

# PATHOLOGY OF INFECTIOUS DISEASES

# **GENITAL CHLAMYDIAL INFECTION**

## **1. Development of Diagnostic Assay for *Chlamydia trachomatis***

**Scientific Staff** : **Dr. Aruna Mittal**  
**In collaboration With** : **Dr. Sudha Salhan, SJH**  
**Technical Staff** : **Mrs. Asha Rani**  
**Duration** : **2002-2007**

### ***Aims, Objectives and Background***

Conventional assays for diagnosis of *Chlamydia trachomatis* have limitations and there is need particularly in developing countries for indigenous, accurate and reproducible diagnostic assay with high rates of sensitivity and specificity. Hence, accurate diagnosis is essential for establishing etiology and instituting the appropriate therapy.

### ***Work done during the year***

Detection of *C. trachomatis* was performed by EIA and cell culture using clones D10.4 (Species specific), H5.6 (D serovar specific) and B2.2 (B serogroup specific) in 200 endocervical specimens. The positivity for clones D10.4, H5.6 and B2.2 in EIA was 45% (90/200), 43% (86/200) and 35% (70/200), respectively when compared with cell culture method [46% (92/200)]. The results of reactivity of those clones in EIA and cell culture methods are shown in table 1.

The sensitivity and specificity for clone D10.4 in EIA when compared with cell culture method was 91.30% (84/92) & 94.44% (102/108), for clone H 5.6 it was 91.30% (84/92) & 98.14% (106/108) and 75.00% (69/92) & 99.07% (107/108) for clone B2.2 (Table 2). The reactivity of clones H5.6, D10.4 & B2.2 in EIA were found statistically significant i.e.  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.01$ , respectively.

<i>EIA</i>	<i>Result of</i>		
	<i>Cell culture</i>	<i>Number</i>	<i>%</i>
<b>D serovar specific (H5.6) MAb</b>			
Negative	Negative	106 <sup>a</sup>	53%
Positive	Positive	84 <sup>b</sup>	42%
Positive	Negative	02 <sup>c</sup>	1%
Negative	Positive	08 <sup>d</sup>	4%
<b>Species specific (D10.4) MAb</b>			
Negative	Negative	102a	51%
Positive	Positive	84b	42%
Positive	Negative	06c	3%
Negative	Positive	08d	4%
<b>B serogroup specific (B2.2) MAb</b>			
Negative	Negative	107a	53.5%
Positive	Positive	69b	34.5%
Positive	Negative	01c	0.5%
Negative	Positive	23 <sup>d</sup>	11.5%

<sup>a</sup>-True Negative, <sup>b</sup>-True Positive, <sup>c</sup>- False Positive, <sup>d</sup>-False Negative

**Table 1: Detection of *C. trachomatis* from endocervical specimens by EIA and cell culture method**

True positive specimens were those that were positive by both the methods i.e., EIA and cell culture. Similarly, true negative specimens were negative with both the methods. False positive specimens were those that were positive in EIA but negative by cell culture method. False negative specimens were those, which were negative in EIA but positive with cell culture method and were considered as being truly positive.

<b>Monoclonal Antibody</b>	<b>Total Positivity (%)</b>	<b>Sensitivity (%)</b>	<b>Specificity (%)</b>	<b>PPV (%)</b>	<b>NPV (%)</b>	<b>Accuracy (%)</b>	<b>P Value</b>
<b>H 5.6</b>	<b>43.00</b>	<b>91.30</b>	<b>98.14</b>	<b>97.67</b>	<b>92.98</b>	<b>95.00</b>	<b>&lt;0.05</b>
<b>D 10.4</b>	<b>45.00</b>	<b>91.30</b>	<b>94.44</b>	<b>93.33</b>	<b>92.72</b>	<b>93.00</b>	<b>&lt;0.01</b>
<b>B 2.2</b>	<b>35.00</b>	<b>75.00</b>	<b>99.07</b>	<b>98.57</b>	<b>82.30</b>	<b>88.00</b>	<b>&lt;0.01</b>

PPV – Positive Predictive Value, NPV – Negative Predictive Value

**Table 2: Comparative analysis for *C. trachomatis* detection by monoclonal antibody based EIA and cell culture method**

## ***2. Humoral Immune Response to Chlamydial Heat Shock Proteins in the Pathogenesis of Genital Tract Infection in Women***

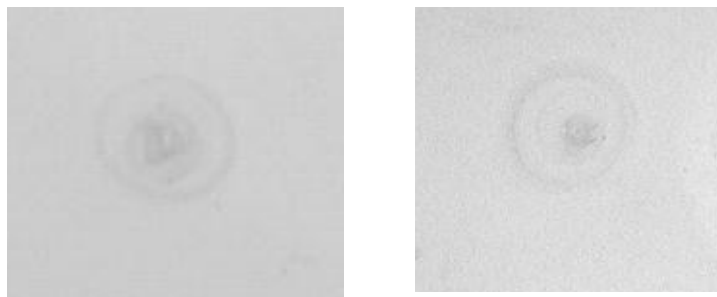
***Scientific Staff*** : ***Dr. Raini Dutta, Mr. Rajneesh Jha, Dr. Aruna Mittal***  
***In collaboration With*** : ***Dr. Sudha Salhan, SJH***  
***Technical Staff*** : ***Mrs. Asha Rani***  
***Duration*** : ***2004-2007***

### ***Aims, Objectives and Background***

The persistent and untreated chlamydial infection causes chronic stimulation of the host immune system against immunogenic antigens such as chlamydial heat shock proteins (cHSP) 60 and 10 and seems to be responsible for wide variety of immunogenic damage to the host.

### ***Work done during the year***

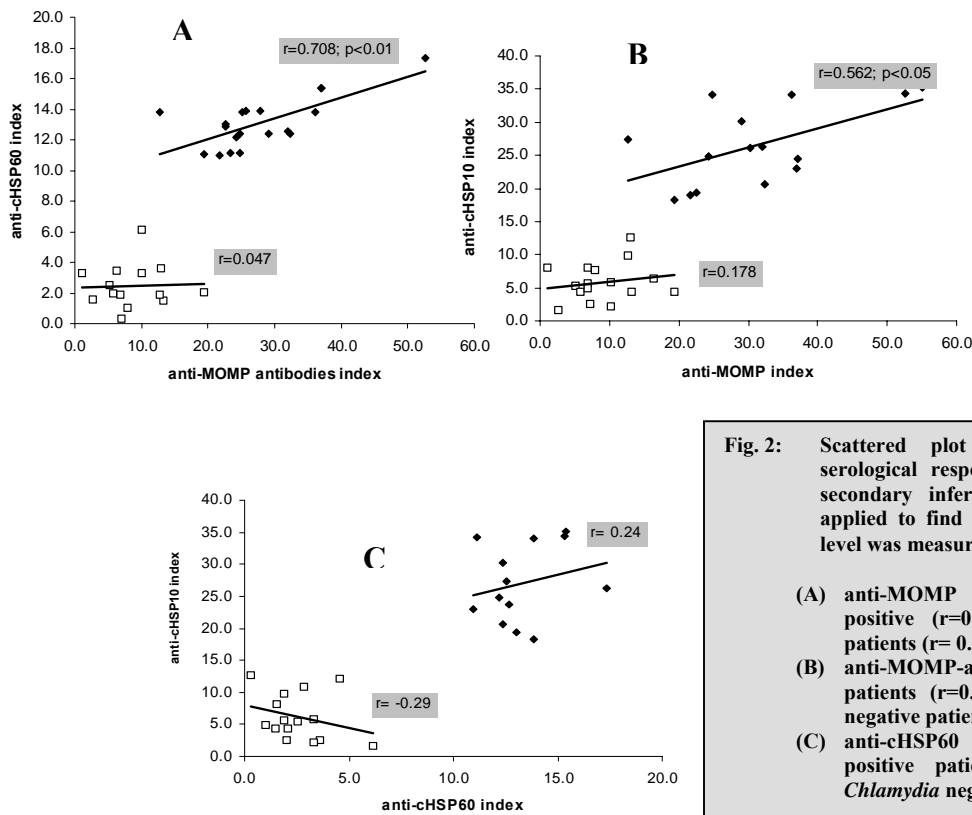
In our earlier study, we detected high antibody titer of both cHSP60 and 10 in *Chlamydia trachomatis* infected infertile patients. Further Dot Blot was done for validation of ELISA (Fig.1). During the year, we further studied the seroprevalence of cHSP60 and 10 antibodies in patients with primary and secondary infertility. Anti-cHSP60 antibodies were present in 54.76% (CI, 51.2%-52.4%) of secondary infertile patients and 30.0% (CI, 28.2%-31.8%) in primary infertility patients. Anti-cHSP10 ELISA showed seroprevalence of 40.5% (CI, 35.3%-45.6%) in secondary infertility patients, which were significantly ( $p \leq 0.05$ ) higher than primary infertility 26.67% (CI, 28.16-35.84) patients.



**Fig. 1: Dot Blot for cHSP60**

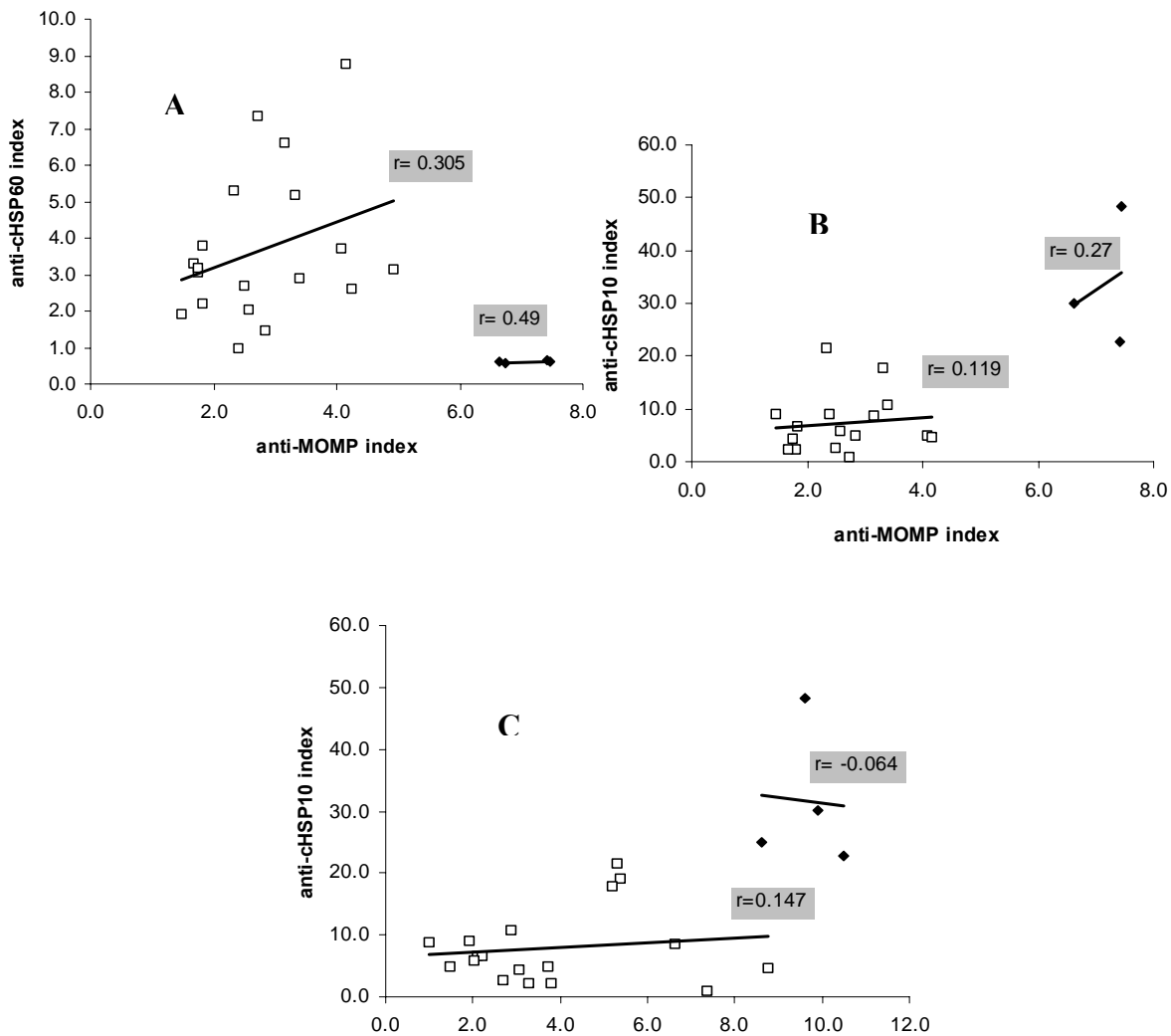
**Dot Blot for cHSP10**

In secondary infertility patients with chlamydial infection, anti-cHSP60 index values were positively correlated both with anti-MOMP ( $r=0.7$ ;  $p\leq 0.01$ ) and anti-cHSP10 ( $r=0.24$ ) index. Anti-cHSP10 index values were also positively significantly correlated with anti-MOMP antibodies ( $r=0.56$ ;  $p\leq 0.05$ ) (Fig. 2). However, in case of *Chlamydia* uninfected secondary infertile patients, the insignificant correlation was observed between the antibodies. In case of primary infertile patients, no correlation was observed between the cHSP 60 and 10 antibodies-neither in chlamydial infected nor in un-infected (Fig. 3).



**Fig. 2:** Scattered plot showing the correlation of serological responses to chlamydial antigens in secondary infertile patients. Pearson test was applied to find correlation and the significance level was measured by  $\chi^2$  test.

- (A) anti-MOMP - anti-cHSP60 in *Chlamydia* positive ( $r=0.708$ ;  $p<0.01$ ) and in negative patients ( $r=0.047$ ).
- (B) anti-MOMP-anti-cHSP10 in *Chlamydia* positive patients ( $r=0.562$ ;  $p<0.05$ ) and in *Chlamydia* negative patients ( $r=0.178$ ).
- (C) anti-cHSP60 -anti-cHSP10 in *Chlamydia* positive patients ( $r=0.24$ ;  $p<0.05$ ) and in *Chlamydia* negative patients ( $r=-0.29$ ).



**Fig. 3:** Scattered plot showing the correlation of serological responses against immunogenic chlamydial antigen in Primary infertile patients. Pearson test was applied to find correlation and the significance level was measured by  $\chi^2$  test.  
 (A) anti-MOMP - anti-cHSP60 in *Chlamydia* positive ( $r=0.305$ ) and in negative patients ( $r= 0.49$ ).  
 (B) anti-MOMP-anti-cHSP10 in *Chlamydia* positive patients ( $r=0.27$ ;  $p<0.05$ ) and in *Chlamydia* negative patients ( $r=0.119$ ).  
 (C) anti-cHSP60 -anti-cHSP10 in *Chlamydia* positive patients ( $r=-0.064$ ;  $p<0.05$ ) and in *Chlamydia* negative patients ( $r=0.147$ ).

Thus early identification of women with high risk of developing secondary infertility is required which may help to prevent long-term sequelae and may improve treatment efficacy during earlier stages of infection. Therefore efforts are being made to develop cHSP60 DOT ELISA for detection of women with secondary infertility.

