siddiqui NA, Verma RB and Das P. Knowledge about sandflies in relation to public and domestic control activities of Kala-azar in rural endemic area of Bihar. *JCD*, 2009, 41(2): 121-128.

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