MISCELLANEOUS PROJECTS

1. Monitoring of Organic Chemical Pollutants in Placental Tissue: A New Approach to Human Environmental Bio-monitoring

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Duration : *2002-2005*

Aims, Objectives & Background

Of late there has been a lot of awareness about pollution, its adverse effects and sources and efforts are being made to control and bring down the levels of pollutants in the environment. Use of pesticides and fertilizers for agriculture, industrial effluents and the increasing numbers of automobiles on road have been worldwide recognized to be the major contributors to environmental pollution. For the implementing agencies to take any decision, it is mandatory that background data on the levels of polluting chemicals should be available through various monitoring programmes. In order to get dynamic information about the changes in the environment, regular monitoring of pollutants at various levels of ecosystem and biosphere assumes great significance. While some attempts are being made to assess levels of pollutants in air, water or vegetables, there have been negligible attempts at Human Environmental Bio-monitoring in India or elsewhere in the world for want of a suitable monitoring model. The limitations faced by HEBM Program and advantages of using Human Placenta for HEBM have already been enumerated in the last report.

This is an extramural research project sanctioned by Department of Biotechnology with an aim to establish the utility of human placenta as a tool for comprehensive bio-monitoring for organic pollutants and to demonstrate the feasibility of monitoring region specific organic pollutants in placenta. It also aims to establishing standard operating procedures (SOP) for the above two objectives.

Work done during the year

This project had been initiated for comprehensive bio-monitoring of Organic Chemical Pollutants in human population using placenta as tool for HEBM. During the year under report approximately 4000 more pregnant women attending Antenatal Out Patient Department Clinic of Safdarjung Hospital were screened for possible exposure to pollutants. A total of 150 women who reported exposure to agricultural chemicals during pregnancy due to their involvement in agricultural activity by themselves or their family members were selected and followed up for subsequent collection of placental sample at the time of delivery. So far a total of 100 samples have been collected which include 51 random samples and 41 samples from high-risk population. In addition fifty samples of blood and milk have also been collected.

During the period under report, recovery experiments were conducted using two different methods, viz., Soxhlet Extraction, extraction using vortex mixer for extraction of pollutants. Both the methods were tried without addition of TCA and after addition of TCA in the placental homogenate. Further all the four extractions procedures were tried with four solvents namely n-Hexane, Dichloromethane, Acetonitrile and methanol again both individually as well as sequentially. It was found that Soxhlet extraction caused more background peaks under GC-MS as compared to extraction using vortex mixer. Further TCA extraction did not gave any additional advantage. Of the four solvents tried it was observed that both hexane as well as DCM gave good recovery 80 to 90%. Accordingly, Standard Operating Procedure for collection of placental sample, extraction of organic chemical pollutants from the placental tissue, cleanup and concentration by nitrogen purging has been finalized. Extracts have been prepared for all the 100 placental samples as per the finalized SOP and 50 extracts were concentrated by gentle nitrogen purge technique.

Further mixtures of pesticide standards consisting of more than 100 pollutants were procured and used for standardizing the separation of each pollutant under GC-MS. The operating conditions for GC-MS analysis by Trace GC-MS (Thermo-Finnigan) have also been finalized (Table 1). The retention times in GC and predominant mass fragments for

all the pesticide/pollutant standard were recorded (Tables 2 and 3) and used for interpretation of retention time and Mass Spectra recorded for the placental samples

On the basis of matching retention time with the known pesticide standards, the Gas chromatography has revealed possibility of presence of pesticide residues in the placental extracts. Figures 1 shows representative GC spectra for two samples. These show presence of peaks at retention time 16.18 to 34.53 minutes, which match with the retention time of several of the pollutants as listed in table 4.

Some of the GC-Mass spectra and Molecular structural formulae for a few representative pesticides and poly aromatic hydrocarbons are given in Table 5.