### ***3. Effect of Sex Hormones on Induction of Immunity by Dendritic Cells in Female Reproductive Tract during Chlamydia trachomatis Infection***

***Scientific Staff*** : ***Tanvi Agrawal, Vikas Vats,  
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***Technical Staff*** : ***Mrs. Asha Rani, Mrs Madhu Badhwar***  
***Duration*** : ***2005-2008***

#### ***Aims, Objectives and Background***

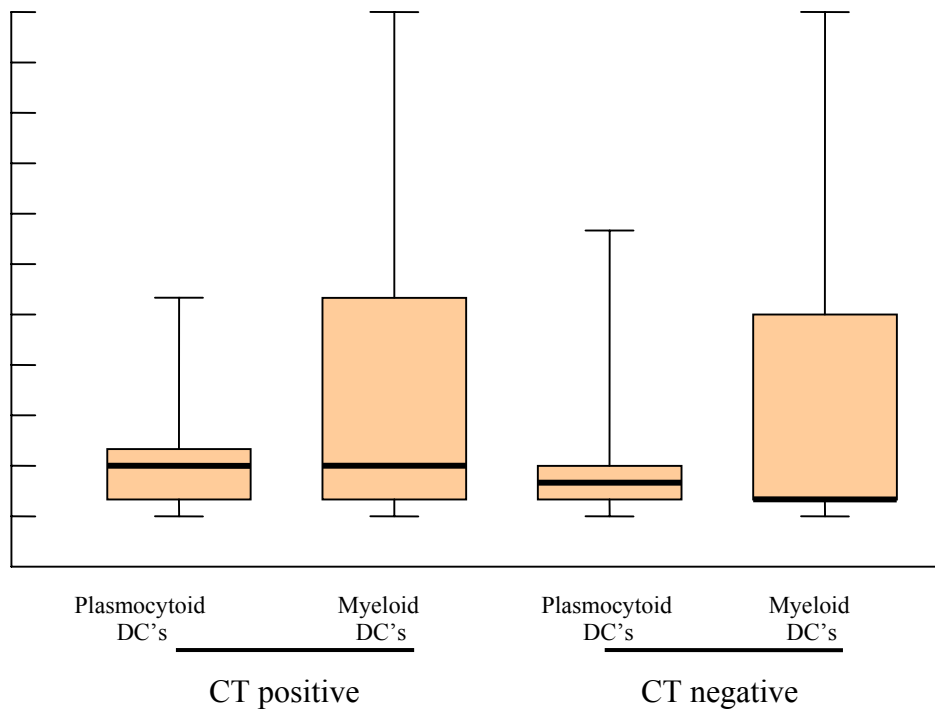
Antigen presenting cells, particularly dendritic cells (DCs) at the mucosal surfaces are central to the generation of immune protection against pathogens. Understanding the mechanism of DC antigen presentation and in directing a T<sub>H</sub>1 protective immune response during chlamydial infection will help in understanding of immunopathogenesis of *Chlamydia trachomatis*.

#### ***Work done during the year***

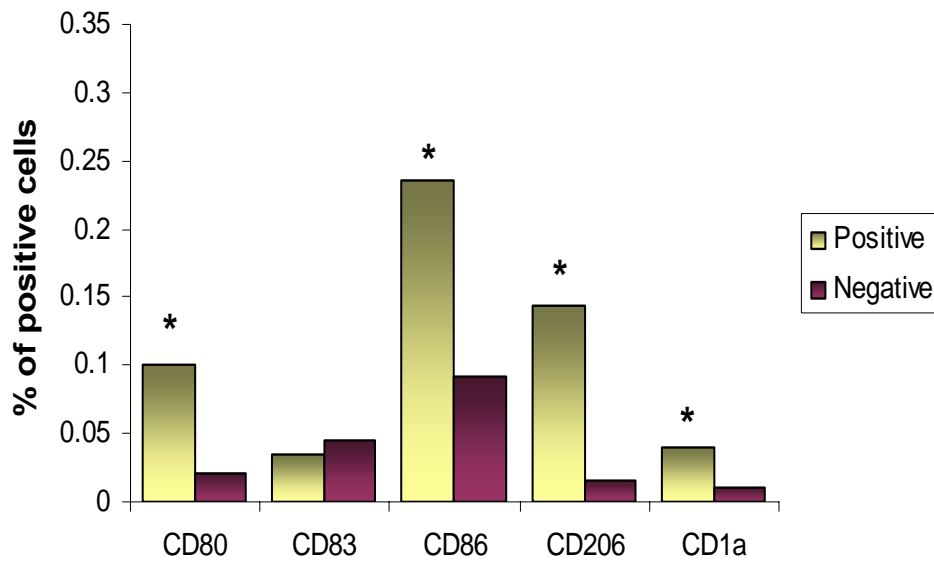
Cervical lavage and peripheral blood were collected from symptomatic female patients (age range: 20-40 years) attending the Gynecology Outpatient Department of Safdarjung hospital, New Delhi, India. Four-color flow cytometry analysis was performed on cells obtained from cervical lavages and blood samples. Antibodies used were fluorescein isothiocyanate conjugated lineage cocktail MoAbs (CD3, CD14, CD16, CD19, CD20, and CD56 for antigens that are expressed by T and B lymphocytes, macrophages, and NK cells, respectively); phycoerythrin conjugated mouse IgG, anti-human CD80, CD83, CD206, HLA-ABC, CD1a, CD123, and CD86 peridinin chlorophyll protein conjugated anti- HLA-DR; allophycocyanin conjugated CD11c. DCs were identified as those cells that expressed major histocompatibility class II DR but did not label with the lineage cocktail. Cell preparations were labeled in parallel with the appropriate isotype

control antibodies. A minimum of 50,000 events was acquired within a leukocyte acquisition gate. Data were recorded and counted using FACS Calibur software.

The percentage of myeloid DC's ( $\text{Lin}^- \text{HLA-DR}^+ \text{11C}^+$ ) in cervical lavages of *C. trachomatis* positive women was found to be significantly increased ( $p < 0.05$ ) than *C. trachomatis* negative women. No significant difference was observed between percentages of Plasmacytoid DC's ( $\text{Lin}^- \text{HLA-DR}^+ \text{123}^+$ ) (Fig. 1). In blood, myeloid DC's were found to be 0.2% and plasmacytoid DC's were 0.14%. In comparison to this, the lavages have equal percentage of plasmacytoid and myeloid dendritic cells. On assessing the expression of various dendritic cell markers in cervical lavages, the percentages of dendritic cells expressing CD80, CD86, CD206 and CD1a were significantly higher in cervical lavages of *C. trachomatis* positive women. No significant difference was observed between percentages of cells expressing CD83 (Fig. 2).

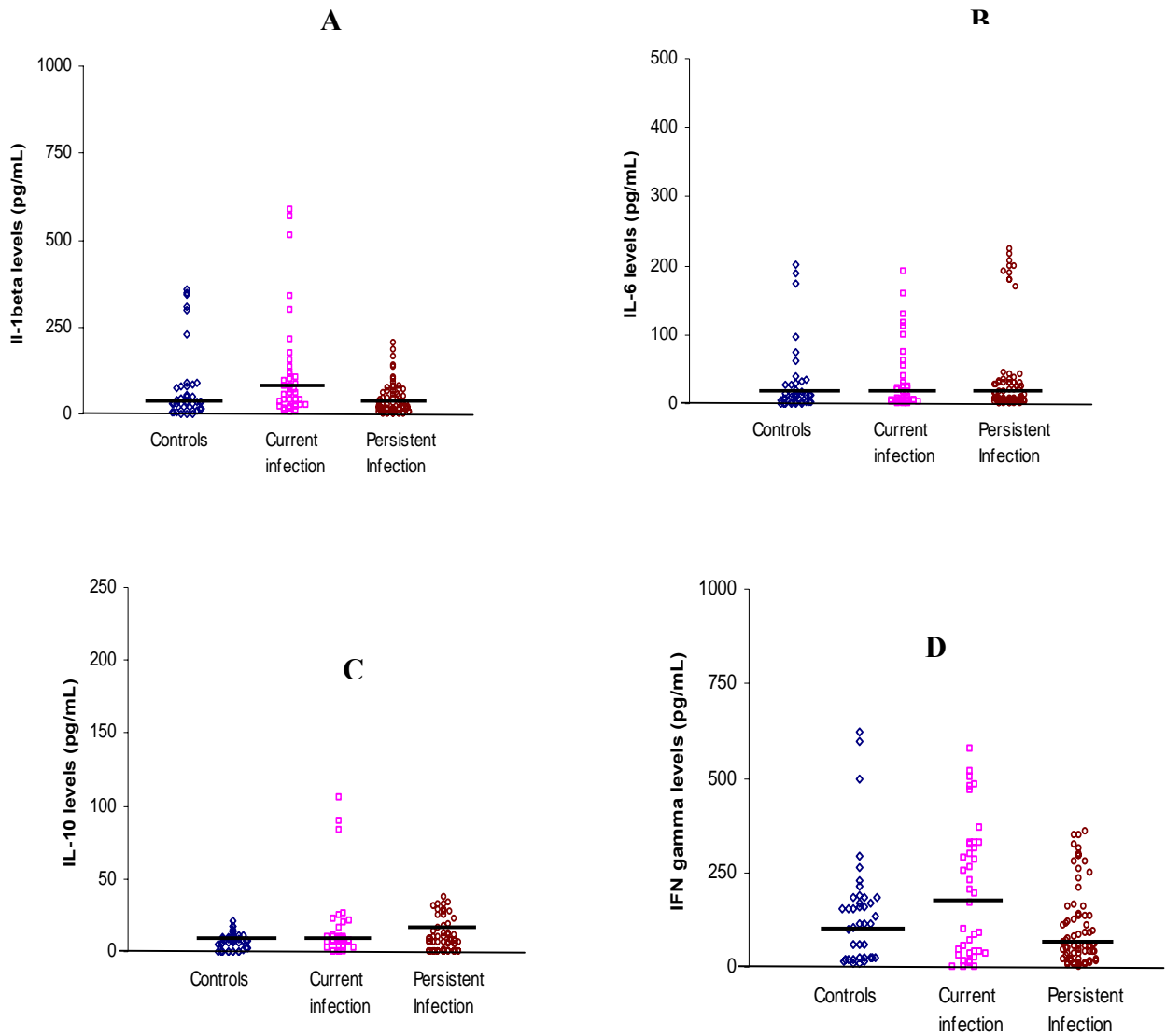


**Fig. 1: Percentage positivity of plasmacytoid and myeloid DC's in CT positive vs CT negative patients**



**Fig. 2: Expression of various dendritic cell markers in CT positive vs CT negative patients**

Concentrations of IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , and IFN- $\gamma$  cytokines in cervical lavages were detected simultaneously using the human Th1/Th2 cytokine cytometric bead array (CBA) kit II. Based on clinical history and serum antibodies against *C. trachomatis*, these patients were categorized into two groups: Group I comprised of patients with recent complaints of discharge and/or abdominal pain. Group II comprised of patients having persistent infections and being tested positive for infection on > 2 consecutive visits. Median IL-1 $\beta$  concentrations were 35, 53 and 23 pg/ml respectively. IL-1 $\beta$  levels were high in patients with current infections as compared to controls or patients with persistent infections but differences were not significant. Median IL-6 concentrations were 13, 9 and 14 pg/ml respectively. Median IL-10 concentrations were 7, 7 and 9 pg/ml respectively. Median IFN- $\gamma$  concentrations were 114, 204 and 69 pg/ml respectively. IFN- $\gamma$  concentrations were significantly higher in patients with current infections than with persistent infections ( $p < .05$ ). Median TNF- $\alpha$  concentrations were 0 pg/ml for all groups. IL-4 was below detection limits in all samples (Fig. 3).



**Fig.3: Concentrations of (A) IL-1 $\beta$ , (B) IL-6 (C) IL-10 and (D) IFN- $\gamma$  in cervical wash samples from controls, patients with current infection and patients with persistent infection.**

## ***4. Inclusion Proteins and their Contribution Towards Chlamydial Pathogenesis***

***Scientific Staff*** : ***Mr. Rishen Gupta, Dr. Aruna Mittal***  
***Technical Staff*** : ***Mrs. Madhu Badhwar***  
***Duration*** : ***2004-2007***

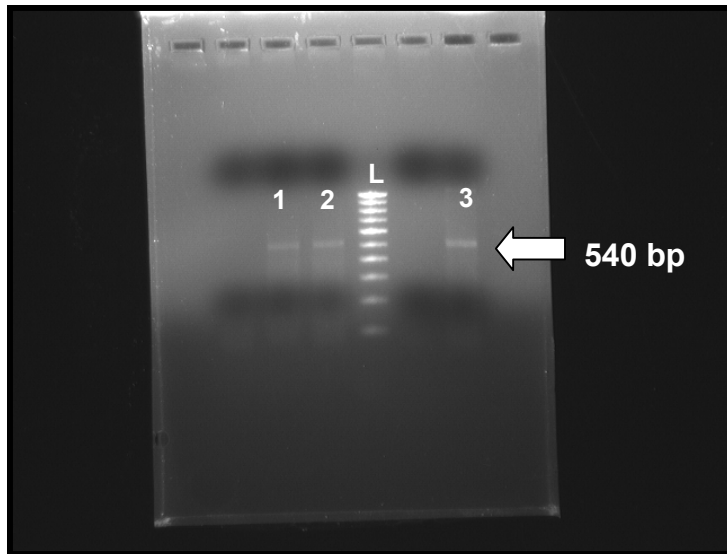
### ***Aims, Objectives and Background***

The diverse Inclusion proteins (*Inc*) encoded by *Chlamydia trachomatis* evokes queries related to evolutionary biology. The mechanisms used namely gene duplication or mutagenesis that adds new *Inc* genes within a genome or possibly a mechanism for random insertion of a significant nucleotide sequence encoding the characteristic hydrophobicity profiles, is of interest. Proteins that acquire this tag may subsequently be transported to the inclusion membrane. Localization of these proteins to the inclusion membrane environment offers a selective advantage, in which the mutant would survive and the mutation would be propagated.

Microinjection studies showed that Inclusion proteins contact the host cell cytosol and that selected inclusion proteins are phosphorylated, likely by host cell phosphokinases. Studies indicate a hypothesis that places inclusion proteins at a central point in the interaction between the host cell and the intracellular, intravacuolar chlamydial developmental forms.

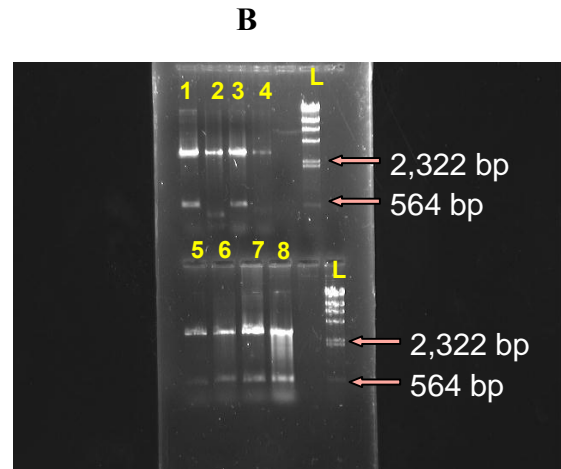
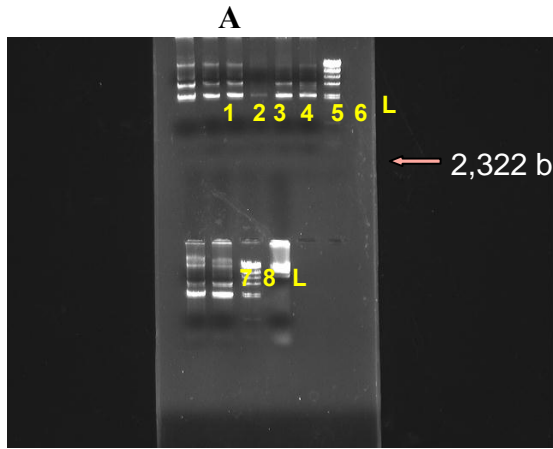
### ***Work done during the year***

During the year, standardization of the optimum conditions for amplification of candidate Inclusion gene has been achieved (Fig. 1).