Condition	**Value**
Column	BP 5 (Polar) 30m length from M/s SGE
Detector	Mass from M/s Finnigan
Mode	Splitless
Injection volume	1 µl
Injection Port Temperature	80°C
Source Temperature	200°C
Gas	Helium 1.2 ml/hour in constant flow mode
Software	X-calibur
Standard Library	Wiley and NIST
Temp. Program - Fast	80°C hold 3 minutes followed by ramp of 15°C/min up to 300°C and hold at 300°C for 10 minutes (Total Run Time 40.5 minutes)
Temp. Program Standardized	70°C hold 2 minutes followed by ramp of 25°C/min up to 130°C, followed by ramp of 2°C/min up to 220°C followed by ramp of 10°C/min up to 280°C and hold at 280°C for 6.6 minutes (Total Run Time 62 minutes)

Table 1: GC MS Conditions

Constituents	CAS No.	Formula	Mol. Wt.	RT on Trace GC	Mass Profile
Anthracene	120-12-7	$C_6H_4(CH)_2C_6H_4$	178.23	18.820	76, 89, 126,152,178
Pyrene	129-00-0	C16H10	202.26	23.190	87, 101, 150, 202
Benzo(g,h,i)perylene	191-24-2	C22H12	276.34	35.300	137, 274, 276
Indeno(1,2,3-cd)pyrene	193-39-5	$C_{22}H_{12}$	276.33	34.350	137, 138, 274, 276
Fluoranthene	206-44-0	C16H10	202.26	22.530	63, 101, 202
Benzo(k)fluoranthene	207-08-9	$C_{20}H_{12}$	252.31	29.970	125, 126, 224, 248, 252
Acenaphthylene	208-96-8	$C_{12}H_8$	152.19	13.780	76, 98, 126, 152
Chrysene	218-01-9	C18H12	228.29	26.990	88, 111, 113, 125, 126, 252
Dibenz(a,h)anthracene	53-70-3	C22H14	278.35	34.500	113, 139, 278
Benz(a)anthracene	56-55-3	C18H12	228.29	26.880	101, 114, 200, 224, 228
Acenaphthene	83-32-9	$C_{10}H_6(CH_2)_2$	154.21	14.380	63, 76, 126, 153, 154
Phenanthrene	85-01-8	$C_{14}H10$	178.23	18.960	63, 75, 76, 89, 98, 152,178
Fluorene	86-73-7	$C_{13}H_{10}$	166.22	15.940	63, 82, 83, 139, 166
Naphthalene	91-20-3	$C_{10}H_8$	128.17	8.840	51, 64, 77, 102, 128

Table 2: Retention Time and Characteristic Mass Peaks of Some of PAHs

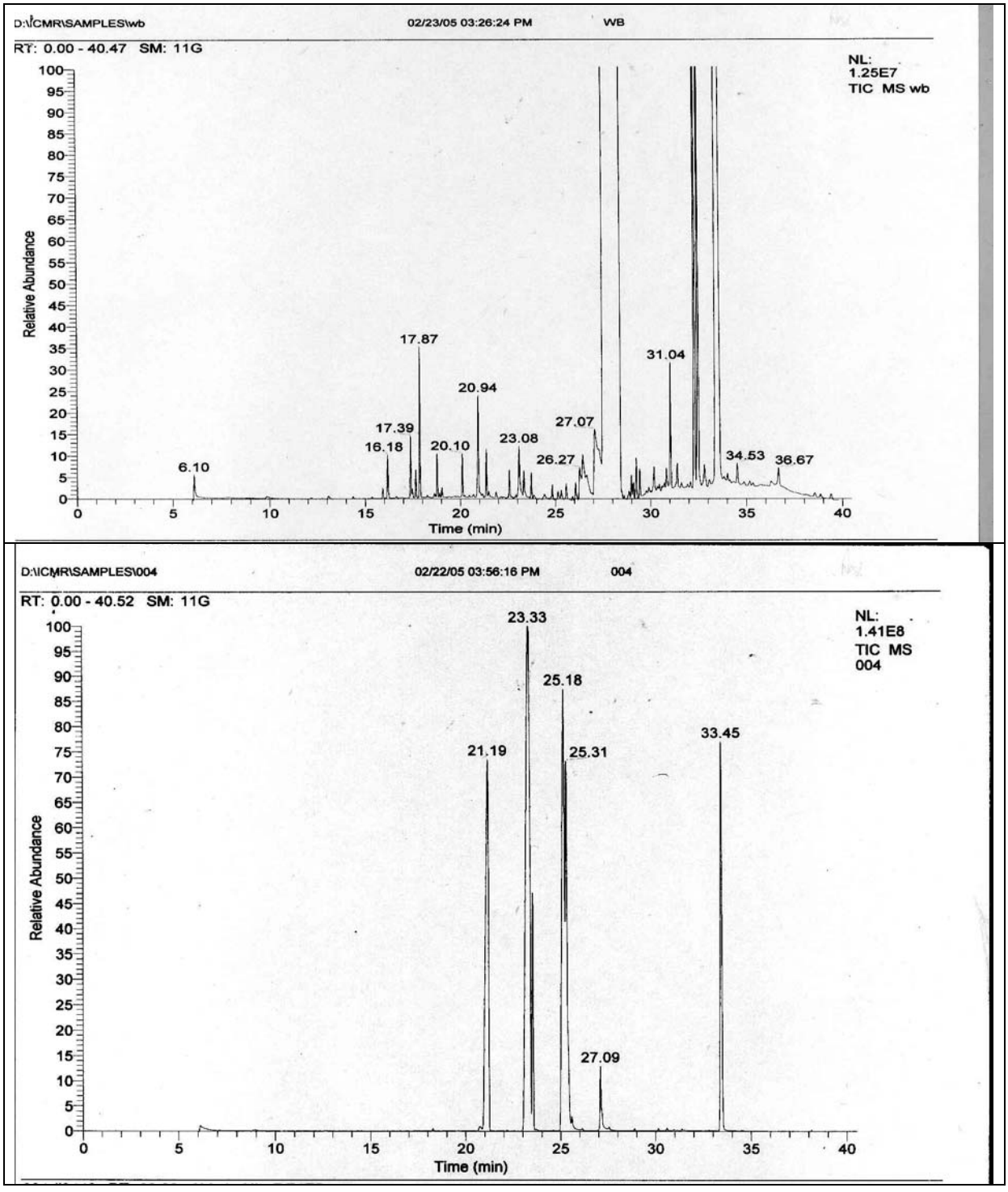


Fig. 1: GLC Spectra showing presence of several peaks in the placental sample as well as in whole blood sample