**Fig. 1: 540bp band showing amplification of *C.trachomatis* specific *inc C* gene.**

The candidate inclusion gene was cloned into pGEM T vector and confirmation of positive inserts was determined using restriction digestion analysis (Fig. 2 (A-B)). pET vector series was chosen as the expression vector for the Inc gene of interest. The samples were confirmed using suitable restriction enzymes choosing internal sites in the MCS of the vector. Expression of Inclusion proteins of interest is underway.



**Fig. 2** A : Lanes 1-8 - white colonies containing Inc C clones in T/A vector. L-Lambda DNA/Hind-III digested Ladder 0.7 % agarose gel  
 B : Lanes 1-8 - white colonies containing Inc C clones in T/A vector which have been digested using ECORI enzyme to confirm size of insert L-Lambda DNA/Hind III digested Ladder 0.7 % agarose gel

## 5. *Role of Iron in Pathogenesis of Chlamydia trachomatis Infection*

*Scientific Staff* : Mr. Harshvardhan, Dr. Aruna Mittal  
*Technical Staff* : Mrs. Madhu Badhwar  
*Duration* : 2004-2007

### *Aims, Objectives and Background*

Iron deprivation synergistically helps *Chlamydia trachomatis* to decrease levels of ROS (Reactive oxygen species) in late stage of development of *C. trachomatis*, thereby

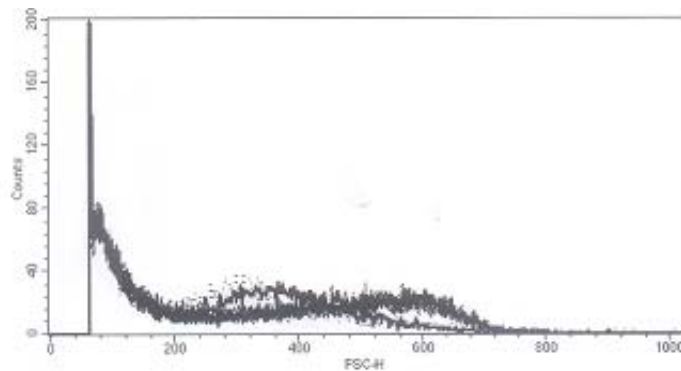
helping chlamydiae to persist. Therefore, to study the role of Iron in persistence of chlamydial infection, levels of ROS *in vitro* in culture system were evaluated in different conditions.

### ***Work done during the year***

In continuation of earlier studies to establish ‘relationship between Iron restriction and apoptosis in *C. trachomatis* infection *in vitro*’ during the year, expression profile analysis of iron and apoptosis related proteins of HeLa cells infected with *Chlamydia trachomatis* in iron restriction and supplementation condition was undertaken. The transition in expression of transferrin receptor (TfR) was not significant in different conditions, whereas Bcl-2 expression was elevated in DFO treated (25µM) HeLa cells infected with *C. trachomatis*.

Cell viability was assessed by MTT assay which revealed that up to 50 µM concentration of Deferoxamine mesylate is well tolerated by *C. trachomatis* infected HeLa cells but not resisted by mock infected cells.

Cellular status of ROS was determined by the intensity of dichlorodihydroflourecein (DCF), resultant product of DCFH-DA and ROS. The intensity of dichlorohydroflourecein (DCF) decreased in *Chlamydia* infected HeLa cells and DFO treated HeLa cells alike but at greater extent in *C. trachomatis* infected HeLa cells treated with Deferoxamine mesylate as assessed by fluorescence microscopy and flow cytometric techniques (Fig. 1).



**Fig. 1: Flow cytometric analysis of ROS generated in infected and mock infected HeLa cells.**

## **6. Screening for *C. pneumoniae* in Coronary Artery Disease Patients by Nucleic Acid Amplification Assays**

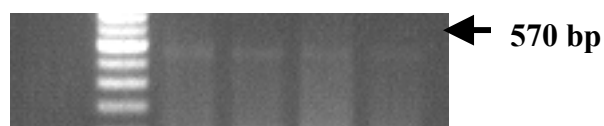
**Scientific Staff** : **Mr. Hem Chandra Jha, Dr. Aruna Mittal**  
**In collaboration With** : **Dr. Jagdish Prasad, SJH**  
**Technical Staff** : **Mr. Yoginder Kumar**  
**Duration** : **2006-2008**

### ***Aims, Objectives and Background***

*C. pneumoniae* has been reported to be responsible for >10 % of case of community acquired pneumonia and to be an etiologic agent of bronchitis & other respiratory tract illness. Various reports have suggested a possible link between *C. pneumoniae* infection and atherosclerotic vascular disease. The emergence of *C. pneumoniae* as a leading causative pathogen in atherosclerosis in India is a cause of concern. Detection of *C. pneumoniae* in circulating blood by nucleic acid amplification assay is required.

### ***Work done during the year***

To understand the role of *C. pneumoniae* in CAD patients, we initially screened CAD patients for presence of *C. pneumoniae* in circulating blood by nucleic acid amplification assays. For this, isolation of genomic DNA from non-heparinized blood was done and later, it was quantified. For amplification, Nested PCR using 16sr- RNA gene was standardized which gave final product of 570 bp (Fig. 1). So far 91 CAD patients were screened of which 23.7% were found positive for *C. pneumoniae* DNA.



**Fig. 1: 570bp showing specific band for *C. pneumoniae***

## ***7. Immune Responses to Chlamydia trachomatis Infection in Spontaneous Aborters***

***Scientific staff*** : ***Dr. Sangita Rastogi, Dr. Aruna Mittal, Ms. Chanchal Yadav***  
***In collaboration with*** : ***Dr. Sudha Salhan, Dr. Banashree Das, SJH***  
***Duration*** : ***Dr. MM Misro, NIHFW, New Delhi***  
***2004-07***

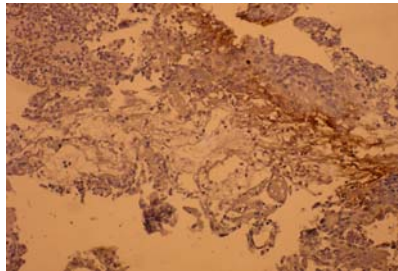
### ***Aims, Objectives & Background***

Infection with *C. trachomatis* is an important public health problem, especially in third world and developing countries. Women largely bear the brunt of *C. trachomatis* infection. During pregnancy, *C. trachomatis* infection may cause a variety of perinatal complications and several studies report that upto 35% of women harbour *C. trachomatis* infection in the endocervix. Pregnant women with *C. trachomatis* infection are ten-fold more likely to suffer an adverse outcome and gestation periods are also significantly shorter. Several studies have reported the presence of chlamydial antigen/DNA/antichlamydial bodies by culture and non-culture techniques in the endometrial curettage tissue (ECT)/endocervical secretions/urine of women undergoing  $\geq 1$  spontaneous abortion/in vitro fertilization/embryo transfer. Despite these associations, the precise immunological mechanism whereby *C. trachomatis* adversely affects early stage pregnancy leading to spontaneous abortion remains to be established.

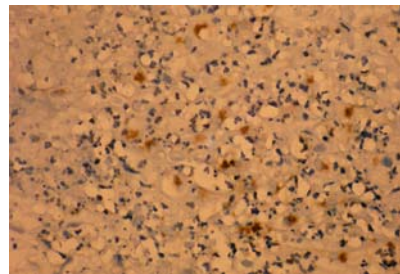
### ***Work done during the year***

During the reporting year, 38 spontaneous aborters (*gestational age*: 04-20 weeks) presenting in Gynecology Receiving Unit (GRR) at Safdarjung hospital, New Delhi were further enrolled for the study after obtaining their informed written consent and with hospital ethical permission. Data has been summarized in 69 cases after including 31 cases reported in last year's Annual Report. Patients were selected as per evaluation by the gynecologist and those with local causes of bleeding were excluded. Non-macerated ECT was collected from spontaneous aborters during dilatation and evacuation (D & E). Blood

was also collected from spontaneous aborters while urine samples were collected from their male partners. Semen sampling and sperm function tests were done in partners of spontaneous aborters at National Institute of Health & Family Welfare, New Delhi and cases showing evidence of male factors were excluded from the study. Spontaneous aborters showing the presence of other STD pathogens, viz.: *Listeria*, streptococcus, microorganisms of TORCH complex, *Mycoplasma hominis*, *Neisseria gonorrhoeae* and *Ureaplasma urealyticum* in ECT/sera were further excluded. The prevalence of *C. trachomatis* was 16.6% and 10.7% by IHC and PCR, respectively in ECT of spontaneous aborters without any previous h/o miscarriage (Group I). In spontaneous aborters with h/o  $\geq 1$  abortion (Group II), the presence of *C. trachomatis* infection was 12.5% by IHC/PCR in ECT. Immunohistochemical staining was standardized for *in situ* localization of cytokines, viz.: TNF- $\alpha$  and IL-8 in formalin fixed, paraffin-embedded ECT sections (Figs. 1-2). Studies on pro-inflammatory cytokine expression in Groups I-II are underway. Also, dual immunostaining for *in situ* localization of chsp60/CD68 positive macrophages in ECT is under standardization.



**Fig. 1:** *In situ* localization of TNF- $\alpha$  in endometrial curettage tissue



**Fig. 2:** *In situ* localization of IL-8 in endometrial curettage tissue

# **LEISHMANIASIS**

## **1. Identification of Virulence-Related Genes in *Leishmania donovani* Using Microarray**

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**In collaboration with** : **Dr. Hira L Nakhasi, Dr. NS Negi**  
**Duration** : **2004- 2008**

### ***Aims, Objectives & Background***

In the *Leishmania* life cycle, the motile promastigote forms that reside in the alimentary canal of the sand-fly vector are transmitted to a mammalian host during a blood meal. The differentiation of *Leishmania* from promastigote to amastigote stage in the mammalian host is associated with differential gene expression important for the parasite's survival in the host and could be targeted therapeutically to block new infection. We carried out studies to identify virulence-related genes in *L. donovani* by studying gene expression at promastigote and amastigote stage and at an intermediate stage of differentiation. In order to look for any change in gene expression during the process of differentiation, we carried out microarray hybridization using RNA from various stages. This is a collaborative study, funded by Indo-US Vaccine Action Program.

Parasites isolated from KA patient were adapted to grow as promastigotes and as axenic amastigotes. RNA was prepared from *L. donovani* parasites cultured in vitro as promastigotes at zero hour, or at an intermediate differentiation time point of 24 hrs (referred to as PA24) and as axenic amastigotes. A prototype chip containing a total of 13584 spots (4224 clones in triplicate with suitable positive and negative controls) was used for microarray hybridization with promastigotes at zero hour vs PA24 RNA in triplicates and with promastigotes vs amastigotes. All details of microarray hybridization, scanning and data analysis were described in last year's report.

## *Work done during the year*

During the year under report, we carried out sequencing of the 57 differentially expressed clones in either of the life cycle stages of *Leishmania* and BLAST analyzed in *Leishmania* geneDB. The identities of these clones and their mean expression ratios along with standard deviation are given in Tables 1-4. The microarray data were normalized based on medians of ratios as well as by Z score transformation and the Z scores of individual genes are given in Tables 1-4. Z score is an index of reproducibility between arrays and a score of 1.5 and above is considered significant. In addition to *L. major*, the clone sequences were also searched by BLAST in the *L. infantum* genome. Among the 30 clones over-expressed at PA24 and Am stages shown in Tables 1 & 3, it is seen that 16 clones showed continuous increase in their expression during the differentiation into Amastigotes. Maintenance genes such as NAD/FAD dependent dehydrogenase, calpain-like cysteine proteinase, serine-carboxypeptidase, aldose-1-epimerase, phosphomannomutase, heat shock protein 83, surface molecules like amastin, proteophosphoglycan (PPG4) were expressed in this pattern. The expression of 14 clones showed a transient increase at PA24 but their expression declined in Am though their expression in Am still remained significantly higher compared to Pro. Most of the protein kinases, HSP 10, surface gene ABC-1 like protein, few hypothetical proteins were found to follow this pattern. Also, few genes showing apparently no homology to any known genes in *L. major* gene database showed increased expression at PA24 and amastigote stages.

**Table 1: PA24 Upregulated clones : identity and mean expression ratios**

Gene category	Clone ID	Gene Identity	Accession no. <i>L.major</i> DB	PA24/Pro ratio (± SD)	Am/Pro ratio (± SD)	Z-ratio
<u>Hypothetical proteins</u>	46G8	3'UTR hypothetical	LmjF28.0980	3.53± 0.10	3.61± 0.26	2.94
	15B2*	3'UTR Hypothetical	LmjF23.1665	3.1± 0.04	2.19± 0.07	4.72
	45E11	Conserved hypothetical protein	LmjF29.2760	2.76± 0.10	2.89 ± 0.20	3.42
	36G8	Conserved hypothetical	LmjF19.0490	2.57± 0.13	1.90 ± 0.12	3.02
	45C12	Conserved hypothetical	LmjF29.1810	2.41± 0.06	1.07 ± 0.11	2.70
<u>Intergenic region*</u>	53H4	Intergenic region	LmjF34	3.36± 0.18	7.27 ± 0.32	3.48
	43E10	Intergenic region b/w histone H4 & Phosphoglycan β-1,3-galactosyltransferase	LmjF36.0010 LmjF35.0010 LmjF31.3190 LmjF25.2450	2.85± 0.09	2.24 ± 0.11	2.07
	61B6	Intergenic region	LmjF31	2.22± 0.18	1.70 ± 0.20	2.32
	34E12	Intergenic region	LmjF33	2.02± 0.10	2.18 ± 0.11	1.98

<u>Kinase</u>	28F11	MAP Kinase homolog	LmjF36.6470	2.56± 0.13	1.48 (0.17)	1.56
	31C2	3'UTR of protein kinase	LmjF31.1530	2.49± 0.07	1.97 (0.17)	2.43
	45F10	3' UTR of mitogen-activated protein kinase	LmjF36.6460	2.42± 0.07	1.98 (0.35)	2.77
	63C8	Protein kinase, putative	LmjF15.1550	2.08± 0.07	0.72 (0.19)	2.34
<u>Heatshock proteins</u>	28F12	Heat shock protein, putative	LmjF26.0620	3.14± 0.15	2.44 (0.20)	3.31
	16C11	HSP83	LmjF33.0314	2.30± 0.19	2.36 (0.13)	2.51
	41E3	Tetratricopeptide repeat protein	LmjF34.3820	1.87± 0.12	0.79 (0.17)	1.41
<u>Surface genes</u>	65G7	3'UTR of ABC1-like protein	LmjF36.0430	3.28± 0.17	2.27 (0.16)	2.00
<u>Maintenance genes</u>	29C8	NAD/FAD dependent dehydrogenase, putative	LmjF15.0970	2.03± 0.10	3.49 (0.24)	3.21