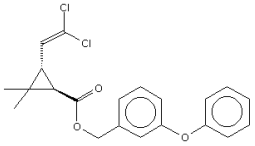
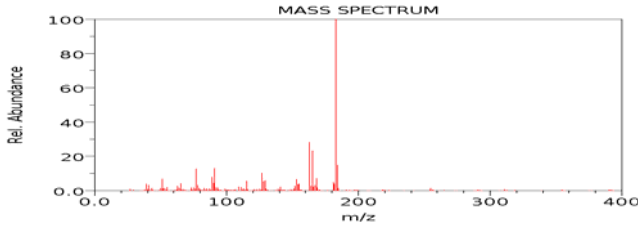
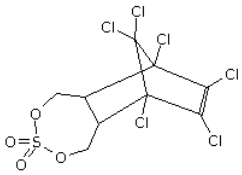
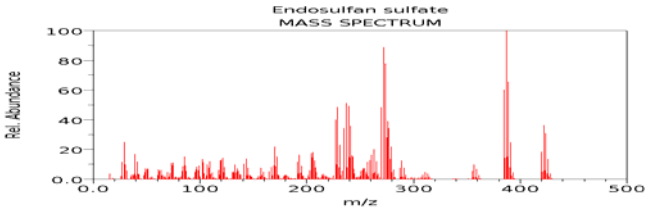
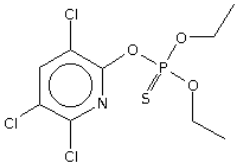
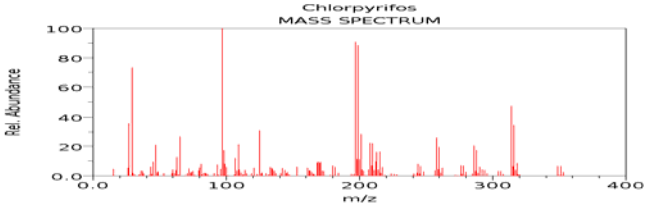
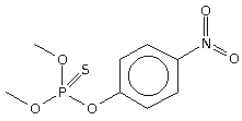
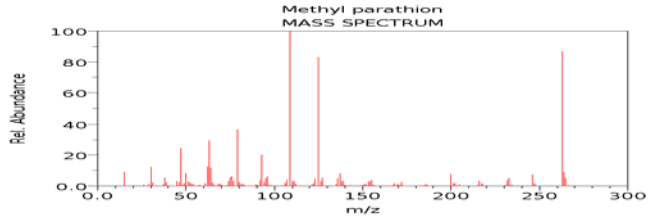
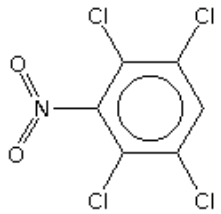
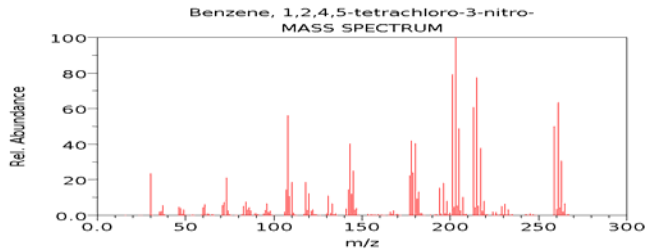
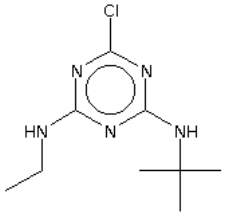
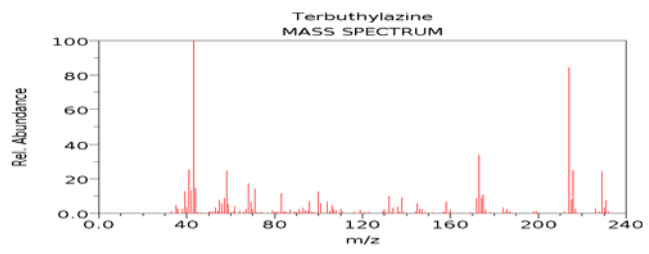
Constituents	CAS No.	Formula	Mol. Wt.	RT on Trace GC	Mass Profile
Dichlorvos	62-73-7	$C_4H_7Cl_2O_4P$	220.98	10.270	79, 109, 145, 185
Methyl parathion	298-00-0	$C_8H_{10}NO_5PS$	263.21	20.270	109, 125, 233, 263
Chlorpyrifos-methyl	5598-13-0	$C_7H_7Cl_3NO_3PS$	322.60	20.275	109, 125, 286
Fenitrothion	122-14-5	$C_9H_{12}NO_5PS$	277.24	20.970	109,125, 260, 277
Phosalone	2310-17-0	$C_{12}H_{15}ClNO_4PS_2$	367.81	27.765	97, 111, 121, 182, 367
α-BHC	319-84-6	$C_6H_6Cl_6$	290.83	13.960	109, 181, 219
g-BHC	58-89-9	$C_6H_6Cl_6$	290.83	16.060	109, 181, 219
Aldrin	309-00-2	$C_{12}H_8Cl_6$	364.91	21.390	66, 79, 91, 101, 263, 293
Heptachlor	76-44-8	$C_{10}H_5Cl_7$	373.32	20.490	100, 237, 272
α-Chlordane	5103-71-9	$C_{10}H_6Cl_8$	409.78	22.980	75, 109, 237, 272, 373
o,p'-DDE	3424-82-6	$C_{14}H_8Cl_4$	318.02	23.090	105, 176, 210, 246, 316
p,p'-DDE	72-55-9	$C_{14}H_8Cl_4$	318.02	23.850	176, 210, 246, 316
p,p'-DDD	72-54-8	$C_{14}H_{10}Cl_4$	320.04	24.860	88, 101, 165, 176, 199, 235,
p,p'-DDT	50-29-3	$C_{14}H_9Cl_5$	354.48	25.730	165,199, 235,
Simazine	122-34-9	$C_7H_{12}ClN_5$	201.66	18.200	68, 96, 138, 173, 186, 201
Atrazine	1912-24-9	$C_8H_{14}ClN_5$	215.68	18.340	58, 68, 173, 200, 215
Terbuthylazine	5915-41-3	$C_9H_{16}ClN_5$	229.71	18.730	173, 214, 229
Pentachloroaniline	527-20-8	$C_6H_2Cl_5N$	265.35	19.830	263, 265, 267,
Cyanazine	21725-46-2	$C_9H_{13}ClN_6$	240.70	21.530	68, 172, 198, 225, 240
Alachlor	15972-60-8	$C_{14}H_{20}ClNO_2$	269.77	20.500	146, 160, 188
Metazachlor	67129-08-2	$C_{14}H_{16}ClN_3O$	277.75	22.320	81, 133, 209
Bentazon	25057-89-0	$C_{10}H_{12}N_2O_3S$	240.30	21.780	92, 119, 161, 198
Endosulfan I	959-98-8	$C_9H_6Cl_6O_3S$	406.92	24.665	85, 170, 195, 207, 241, 267

Endosulfan II	33213-65-9	$C_9H_6Cl_6O_3S$	406.92	23.280	85, 160, 170, 195, 207
Endosulfan sulfate	1031-07-8	$C_9H_6Cl_6O_4S$	422.92	25.695	229, 237, 272, 387, 422
Piperonyl butoxide	51-03-6	$C_{19}H_{30}O_5$	338.44	26.240	147, 176, 221, 281, 355, 429
Bromopropylate	18181-80-1	$C_{17}H_{16}Br_2O_3$	428.12	26.920	155, 183, 341
Dieldrin	60-57-1	$C_{12}H_8Cl_6O$	380.91	23.925	79, 108, 237, 263, 345
Endrin	72-20-8	$C_{12}H_8Cl_6O$	380.90	24.470	67, 81, 263, 281, 317
trans-Permethrin	51877-74-8	$C_{21}H_{20}Cl_2O_3$	391.29	29.130	163, 165, 183
cis-Permethrin	54774-45-7	$C_{21}H_{20}Cl_2O_3$	391.31	29.290	163, 165, 183

Table 3: Retention Time and Characteristic Mass Peaks of Some of Organo-Phosphorus, Organo-Chlorine and Pyrethroid Pesticides

Retention Time of the sample	Retention Time of the standard	Standard
16.18	16.23	2,3,5,6-Tetrachloronitrobenzene
17.87	17.89	Hexachlorobenzene
20.10	20.08	2,4,4'-Trichlorobiphenyl
20.94	20.97	2,2',5,5'-Tetrachlorobiphenyl & Fenitrothion
21.19	21.239	Malathion
23.08	23.09	o,p'-DDE
23.33	23.3	Endosulfan II
25.18	25.17	2,2',3,4,4',5'-Hexachlorobiphenyl
26.27	26.24	Piperonyl butoxide
27.07	27.06	Methoxychlor
34.53	34.5	Dibenz(a,h)anthracene

Table 4: GC Retention Times of the samples that matched with the GC retention times of the standards under similar conditions

Constituents	Structural Diagram	Mass Spectrum
trans-Permethrin		
Endosulfan sulfate		
Dursban		
Methyl parathion		
2,3,5,6-Tetrachloro-nitro-benzene		
Terbuthylazine		