Table 2: PA24 Down regulated clones : identity and mean expression ratios

Gene category	Clone ID	Gene Identity	Accession number <i>L. major</i> Gene DB <sup>a</sup>	PA24/Pro ratio (± SD)	Am/Pro ratio (± SD)	Z-ratio
<u>Hypothetical proteins</u>	67D9	Hypothetical	LmjF27.0350	0.28± 0.59	1.094± 0.09	1.44
	61C6	Hypothetical	LmjF15.1260	0.29 ± 0.76	1.046± 0.03	2.54
	40F12	Hypothetical	LmjF07.1080	0.37 ± 0.38	1.487± 0.18	2.40
	51G11	Hypothetical	LmjF14.1440	0.37 ± 0.66	1.207± 0.02	2.22
	61C9	Hypothetical	LmjF36.6465	0.44 ± 0.49	1.602± 0.25	2.51
<u>Kinase</u>	40E10	Protein kinase A-like pr.	LmjF34.2810	0.33± 0.67	1.270± 0.01	3.11
<u>Chaperone</u>	35G2	T-complex protein	LmjF23.1220	0.33 ± 0.41	1.128± 0.03	1.47
<u>Surface genes</u>	37G1	AA permease,	LmjF27.0680	0.22 ± 0.76	3.12 ± 0.29	4.34
	42A11	PSA 2	LmjF12.0910.	0.25 ± 0.54	3.27 ± 0.07	1.87
	49A11	Surface antigen protein 2	LmjF12.0730	0.25 ± 0.88	1.85± 0.051	1.65
	49A10	AA transporter	LmjF31.0320 LmjF27.0680	0.30 ± 0.87	0.882± 0 .05	1.93
	<u>Maintenance genes</u>	30G11	Universal minicircle-binding protein	LmjF36.1610	0.27± 0.95	0.754± 0.03
37H3		Sn RNA	LmjF27.Sn.RNA.0002	0.36 ± 0.25	1.161± 0.07	2.54
14H6		Glutamate DH	LmjF15.1010	0.37 ± 0.52	1.007± 0.17	2.04
46C4		SL-RNA	LmjF05.SnRNA.02	0.52 ± 0. 29	0.837± 0.02	2.09
45E8		3-hydroxyacyl-coa- DH	LmjF36.1140	0.54 ± 0.49	1.079± 0.05	1.79
48A6		Histone putative H2A,	LmjF17.1550	0.54 ± 0.36	1.139± 0.07	2.08

Table 3: Amastigote Upregulated clones : identity and mean expression ratios

Gene ontology category	Clone ID	Gene identity	Accession number <i>L. major</i> Gene DB <sup>a</sup>	Am/Pro Microarray (±SD)	PA24/Pro microarray (±SD)	Z ratio
<u>Hypothetical proteins.</u>	16D8 ED004269	3'UTR of conserved hypothetical	LmjF19.0980	4.11 ±0.46	2.12 ± 0.07	1.64
	63C5* ED004316	Hypothetical conserved	LmjF09.0640	2.80 ±0.56	1.97 ± 0.05	2.15
	14C7 ED004266	3'UTR of conserved hypothetical	LmjF22.0640	2.53 ±0.34	2.77 ± 0.04	2.00
<u>Kinase</u>	65F10 ED004318	Protein kinase, putative	LmjF04.1210	2.98 ±0.51	1.81 ± 0.03	2.08
<u>Surface genes</u>	18D8 ED481185	Proteophosphoglycan PPG4	LmjF35.0520	4.83 ±0.63	2.47 ± 0.07	2.30
	14D7 CL844745	Amastin-like protein	LmjF08.0670	3.10 ±0.53	2.355± 0.05	3.64
	29B8 ED004283	Amastin, putative	LmjF31.0450	2.95 ± 0.38	2.20 ± 0.11	3.24
<u>Maintenance genes</u>	35C8 ED004287	Calpain-like cysteine Proteinase, putative	LmjF31.0410	3.34 ± 0.34	2.48 ± 0.02	2.38
	22C10 ED004275	Aldose-1 epimerase like protein	LmjF35.0980	3.1±0.29	2.60 ± 0.01	3.38
	46F7 ED004306	Spliced leader RNA	LmjF35.SnoRNA.0053	2.73 ±0.48	2.34 ± 0.05	3.00
	41B10 ED004295	Serine carboxypeptidase CBP-1, putative	LmjF18.0450	2.48 ±0.39	1.37 ± 0.06	2.03
	40B11 ED004292	Phosphomannose mutase, putative	LmjF36.1960	2.25 ±0.33	2.64 ± 0.02	1.31

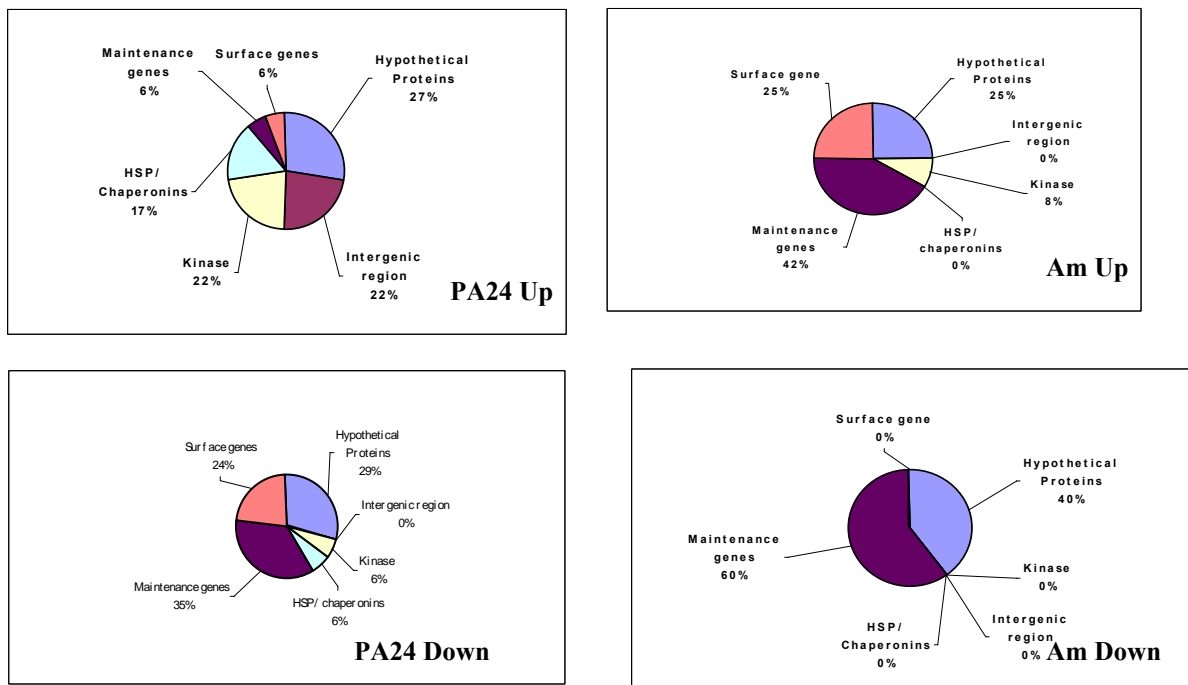
Table 4: PA24 Down regulated clones : identity and mean expression ratios

<u>Gene ontology category</u>	Clone ID	Gene identity	Accession number L.major Gene DB	Am/Pro Microarray ( $\pm$ SD)	PA24/Pro microarray ( $\pm$ SD)	Z ratio
<u>Hypothetical proteins</u>	23G7 ED004280	Hypothetical conserved	LmjF27.0600	0.219 $\pm$ 0.32	0.341 $\pm$ 0.16	2.70
	23A11 ED004276	Conserved hypothetical	LmjF34.	0.278 $\pm$ 0.20	0.377 $\pm$ 0.02	2.51
	23B8* ED004278	Conserved Hypothetical	LmjF25.1445	0.359 $\pm$ 0.63	0.555 $\pm$ 0.05	1.75
	23A3 ED004277	Conserved hypothetical	LmjF34.1390	0.392 $\pm$ 0.43	0.366 $\pm$ 0.01	2.26
<u>Maintenance genes</u>	41D6 ED004296	Eukaryotic translation initiation factor 3 subunit, putative	LmjF36.3880	0.338 $\pm$ 0.27	0.465 $\pm$ 0.04	1.88
	13H8 ED004265	Stomatin-like protein	LmjF05.1040	0.354 $\pm$ 0.27	0.364 $\pm$ 0.03	1.32
	23G11 ED004279	Kinesin, putative	LmjF09.0120	0.378 $\pm$ 0.57	0.316 $\pm$ 0.03	1.78
	44D6 ED004300	V-type ATPase, subunit, putative C	LmjF30.3660	0.4 $\pm$ 0.98	0.411 $\pm$ 0.04	2.72
	68D7 ED004322	Methyltransferase, putative	LmjF22.0090	0.434 $\pm$ 0.92	0.578 $\pm$ 0.03	1.85
	67B10 ED004320	40S ribosomal protein S3a, putative	LmjF35.0420	0.485 $\pm$ 0.47	0.411 $\pm$ 0.01	2.06

Among the 27 clones showing down regulation in PA24 stage (clones showing up regulation at Pro), the expression of 6 clones decreased further in Am. Most of the maintenance genes were found to belong to this category. The lower expression of 4 clones at PA24 remained uniform in Am. However, in 13 clones though the expression was low in PA24, their expression was regained as the parasite fully differentiated into Am. Of particular interest were 4 clones which showed transient decrease at PA24 stage while their expression level increased by > 1.5 fold in Am in comparison to Pro. The identity of these clones revealed them to be surface molecules such as antigen proteins and amino acid transporters.

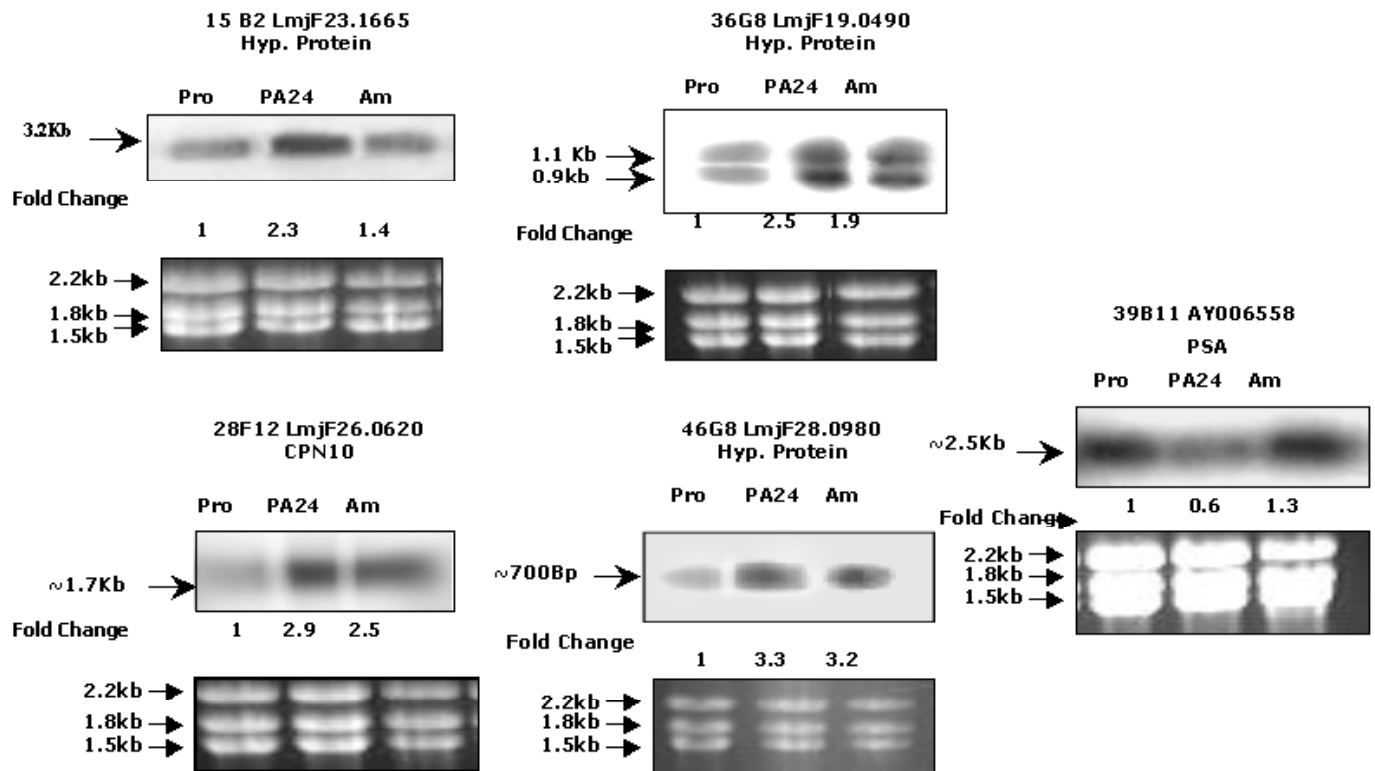
Based on the identities of the 57 differentially expressed clones, putative functions were assigned by homology to proteins of known function. These genes were classified into various groups based on their function. The genes coding for proteins involved in basic cell metabolism for maintenance of life were grouped as maintenance genes whereas those coding for surface proteins were classified as surface genes. Kinases include the genes coding for protein kinases and HSP/ Chaperonins represents the heat shock proteins and other chaperone

proteins. The category, intergenic region, represents the portion of clones that apparently do not code for any protein and are located between two ORFs. The category assignments are given in Tables 1-4, and the proportion of genes in each category is represented in pie charts (Fig. 1). Analysis of Fig. 1 reveals that the largest category of identified genes upregulated in the PA24 stage was the kinase category (22% in PA24 compared to 8% in amastigote up regulated and 6% in PA24 downregulated clones), while the largest category downregulated was the maintenance genes (35% of PA24 downregulated genes). HSP/Chaperones were the other major category showing upregulation at PA24 stage (17% in PA24 and 6% in the PA24 downregulated clones).

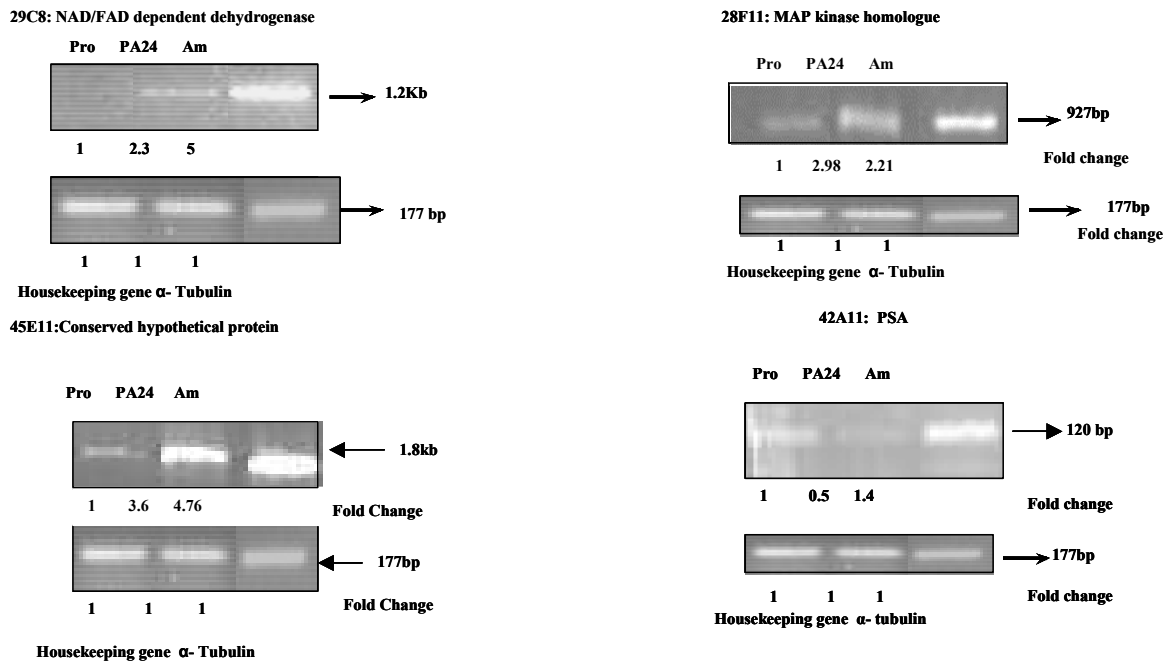


**Fig. 1: Pie charts showing distribution of differentially regulated genes in PA24 vs Pro and Am vs Pro microarray experiments. 57 clones showing  $\geq 1.7$  fold change in the expression levels in either PA24 vs Pro or Am vs Pro microarrays were assigned to functional categories by homology with *L. major* annotated genes and the proportion of differentially expressed genes in each category represented in pie charts.**

The expression changes in representative clones from each category were verified by Northern and RT-PCR experiments. The gene expression changes in 5 clones representing different categories were validated by northern blots in two different patient isolates of *L. donovani* (Fig. 2) and 5 other clones were tested by RT-PCR in three field isolates (Fig 3). Northern blots and RT-PCR with different parasite lines gave similar expression levels and the expression pattern observed by RT-PCR and Northern blots were found to be similar to microarray results. PCR reactions were also carried out with parasite RNA with all of the above mentioned primer sets but no amplification was seen with RNA indicating the absence of DNA contamination in the RNA samples.