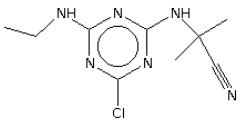
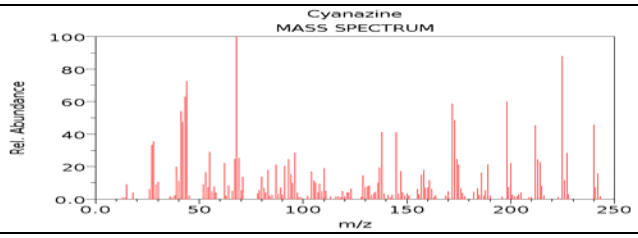
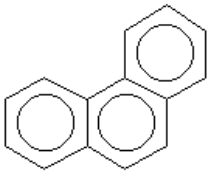
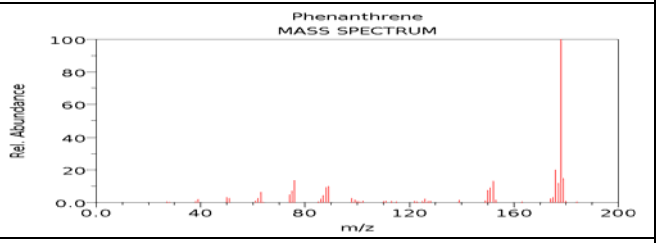
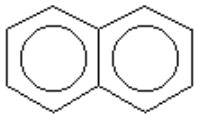
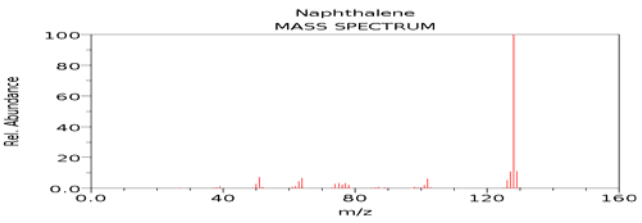
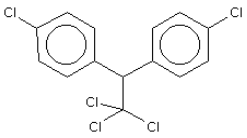
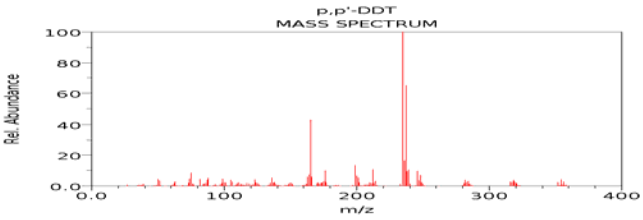
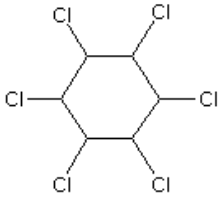
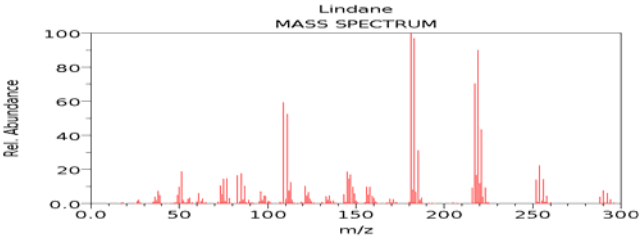
Cyanazine		
Phenanthrene		
Naphthalene		
p,p'-DDT		
g-BHC		

Table 5: Molecular Structural Diagrams and GC-Mass Spectrums of a few representative pesticides and PAHs

GC- MS analysis of the placental extracts showed presence of some normal biological constituents such as cholesterol (Fig.2). In few cases the GC-MS analysis revealed presence of pollutants like Cycloheptatrienylium Bromide (Fig. 3) and Naphthalene. In the latter case the peaks matched both the GC retention time as well as Mass Fragments Profile (Fig. 4). Further confirmation of these residues by GC-MS is still under progress.

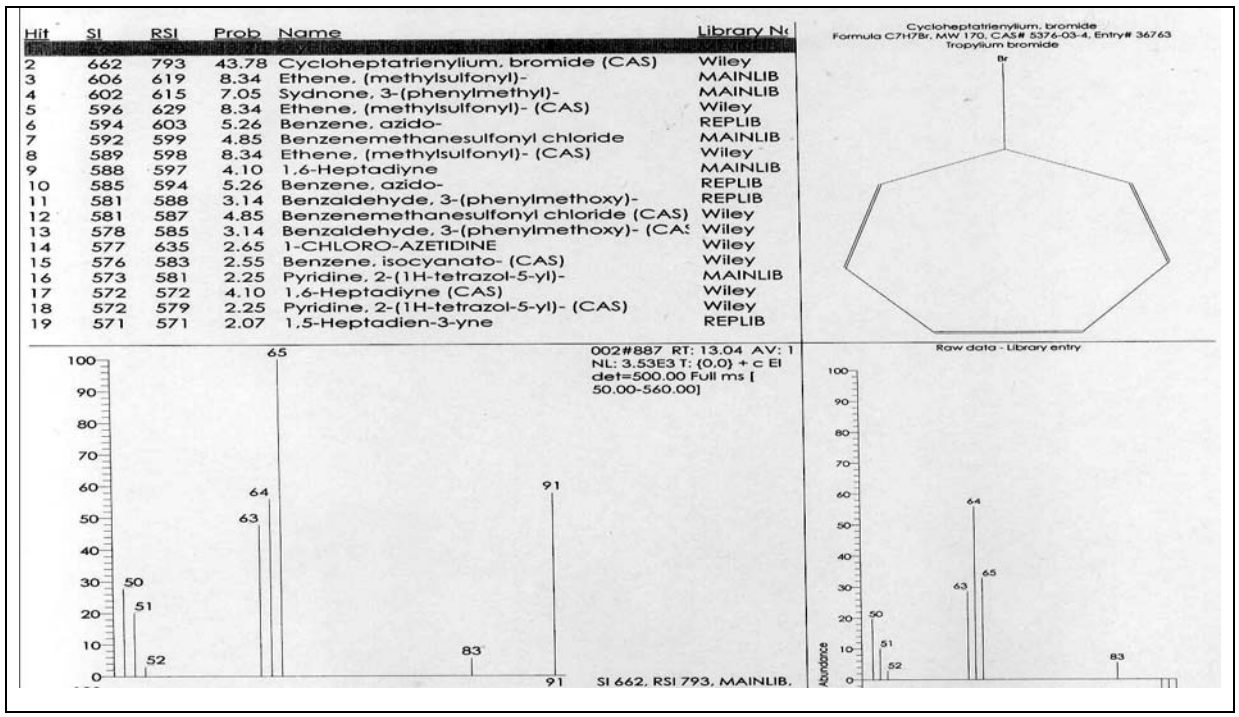


Fig. 2: GC- MS spectrum from another sample showing presence of Cycloheptatrienylium Bromide Peak

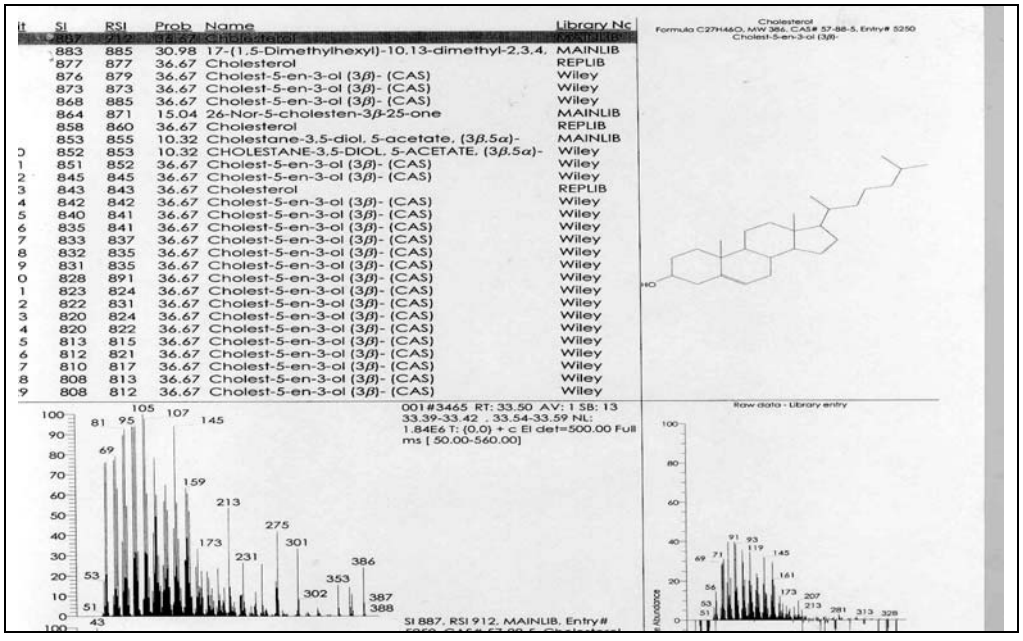


Fig. 3: GC-MS spectrum from a placental extract shows presence of cholesterol a normal constituent of the tissue