**Fig. 2: Northern blots for Validation of microarray data. Ethidium bromide stained rRNA bands were used as loading controls.**



**Fig. 3: Validation of microarray data by RT-PCR. PCR amplified  $\alpha$ - tubulin was used for normalization. For negative control, RT-PCR was done with total RNA to check for DNA contamination in RNA samples.**

### *Future Plan of Action*

Based on the microarray results, we have selected a few genes representing each category for further characterization. These include calpain (showing transient upregulation at PA24 stage), NAD/FAD dependent dehydrogenase (showing continuous increase at both PA24 and amastigote stages), a trypanosomatid specific gene showing upregulation at PA24 and amastigote stages and PSA showing transient decreased expression at PA24 stage. The presence of these amastigote upregulated gene transcripts will also be checked in human bone marrow samples and in true amastigotes derived from cultured macrophages or infected animals. The expression of these genes will be further tested at the protein level to check for their differential expression. Recombinant proteins to these genes will be produced and antibodies will be raised. The subcellular localization of these proteins will be checked by immunofluorescence assays. The functional study of these genes will be tested by overexpression in parasites. The functional study of some of array-identified genes might provide useful insights in *Leishmania* survival and pathogenesis.

## 2. AP-PCR for Identification of Differentially Expressed Genes in PKDL

**Scientific staff** : **Dr. Poonam Salotra, Dr. Ruchi Singh  
Mr. BV Subba Raju, Mr. Rajesh Kumar**  
**In collaboration with** : **Dr R K Jain, Dr V Ramesh, SJH**  
**Duration** : **2002- 2006**

### *Aims, Objectives & Background*

A correlation between differentiation of *Leishmania* from promastigote to amastigote form and expression of a variety of genes has been established in several species of the parasite, but not in parasite of PKDL origin. We have exploited AP-PCR technique for identification of differentially expressed genes in PKDL isolates on the basis of their expression in different stages of the parasite and comparative expression in PKDL isolates versus KA isolates.

### *Work done during this year*

Studies on DNA fingerprinting with PKDL and different geographical isolates were extended further, using primer AP-16 as shown in (Fig. 4). The analysis with AP-16 was extended to 14 PKDL isolates; and a similar pattern was obtained with all PKDL isolates.

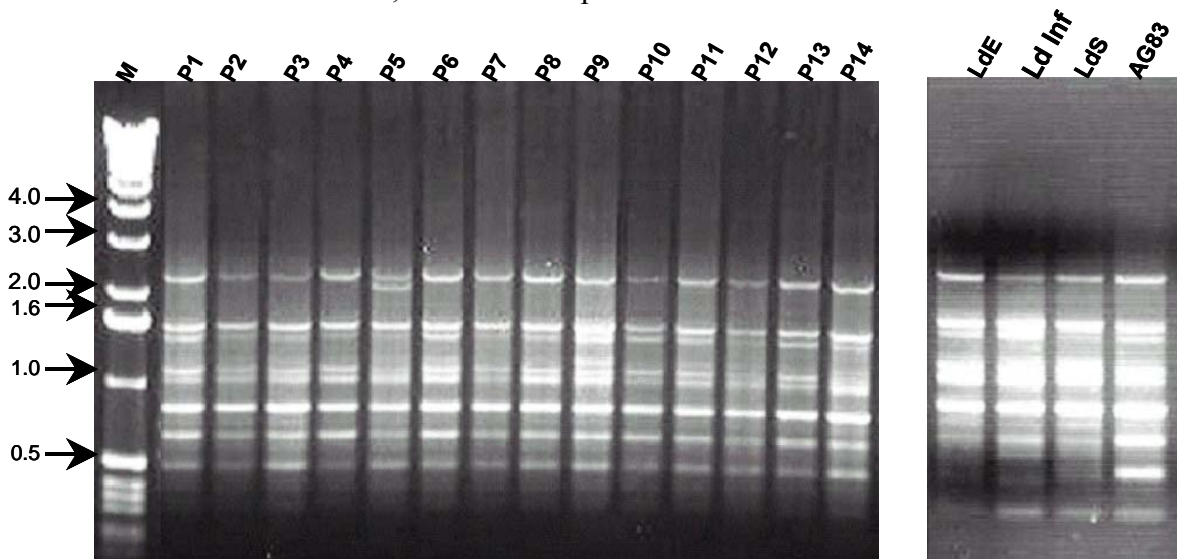
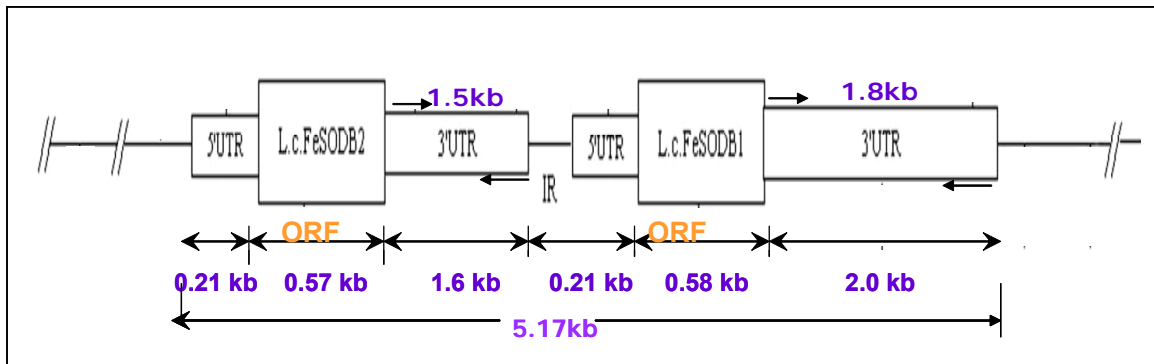


Fig. 4. AP-PCR amplification using DNA 50ng and AP-16 primer with *L. donovani* isolates of PKDL origin( Lane 1-14) and geographical isolates of *L. donovani* Ethiopia (Ld E); L.infantum (Ld Inf); Sudan (Ld S); and India (AG83) (Lane 15-18) ; M -1kb DNA ladder.

Four polymorphic fragments were reported last year in pro and amastigote stages of PKDL isolates using oligos AP-9 and AP-10 by altering primer and template DNA concentration since the amplification patterns in AP-PCR are sensitive to such alterations. In last year's report, northern analysis for these 4 fragments was given. In the current year, one more polymorphic band at 0.3 kb was identified in PKDL isolates with AP-9 using 100 ng DNA. Cloning and sequencing of the polymorphic band (0.3 kb) was done and analysis of the sequence in GeneDB revealed its identity with 3'UTR of phosphodiesterase (LmjF18.1090; LinJ18.1100). Northern analysis revealed 2-fold higher expression in promastigote stage. RT-PCR analysis was conducted using primers from full length ORF and normalization with alpha tubulin (Fig. 5A). The product of 1.8kb showed higher expression in pro compared to amastigotes. Results were comparable with northern data with both PKDL and KA isolates showing 2 fold higher expressions in promastigote. The gene expression was validated in 3 different KA isolates. Last year, we had shown Northern analysis with UTR of RNA helicase DEAD box depicting differential expression in pro and am stages. This was extended to carry out RT-PCR from full length ORF region. Results of the RT-PCR analysis with full ORF are having concurrent expression level (fold change) with that of northern blots analysis. RNA helicase DEAD BOX depicted 2.0 higher expressions in amastigote stage compared to promastigotes stage (Fig. 5B). Further attempts were made to detect presence of gene transcripts in parasite infected lesion tissues. The 1.7 kb transcripts corresponding to ORF of *Leishmania* RNA helicase DEAD BOX was demonstrated in human bone marrow and skin lesions cDNA of three PKDL and KA patients tested while no corresponding band was seen in control samples ( Fig. 6). HPRT gene was used for normalization.

### **Cloning and characterization of Iron superoxide dismutase (FeSODB)**

FeSODB are of two forms: *fesodb1* and *fesodb2*. These two genes are having 88% identical ORF amino acid sequences and differ primarily by the presence of a 14-aminoacid extension found at the carboxyl terminus of *fesodb2*. The last three amino acids of *fesodb1* (SQL) and *fesodb2* (SDL) resemble the typical glycosomal localization signal sequence, SKL & SDL, suggesting that these proteins may be localized to the glycosomes and code for Iron Superoxide dismutases (*fesodb*).

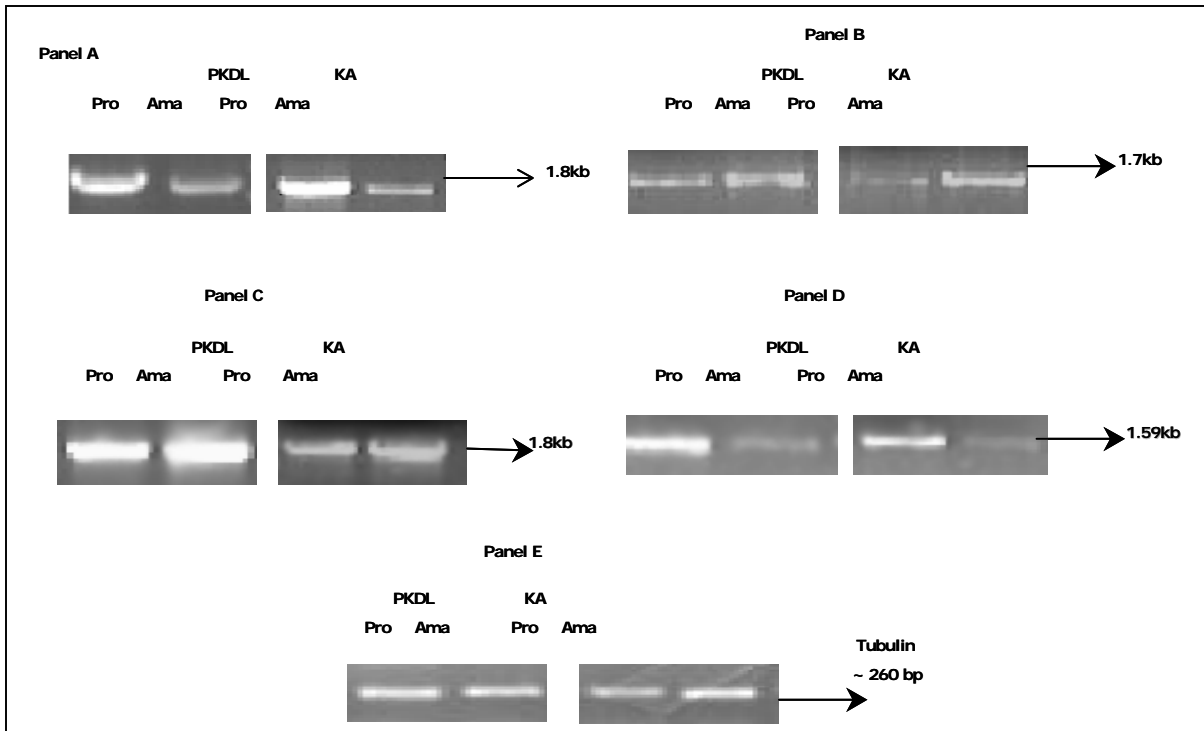


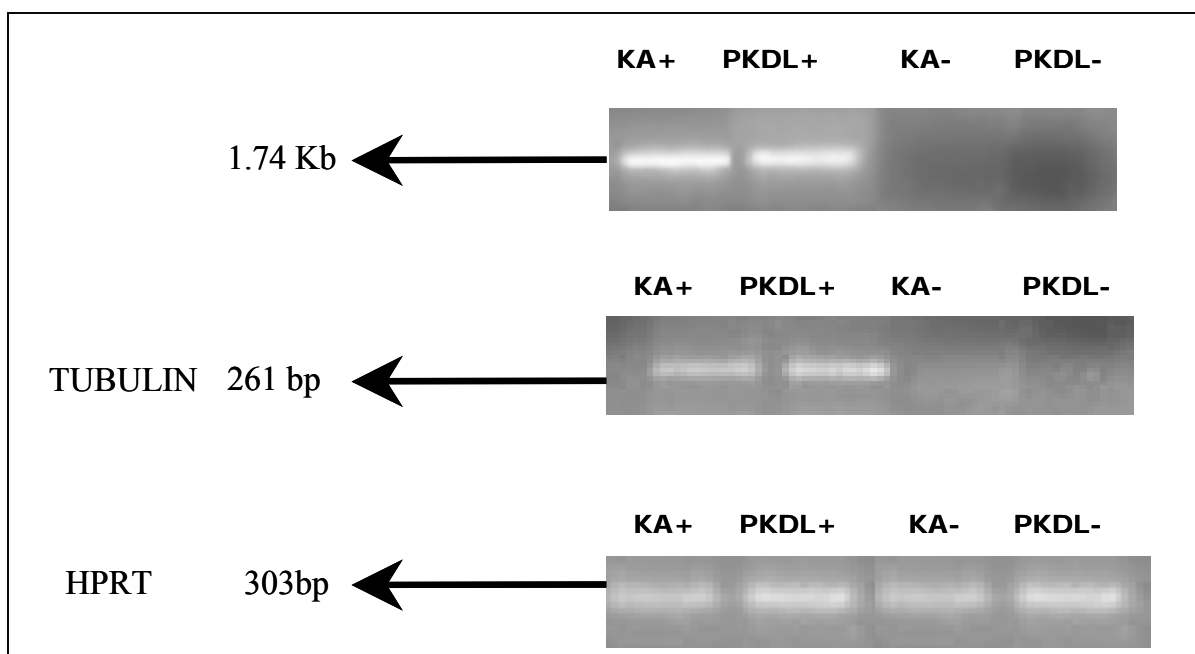
**Schematic representation of Iron Superoxide dismutase (FeSOD) in *Leishmania donovani***

Genomic DNA from PKDL isolate was taken and subjected to amplification of SODB gene for expressing the protein. The amplified product was cloned into pGEM-T easy vector followed by ligation in PGEX-4t-1 expression vector (*Amersham Biosciences*). Colonies were screened using NotI enzyme and confirmed colonies were sequenced to check for correct orientation of the ORF. Gene was expressed with GST tag and subjected to purification using GST-CL Agarose column (*Bangalore Genei*). The protein was subjected to western blot using anti-GST antibodies. GST-SOD fusion protein gave a 43-kDa band on the SDS-PAGE similar to the report for *L. chagasi*. Purified protein with equal amount was subjected to western blot using PKDL, KA and healthy control serum to determine the humoral response in patients. Both KA and PKDL sera reacted positive showing presence of antibodies to *fesod*.

Since sequences of 3' UTR's of SODB1 and B2 are different, these UTR's can be used to differentiate the expression of the gene. To find out the differential expression of this gene, we carried out RT-PCR using gene specific 3' UTR of *fesodb1*. RT-PCR revealed a product of 1.8 kb that showed increased intensity in amastigote stage (2.5 fold) in comparison with the promastigotes (Fig. 5C). In contrast, RT-PCR analysis using 3' UTR of *fesodb2* revealed a band of 1.6 kb that decreased (2.0 fold) in intensity from the amastigote to promastigote stage (Fig. 5D). Results demonstrate that the *fesodb* genes are differentially expressed in the 2 stages of the parasite.

In conclusion, AP-PCR was exploited to identify differentially expressed genes in PKDL isolates; five polymorphic fragments were identified in PKDL in comparison with KA isolates, and were subjected to northern blot analysis. All five represented transcribed sequences; four out of five showed differential expression in pro and amastigote stages of PKDL. BLAST analysis of the transcribed sequences showing differential expression depicted their homology to Iron Superoxide dismutase (*fesodb*), Phosphodiesterase putative, DEAD box RNA helicase and a hypothetical protein.





**Fig. 6:** RT-PCR to detect the expression of DEAD box RNA helicase in bone marrow and skin lesions .  $\alpha$ -Tubulin of *Leishmania* and HPRT human house keeping genes were used for normalizing the expression of RNA. KA- and PKDL- are taken from healthy patients of bone marrow and tissue samples.

### ***3. Characterization of Cellular Immune Responses in KA and PKDL***

***Scientific staff*** : ***Dr. Poonam Salotra, Mr. Nasim A Ansari, Mr. Gajendra Katara***

***In collaboration with*** : ***Dr. V Ramesh, Dr. S. Saluja, SJH***

***Duration*** : ***2002- 2009***

#### ***Aims, Objectives & Background***

In human infections with *L .donovani*, a marked depression in the Th1 responses is characterized by absence of proliferative responses or inability to secrete IL-2 and IFN- $\gamma$  in responses to *Leishmania* antigens. Active leishmaniasis is considered to be driven by parasite induced Th2 responses of which IL-4 is the predominant cytokine. It has been shown in studies in Sudan that high levels of IL-10 production have predictive value for

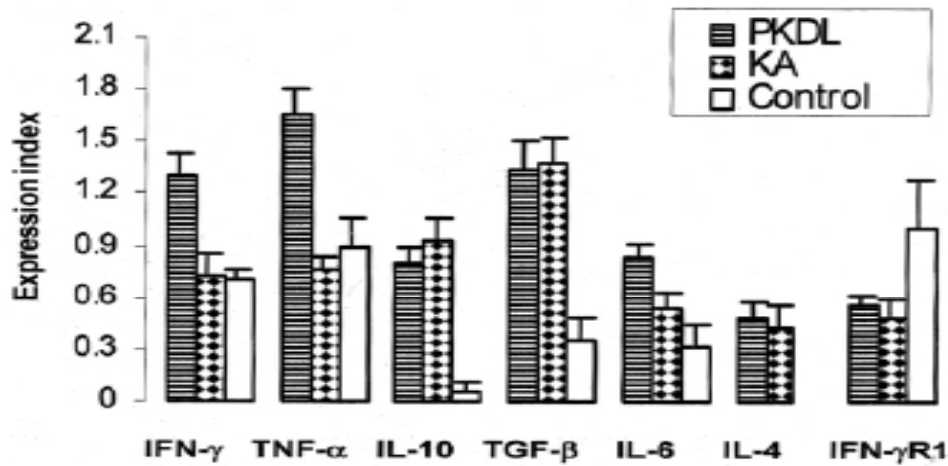
subsequent development of Post kala -azar dermal leishmaniasis (PKDL). Characterization of systemic and localized immune responses were carried out in Indian Kala-azar (KA) and Post kala-azar dermal leishmaniasis (PKDL) patients, to dissect the immunological parameters involved in the pathogenesis of Indian leishmaniasis. Results of circulatory immune responses was given last year and here we give results of localized cellular responses in tissue lesions of both KA and PKDL. This study was carried out under a project funded by LSRB, DRDO.

### ***Work done during this year***

Semi quantitative RT-PCR was exploited to evaluate cytokine microenvironment in lesion tissues collected from 28 PKDL and 14 KA patients (dermal lesion tissue and BMA respectively).

#### ***Cytokine message level for IFN- $\gamma$ & TNF- $\alpha$***

Message for IFN- $\gamma$  & TNF- $\alpha$  was detectable respectively in 100% & 92.8% of PKDL patients and in 92.8% and 100% of KA patients. The expression levels of both cytokines were found significantly elevated in PKDL cases compared to control ( $P < .05$ ,  $P < .05$ ) and compared to KA cases ( $P < .01$ ,  $P < .001$ ; Fig. 7). After treatment, significant decrement in IFN- $\gamma$  ( $P < .05$ ) & TNF- $\alpha$  ( $P < .05$ ) level was noticed in PKDL and the expression of both cytokines was restored to near control values (Fig. 8).



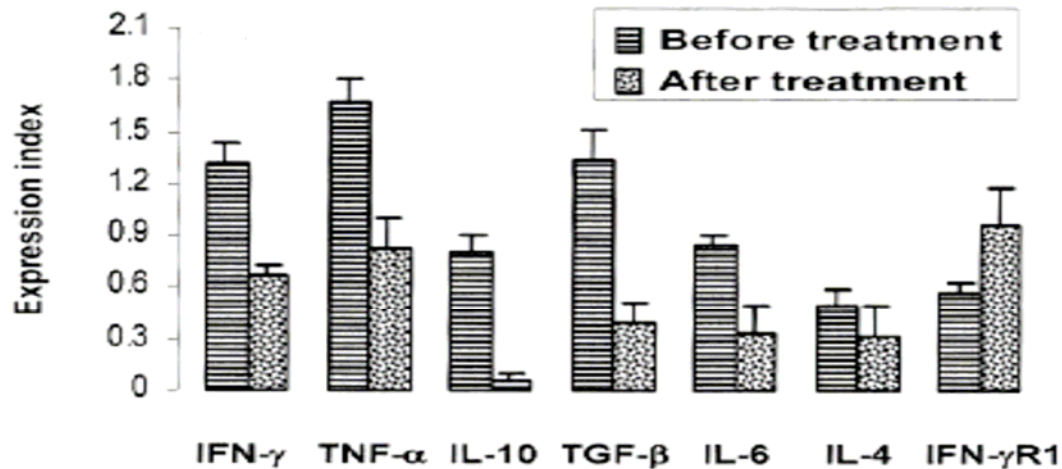
**Fig. 7:** Levels of cytokine (interferon [IFN], interleukin [IL], tumor necrosis factor [TNF], transforming growth factor [TGF], and IFN- $\gamma$  receptor 1 [IFN- $\gamma$ R1]) mRNA in lesion tissues from patients with post-kala azar dermal leishmaniasis (PKDL;  $n = 28$ ) and kala azar (KA;  $n = 14$ ) and in control tissues ( $n = 7$ ). Normalized cDNA was amplified with respective cytokine primers. Polymerase chain reaction products were electrophoresed, and the intensity of signal was determined by densitometry. The graph shows results as an expression index, defined as the ratio of the intensity of cytokine with respect to the HPRT gene. The bars indicate SEs.  $P < .05$  was considered to be statistically significant.

#### ***Cytokine message level for IL-10 & TGF- $\beta$ in tissue lesions***

Message for both IL-10 & TGF- $\beta$  was detectable in 92.8% of PKDL patients and respectively in 92.8% and 100% of KA patients. The level of IL-10 & TGF- $\beta$  was found significantly elevated in PKDL compared to control ( $P < .01$ ,  $P < .05$ ; Fig. 7), whereas the difference in expression was not significant between PKDL and KA cases. Upon treatment, significant decrement in the expression level of IL-10 & TGF- $\beta$  was noticed in PKDL cases ( $P < .01$ ,  $P < .05$ ; Fig. 8).

#### ***Cytokine message level for IL-6 in tissue lesions***

The level of IL-6 was detected in 100% PKDL and 85.7% KA cases and was found significantly elevated in PKDL cases compare to KA ( $p < .05$ ) or control ( $P < .01$ ; Fig. 7). Similar to other cytokines, upon SAG treatment significant decrement in the level of IL-6 was noticed in PKDL cases ( $P < .01$ ; Fig. 8).



**Fig. 8** Levels of cytokine (interferon [IFN], interleukin [IL], tumor necrosis factor [TNF], transforming growth factor [TGF], and IFN- $\gamma$  receptor 1 [IFN- $\gamma$ R1]) mRNA in post-kala azar dermal leishmaniasis (PKDL) tissues before ( $n = 28$ ) and after treatment ( $n = 7$ ). Normalized cDNA was amplified with respective cytokine primers. Polymerase chain reaction products were electrophoresed, and the intensity of signal was determined by densitometry. The graph shows results as an expression index, defined as the ratio of the intensity of cytokine with respect to HPRT gene. The bars indicate SEs.  $P < .05$  was considered to be statistically significant.

#### ***Cytokine message level for IL-4 in tissue lesions***

Involvement of IL-4 in disease progression has been mainly shown in the murine model. However, the role of IL-4 in the regulation of immunity to *L. donovani* infection is still poorly understood. The message level of IL-4 gene was detectable in PKDL 64.2% and KA 57.1% cases, with no significant difference between PKDL and KA cases. Interestingly, IL-4 was not detectable in any of the control tissues. Moreover, we did not find a significant decrement in IL-4 expression level upon treatment (Fig. 8).

#### ***Cytokine message level for IFN- $\gamma$ R1 in tissue lesions***

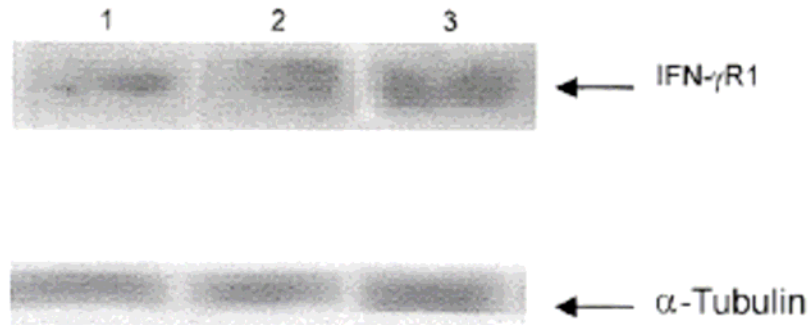
Expression of IFN- $\gamma$ R1 was detectable in 89.2% PKDL and 78.5% KA cases while 85.7% control cases had detectable IFN- $\gamma$ R1. The expression level was significantly lower

in PKDL cases compared to control ( $P < .05$ ; Fig. 7). A significant elevation in the level was noticed in post-treated PKDL cases that approached the control value ( $P < .05$ ; Fig 8). No significant difference was observed in the expression of IFN- $\gamma$ R1 between KA & PKDL cases.

### ***Reduced IFN- $\gamma$ R1 protein expression in tissue lesion***

As the message level of IFN- $\gamma$ R1 was significantly decreased in lesion tissue of PKDL patients, we sought to determine whether comparable reduction in IFN- $\gamma$ R1 could be demonstrated at protein level. Therefore, we investigated the IFN- $\gamma$ R1 protein expression using western blot analysis with a commercially available anti human IFN- $\gamma$ R1. Densitometric analysis of IFN- $\gamma$ R1 protein expression revealed 50 percent decrease in intensity, taking the percent expression in normal tissue as hundred. Thus, data reveals down regulation of IFN- $\gamma$ R1 expression in lesion tissue of PKDL compared to normal tissue. Further, upon treatment with antileishmanial drug SAG the expression of IFN- $\gamma$ R1 protein was restored to 80 percent of the control level (Fig. 9).

Thus, data revealed mixed Th1 & Th2 responses, as reflected by elevated IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$ , IL-10, IL-6 and IL-4 with minimal expression of IFN- $\gamma$ R1 message in PKDL lesions compared to normal skin tissue. In comparison with KA, message for IFN- $\gamma$ , TNF- $\alpha$  and IL-6 were found significantly elevated in PKDL lesions, implying an important role of these cytokines in PKDL pathogenesis. In presence of elevated levels of IFN- $\gamma$  and TNF- $\alpha$ , interference with type 1 effector activity in PKDL may be due to minimal expression of IFN- $\gamma$ R1 gene or simultaneous presence of elevated levels of IL-10, IL-6 & TGF- $\beta$  that have counter acting activities. Upon treatment, the restoration of IFN- $\gamma$ R1 at both message and protein levels, coupled with down regulation of counter acting cytokines, may facilitate the action of signals associated with IFN- $\gamma$ , yielding parasite clearance. The study emphasizes that host immune determinants in PKDL may have a vital role in coordination of effective immune response against the pathogen. Less favorable clinical evolution is not due to the absence of an intralesional Th1-type response, rather it may be associated with presence of counteracting cytokines along with down modulation of IFN- $\gamma$ R1.



**Fig. 9** Western blot analysis for protein level of interferon- $\gamma$  receptor 1 (IFN- $\gamma$ R1) in lesion tissue lysate from patients with post-kala azar dermal leishmaniasis before treatment (*lane 1*) and after treatment (*lane 2*), compared with that in control tissue (*lane 3*). The blot was stripped and reprobbed with anti- $\alpha$ -tubulin antibody (Santa Cruz Biotechnology), to control for protein loading. The experiment was performed with tissue from 3 different patients with PKDL, 3 control tissues, and tissue from 1 patient who was followed up, and representative data are shown.

### ***Future Plan of Action***

It is proposed to evaluate the gene expression of immuno-determinants (cytokines and chemokines/receptors) in KA and PKDL lesion tissues using cDNA microarray. Validation of microarray data will be carried out using real time PCR and at protein level by western blotting and immunohistochemistry. This will pave way for elucidation of defect in signaling pathways using pathway based real time PCR assay.

#### ***4. Studies on Antimony Resistance in Field Isolates of Leishmania donovani from Indian Patients of Kala-azar and PKDL***

***Scientific staff*** : ***Dr. Poonam Salotra, Dr. Ruchi Singh, Mr. Dhiraj Kumar, Ms. Arpita Kulshrestha***  
***In collaboration with*** : ***Dr. V Ramesh, Dr. NS Negi, SJH***  
***Duration*** : ***2005-2008***

#### ***Aims, Objectives & Background***

Kala-azar (KA) is a chronic infection caused by protozoan parasite *Leishmania donovani*, associated with high morbidity and mortality. Post kala-azar dermal leishmaniasis (PKDL) develops in 5-15% of treated KA patients in India. Cases of PKDL are of considerable epidemiological importance, acting as a reservoir of parasite during inter-epidemic periods of KA. Unresponsiveness of KA patients to the first line drug Sodium antimony gluconate (SAG) has reached alarming proportions (>60%) in Bihar, India. Effective treatment of PKDL cases require parenteral SAG treatment for a duration exceeding 4 times of the treatment required for KA, at the same dose. There is evidence that an inherent lack of susceptibility and/or the acquired resistance by the parasite contributes to unresponsiveness to antimonials in KA, however such studies are lacking for PKDL.

Monitoring of antimonial resistance is a crucial issue in the endemic anthroponotic focus in India. The higher drug requirement for effective treatment of PKDL and increasing number of treatment failures in India, led us to investigate the role of anthroponotic transmission in the spreading of drug-resistant parasites as a possible cause of SAG unresponsiveness. With this objective in view, a comparison of the SAG sensitivity of parasites from KA and PKDL patients originating from regions of different endemicity was undertaken. We also sought to study the amplification of genes shown to be involved *in vitro* in conferring resistance i.e. *pgpA*, *ldmdr* and *gsh-1*.

## *Work done during this year*

SAG susceptibility of *Leishmania* isolates was determined in field isolates from KA and PKDL patients, taking care to include SAG responsive and non responsive cases. Susceptibility was initially determined using promastigotes and subsequently in amastigotes using J774 macrophage like cell line. The data with susceptibility at amastigote stage was further analysed.

The patients hailed from different zones of endemic area (Bihar and W. Bengal) with varying degree of endemicity and resistance reported as high (HR), medium (MR) and low endemicity (LR) zones. The profile of the KA patients and the range of SAG sensitivity of the KA *Leishmania* isolates is summarized in the Table 5. Based on a recent comprehensive study on the SAG pharmacokinetics in Indian KA patients, we categorized the parasites with  $ED_{50} > 11 \mu\text{g/ml}$  as resistant. The mean  $ED_{50} \pm \text{SEM}$  for KA isolates (n=19) as amastigotes was  $11.0 \pm 1.38 \mu\text{g/ml}$ . KA isolates from LR or MR zones showed the  $ED_{50}$  in the range of 2.1 to  $10.4 \mu\text{g/ml}$  of SAG. A strong correlation was observed between the isolates from different endemicity zones with *in vitro* susceptibility ( $r_{\text{rank}} = 0.997$ ) as well as the clinical response ( $r_{\text{rank}} = 0.991$ ). 11/19 KA isolates (58%) showed  $ED_{50} > 11 \mu\text{g/ml}$ , all 11 originating from HR zone. The median  $ED_{50}$  of the resistant KA isolates was  $15.7 \mu\text{g/ml}$ .  $ED_{90}$  of sensitive KA isolates ranged between 20-60  $\mu\text{g/ml}$  while for resistant isolates it was  $> 100 \mu\text{g/ml}$ .

The characteristics of the PKDL patients and the SAG susceptibility for the PKDL isolates is summarized in Table 6. The mean  $ED_{50} (\pm \text{SEM})$  for PKDL isolates (n=18) as amastigotes was  $12.0 \pm 2.49 \mu\text{g/ml}$ . Majority (6/7) of PKDL isolates from LR or MR zone displayed high susceptibility to SAG with  $ED_{50}$  in the range of 2.9-  $8.5 \mu\text{g/ml}$  while one isolate showed exceptionally high  $ED_{50}$  of 36  $\mu\text{g/ml}$ . Isolates from HR zones displayed varying degree of SAG sensitivity with the  $ED_{50}$  ranging from 3.0 to  $31.2 \mu\text{g/ml}$ . Only 7/18 PKDL isolates (39%) showed  $ED_{50} > 11 \mu\text{g/ml}$ ; all but one of them belonged to HR region. The median  $ED_{50}$  of the resistant PKDL isolates was  $20.0 \mu\text{g/ml}$  of SAG. A good correlation was evident between the PKDL isolates from different endemicity zones with *in vitro* susceptibility ( $r_{\text{rank}} = 0.97$ ) as well as with the clinical response ( $r_{\text{rank}} = 0.95$ ).  $ED_{90}$  of

sensitive PKDL isolates ranged in between 10-60 µg/ml while for resistant isolates it was >100µg/ml.

**Table:5 Clinical profile of patients with visceral leishmaniasis (VL) who are from different zones of endemicity, and in vitro susceptibility of intracellular amastigotes to sodium antimony gluconate (SAG).**

Patient (age, years; sex)	Region, zone <sup>a</sup>	Response <sup>b</sup>	ED <sub>50</sub> (µg/mL) for amastigotes <sup>c</sup>
K1 (14; M)	Western Bengal, LR	R	2.1 ± 0.28
K2 (20; M)	Western Bengal, LR	R	3.4 ± 0.28
K3 (24; F)	Munger, LR	ND	3.9 ± 0.28
K4 (10; F)	Munger, LR	R	4.7 ± 0.42
K5 40/ F	Bhagalpur, LR	NR	10.4 ± 2.17
K6 (45; M)	Gopalganj, MR	R	4.2 ± 0.38
K7 7/M	Araria, MR	R	4.2 ± 1.03
K8 (36; F)	Siwan, MR	R	5.6 ± 0.57
K9 (22; F)	Madhubani, HR	R	11.3 ± 0.89
K10 (11; M)	Saran, HR	ND	11.8 ± 1.28
K11 (21; F)	Vaishali, HR	NR <sup>d</sup>	14.7 ± 3.29
K12 (7; F)	Madhubani, HR	NR <sup>d</sup>	15.8 ± 1.03
K13 (22; M)	Saharsa, HR	NR	19.4 ± 1.68
K14 (12; M)	Saran, HR	... <sup>e</sup>	13.0 ± 4.12
K15 (7; F)	Dharbhanga, HR	... <sup>e</sup>	13.6 ± 2.51
K16 (20; M)	Saran, HR	... <sup>e</sup>	15.7 ± 4.01
K17 (17; F)	Madhubani, HR	... <sup>e</sup>	17.3 ± 4.32
K18 (23; F)	Saharsa, HR	... <sup>e</sup>	17.8 ± 2.28
K19 (24; M)	Saran, HR	... <sup>e</sup>	20.3 ± 0.84

**NOTE.** HR, high resistance to SAG; LR, low resistance to SAG; MR, moderate resistance to SAG; ND, not determined; NR, not responsive to SAG; R, responsive to SAG.

<sup>a</sup> Based on data reported by Sundar [2].

<sup>b</sup> After 30 days of treatment with SAG at a daily dose of 20 mg/kg body wt. Patients without fever and with reduction in spleen size were classified as R; the other patients were classified as NR. All patients were monitored for 6 months, and, if there were no signs of relapse, were considered to have been cured; during this period, none of the cured patients with VL developed post-VL dermal leishmaniasis.

<sup>c</sup> For 3 assays.

<sup>d</sup> Patient died during treatment.

<sup>e</sup> Patient was treated with amphotericin B; therefore, responsiveness to SAG could not be determined.

There was no significant difference in the overall mean ED<sub>50</sub> (ED<sub>90</sub>) of KA and PKDL isolates indicating that pharmacokinetics of SAG may not be responsible for longer treatment required in PKDL. Comparing the susceptibility of all the resistant strains, PKDL isolates had significantly higher median ED<sub>50</sub> compared to KA isolates (p<0.05); emphasizing the fact that the prolonged exposure to SAG promotes parasitological resistance.

**Table 6 Clinical profile of patients with post-*visceral leishmaniasis* (VL) *dermal leishmaniasis* (PVL) who are from different zones of endemicity, and *in vitro* susceptibility of intracellular amastigotes to sodium antimony gluconate (SAG).**

Patient (age, years; sex)	Region, zone <sup>a</sup>	History of VL, years	Response <sup>b</sup>	ED <sub>50</sub> (µg/mL) for amastigotes <sup>c</sup>
P1 (18; M)	Western Bengal, LR	15	ND	4.8 ± 0.23
P2 (28; M)	Siwan, MR	8	R	2.9 ± 0.13
P3 (20; M)	Munger, MR	8	R	4.2 ± 0.56
P4 (18; M)	Gopalganj, MR	3	R	6.3 ± 0.24
P5 (25; M)	Siwan, MR	1	R	6.4 ± 0.33
P6 (25; M)	Ararhia, MR	6	SR	8.5 ± 0.78
P7 (35; M)	Gopalganj, MR	9	ND	36.0 ± 3.35
P8 (32; M)	Samastipur, HR	3	R	3.0 ± 0.41
P9 (12; M)	Darbhanga, HR	4	R	3.9 ± 0.42
P10 (20; M)	Darbhanga, HR	0.5	R	4.3 ± 0.32
P11 (10; M)	Vaishali, HR	4	R	4.7 ± 1.04
P12 (10; M)	Vaishali, HR	4	R	5.5 ± 1.19
P13 (8; M)	Darbhanga, HR	3	SR	12.4 ± 2.03
P14 (40; M)	Vaishali, HR	3	ND	15.3 ± 2.98
P15 (17; M)	Saharsa, HR	5	SR	17.9 ± 3.32
P16 (23; M)	Darbhanga, HR	3	SR	20.0 ± 2.64
P17 (18; F)	Muzzafarpur, HR	7	Relapsed	28.7 ± 1.02
P18 (15; M)	Madhubani, HR	No history	ND	31.2 ± 4.5

**NOTE.** HR, high resistance to SAG; LR, low resistance to SAG; MR, moderate resistance to SAG; ND, not determined; R, responsive to SAG; SR, slow response to SAG.

<sup>a</sup> Based on data reported by Sundar [2].

<sup>b</sup> All 18 patients were advised to take SAG, at a daily dose of either 10 mL (1000 mg), for patients ≥12 years old, or 5 mL (500 mg), for patients <12 years old. On the basis of a previous study, patients who showed improvements ≤4 weeks after treatment were classified as R; those who did so after this period were classified as SR [3]. All patients were monitored for 1 year, to assess improvement (e.g., such as a decrease in the size of papules and nodules and/or a regression of indurated areas) after treatment; because repigmentation is a slow process, improvement in those with a predominantly hypochromic macular presentation could be ascertained only toward the end of or after completion of the 4-month treatment period.

<sup>c</sup> For 3 assays.

### Gene amplification

Investigation of gene amplification for *ldmdr*, *gshI* and *pgpA* was undertaken by southern blot analysis. Similar band intensity on radiograph was observed in all KA and PKDL isolates for all the probes tested, indicating that the resistance mechanism in clinical isolates is different than laboratory selected SAG resistant isolates.

The preponderance of SAG resistance observed in the PKDL patients and the *in vitro* correlation of parasite susceptibility implicates PKDL patients as a source of SAG refractory parasites, which may circulate in the community efficiently as SAG sensitive parasite get eliminated by the drug, and the proportion of patients with SAG refractory parasite rises.

## ***Future Plan of Action***

It is proposed to analyze differential gene expression in SAG resistant and sensitive *Leishmania donovani* using genomic microarrays in order to identify genes involved in resistance. The natural sensitivity towards other available anti-leishmanial drugs (Miltefosine, Paromomycin, Amphotericin B and Sitamaquine) will be evaluated and compared with susceptibility to SAG. Further screening of parasite susceptibility towards combination of available drugs will be undertaken for determining suitable combination.

## ***5. Molecular Characterization of Leishmania Parasites Isolated from Dermal Lesions of PKDL Patients***

***Scientific staff*** : ***Dr. Poonam Salotra, Mr. BV Subba Raju, Mr. Rajesh Kumar***  
***In collaboration with*** : ***Dr. Gabriele Schonian***  
***Dr RK Jain, SJH***  
***Duration*** : ***2005- 2008***

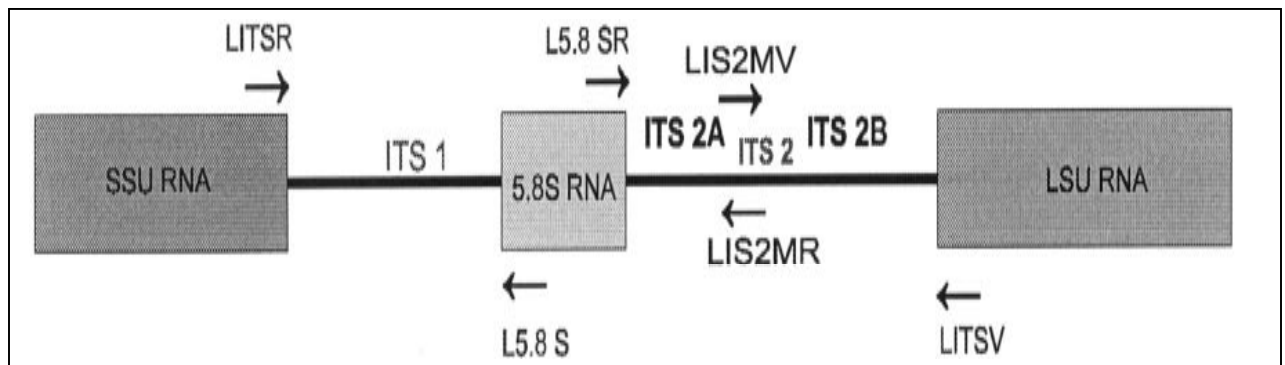
## ***Aims, Objectives & Background***

This work was initiated as an Indo-German project with the aim to carry out molecular characterization of *Leishmania* parasites isolated from dermal lesions of Indian PKDL patients. The genetic loci of hypervariable nature will be targeted since distinction at molecular level has been reported within species and strains of *Leishmania* on these loci. Highly polymorphic loci, such as non-coding gene spacers like ITS, and anonymous genomic sequences as well as different micro-satellite loci, will be analysed in Indian strains of *L. donovani* in order to develop genetic markers for strain typing in *L. donovani*. Ribosomal DNA has been used in phylogenetic studies at several taxonomic levels, ranging from major phyla to populations. This broad utility of rDNA is because the multiple copies per genome are usually tandemly repeated and the non-coding spacers evolve faster than

the coding regions. Like other multigene families individual rDNA copies are not believed to accumulate mutations independently, thus resulting in little intragenomic or intraspecific variation but substantial inter-specific differentiation. The homogenization of multigene families, called concerted evolution, is thought to be achieved by several molecular mechanisms (collectively called molecular drive), such as gene conversion (biased and unbiased), unequal crossing over and transposition. Intergenic region typing of the ribosomal internal transcribed spacer (ITS) was carried out by PCR-SCCP using a few isolates of KA and PKDL

Initially, all PKDL and KA isolates used in the study were characterized as *L. donovani* by species specific PCR, and reaction with species specific monoclonal antibodies. Intergenic region typing by PCR-RFLP, PCR-SCCP of the ribosomal internal transcribed spacer (ITS) was tested as genetic maker for differentiating PKDL and KA strains of *Leishmania donovani* species. The results of the analysis are given below.

### Internal transcribed spacers



Internal transcribed spacers (ITS) in the ribosomal operon and the position of primers to amplify ITS sequences

### *Amplification and restriction analysis of the ITS-1 region.*

The internal transcribed spacer (ITS-1) in the ribosomal operon were amplified using template DNA of PKDL and KA, *L. donovani* from Sudan, *L. d. infantum*, *L. tropica*, and *L. major* using the following primers

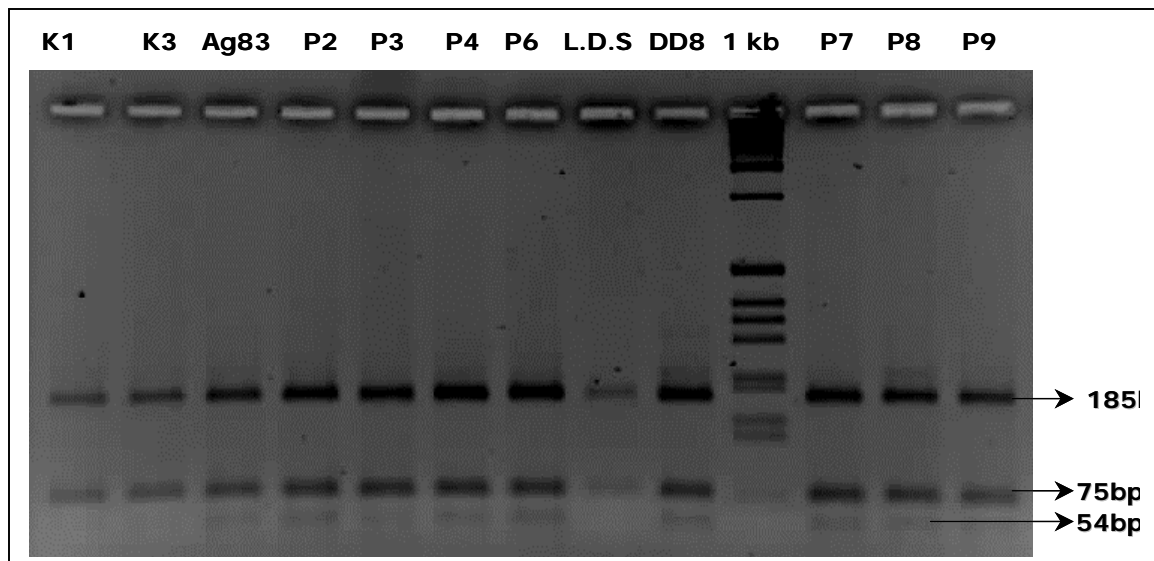
LITSR- 5' CTGGATCATTTCGATG 3' (forward) and

L5.8SR 5'GATACCACTTATCGCACTT-3 (reverse).

Amplification products were subjected to electrophoresis in 1% agarose at 100 V in 1x TBE buffer and visualized under ultraviolet light after staining for 15 min in ethidium bromide (0.5 mg/ml). PKDL and KA isolates of *L.donovani* (10 each) were subjected to amplification of the ITS-1 region with amplicon size of 320bp. All PKDL and KA isolates gave amplicons of same size as standard *L donovani* isolates.

### **PCR RFLP**

Amplified products were used for subsequent RFLP analysis using the restriction enzyme *HaeIII* (*Fermentas*). Restriction products were subjected to electrophoresis in 1.3% metaphor agarose (*Cambrex's MetaPhor Agarose*) for 2 h at 80 V in 0.5 TBE buffer and visualized under UV light after staining for 15 min in ethidium bromide 0.5 mg/ml. There was no discernible difference in the banding patterns when digested with *HaeIII* enzyme as shown in Fig. 10.



**Fig.10: PCR-RFLP. Restriction Digestion of L.d (P1-P9);L.d KA1 KA3; L.d AG83; L.d DD8; L.d Sudan isolates of *Leishmania* with *HaeIII* enzyme. Band pattern showing no differences among *L donovani* isolates of Indian origin.**

### ***Future Plan of Action***

Sequencing and SSCP analysis of ITS-1 from PKDL and KA isolates will be carried out. Microsatellite typing of PKDL and KA culture isolates will be performed using primers specific for *L donovani*.

## ***6. Identification and Characterization of L. tropica Isolated from Indian Patients of Cutaneous Leishmaniasis***

***Scientific staff*** : ***Dr. Poonam Salotra, Mr. Rajesh Kumar, Mr Nasim Ansari***  
***In collaboration with*** : ***Dr. RA Bumb, SPMC, Rajasthan***  
***Duration*** : ***2005- 2008***

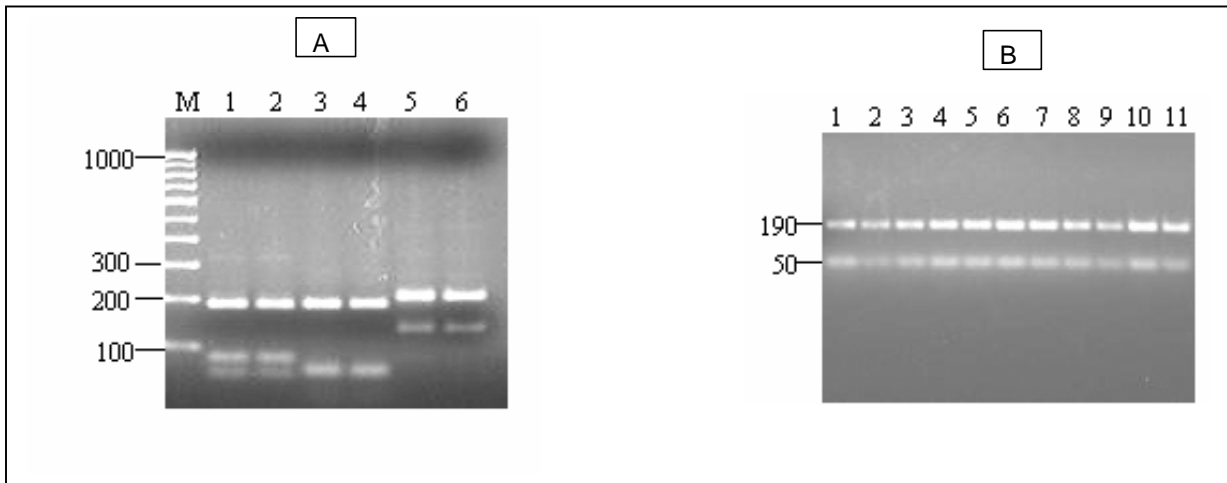
### ***Aims, Objectives & Background***

The current study was carried out for molecular and immunological characterization of species causing Cutaneous Leishmaniasis (CL) in Rajasthan. There is a substantial focus of CL, in Bikaner district, Western Thar Desert of Rajasthan state. CL exhibits symptoms that are very similar to those seen in several other skin diseases, and thus confirmation of parasites is mandatory when the diagnosis is suspected. The diagnosis of CL in regional clinics relies on demonstration of parasite in the smears or skin biopsy specimens by direct microscopic examination. These classical methods lack high sensitivity and specificity and do not provide any clues regarding the species involved in the disease pathogenesis. The species characterization in Indian CL is an important aspect, particularly in view of the recent identification of *L. donovani* causing CL in some parts of India. We have carried out the first comprehensive study of identification and characterization of species causing CL in Rajasthan, India, confirming *L. tropica* as the prevalent species in the endemic area, which has immense importance from epidemiological, transmission and treatment point of view.

### ***Work done during this year***

**ITS1 PCR-RFLP.** DNA from culture isolates (n=14) were subjected to ITS1 PCR-RFLP for confirmation of *Leishmania* species. Standards isolates of *L. donovani*, *L. tropica* and *L. major* (2 strains of each) were used as controls. PCR to amplify ITS 1 region gave an amplified fragment of about 300-350bp with all *Leishmania* species and its

subsequent digestion with the restriction enzyme HaeIII revealed three bands for *L. donovani* (50bp, 80bp, 190bp), two bands for *L. tropica* (50bp, 190bp) and two for *L. major* (160bp, 210bp), clearly differentiating the *Leishmania* species (Fig. 11A). Consistent band pattern corresponding to *L. tropica* was noticed in all the 14 isolates examined (Fig. 11B). The assay was further extended to DNA isolated from dermal lesions, the pattern corresponding to *L. tropica* was obtained in all cases and no amplification was observed using DNA from healthy skin tissues (n=5).

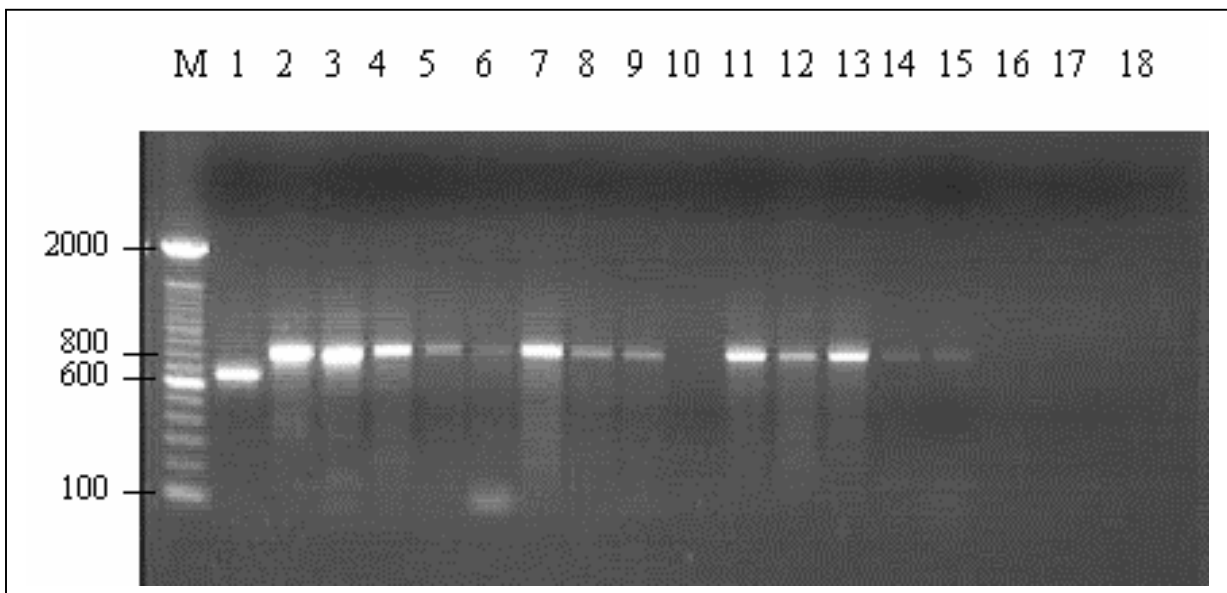


**Fig. 11:** The restriction analysis pattern of nested ITS1 PCR product digested with HaeIII. (A). DNA (10 ng) isolated from culture of different *Leishmania* species was subjected to PCR-RFLP and analyzed on 2% agarose gels. Lane 1, *L. donovani* DD8 (MHOM/IN/80/DD8); lane 2, *L. donovani* AG83 (MHOM/IN/83/AG83); lane 3, *L. tropica* WR683 (MHOM/SU/58/OD); lane 4, *L. tropica* WR 664 (MHOM/SU/74/K27); lane 5, *L. major* 662 (MHOM/IL/67?Zericho II/ WR662); lane 6, *L. major* LV39 (MHOM/SU/59/P/LV39); lane 7, no DNA. Lane M, 100 bp ladder as molecular size marker. (B) Characterization of *Leishmania* species in clinical isolates of CL patients using nested ITS1 PCR and restriction enzyme (HaeIII) analysis. Lane1, *L.tropica* WR 664 (MHOM/SU/74/K27); lane 1-11, cultures from tissue biopsies of different CL patients.

**Sequence analysis.** To confirm authenticity of the amplicon, the 340bp ITS-1PCR product obtained with DNA from clinical tissue samples of CL patients (n=5) as well as from parasites isolates (n=2) were subjected to sequence analysis. NCBI BLAST analysis revealed 99% homology with corresponding *L. tropica* sequence in Database (accession number AJ000301).

**kDNA PCR.** We also exploited primers (Uni21/Lmj4) suitable for amplification and identification of parasite DNA of *Leishmania* species. PCR with all the culture isolates

(n=14) gave a consistent species-specific pattern of *L. tropica* (850bp), clearly differentiated from *L. donovani* (800bp) and *L. major* (650bp) (Fig. 12). Standard WHO reference strains of all 3 species were used as positive controls. The assay was extended to DNA isolated directly from dermal lesions of patients with CL and suitable controls to determine its sensitivity and specificity for clinical utility. Most of the CL cases (29/32) gave positive result, whereas the DNA from healthy skin tissue (n=5) were all negative. The size difference between the PCR products of *L. donovani* and *L. tropica* with *L. major* allowed differential diagnosis.



**Fig. 12:** kDNA PCR assay with various *Leishmania* strains and clinical samples of CL. DNA isolated from parasite culture (10 ng) or clinical samples (200 ng) was used for amplification and analyzed on 1% agarose gels. Lane 1, *L. major* LV39 (MHOM/SU/59/P/LV39); lane 2, *L. tropica* WR 664 (MHOM/SU/74/K27); lane 3, *L. donovani* AG83 (MHOM/IN/83/AG83); lane 4-15, tissue biopsies from skin lesions of CL cases; lane 16 and lane 17, healthy skin tissues (n=2) from CL patients; lane 18, negative control, no DNA. Lane M, 100 bp ladder.

### *Future Plan of Action*

It is proposed to study cellular and humoral immune responses in Indian CL patients belonging to Bikaner region.

## ***7. Analysis of Cytokine Expression in Post Kala - Azar Dermal Leishmaniasis Biopsies***

<b><i>Scientific staff</i></b>	<b>:</b>	<b><i>Dr Avninder Singh, Dr Nidhi Bajaj, Dr. Sunita Saxena</i></b>
<b><i>In collaboration with</i></b>	<b>:</b>	<b><i>Dr. V Ramesh,, SJH</i></b>
<b><i>Technical staff</i></b>	<b>:</b>	<b><i>Mrs. Valsamma Mathews, Mrs. Karuna</i></b>
<b><i>Duration</i></b>	<b>:</b>	<b><i>2005-06</i></b>

### ***Aims, Objectives & Background***

Post kala-azar dermal leishmaniasis is a cutaneous sequel to visceral with various T- cell subsets involved in cell-mediated immunity with reference to host-parasite interactions. When T- cells are activated by antigen or its co- stimulators, they secrete locally acting proteins called cytokines. Under the influence of these cytokines, the T-cells differentiate into effector and memory cells, thereby deciding the course of the disease process. Our objective in this study is to analyze the expression of Th1/Th2 cytokines by immunohistochemistry with monoclonal antibodies, namely; IFN-gamma, TNF-alpha, IL-10 and IL-12 on formalin fixed-paraffin embedded archival tissue in 50 cases of previously diagnosed cases of PKDL and correlate the expression of these cytokines with the morphological subtype of PKDL.

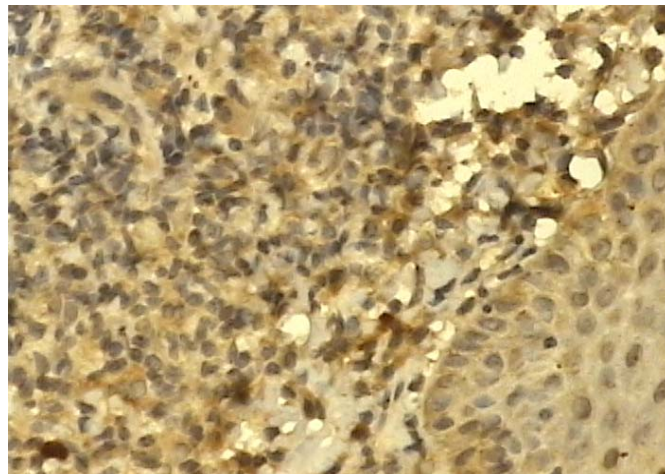
### ***Work done during this year***

IL-10 cytokine expression increased as the clinical presentation of PKDL progressed from macule to plaque to nodule suggesting that Th 2 response which is the disease promoting cytokine and plays a dominant role as compared to Th1 which are the disease protecting cytokines.

<b>Lesion Type</b>	<b>TNF-alpha</b>	<b>IFN-gamma</b>	<b>IL-10</b>	<b>IL-12</b>
Macular (n=13)	8/13(61.5%)	8/13(61.5%)	10/13(77%)	5/13(38.4%)
Polymorphic(n=32)	17/32(53.1%)	23/32(71.8%)	26/32(81.2%)	15/32(46.8%)
Nodular (n=5)	2/5(40%)	3/5 (60%)	5/5(100%)	3/5(60%)

## *Future Plan of Action*

Role of Stem cell factor/c-KIT protein interactions and its downstream regulator melanocyte specific microphthalmia-associated transcription factor, MITF-M may have possible implications in causation of hypopigmentation in PKDL



**Immunohistochemical expression of IL-10 in skin biopsy of Post Kala-azar Dermal Leishmaniasis patient**