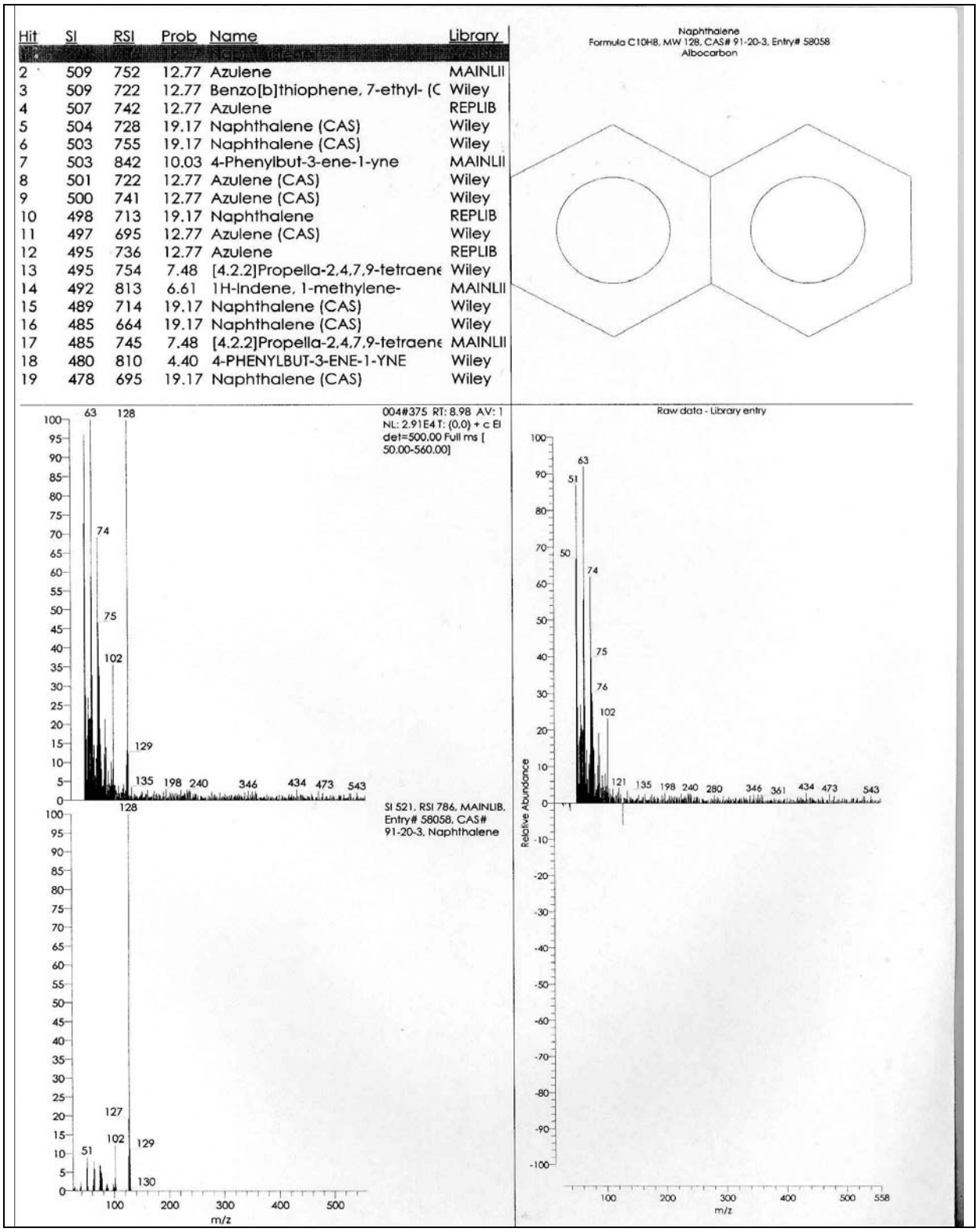


Fig 4: GC MS Spectrum form a sample of human placenta showing presence of characteristic peak for Naphthalene

2. Cell Culture Contamination with Mycoplasma in Basic and Applied Biomedical research

Scientific Staff : *Dr. LK Yerneni, Mr. Ashok Kumar*
Duration : *2005-2008*

Aims, Objectives and Background

This project has been envisaged looking at the lack of awareness about *Mycoplasma* contamination in cell culture laboratories in our country and its significance in basic and applied biomedical research.

The project has the following objectives :

(a) To study the frequency of *Mycoplasma* contamination in various reputed Institutes of Delhi and to identify a protocol for detection of *Mycoplasma* in human and other mammalian cell cultures.

(b) To verify effectiveness of various antibiotics and mycoplasma removal agents for elimination of *Mycoplasma* contamination from infected cell lines.

(c) To find out an ideal dose of anti-*Mycoplasm*a antibiotics like Ciprofloxacin and Gentamycin for their routine use in cell cultures in place of Penicillin and Streptomycin that have least anti-*Mycoplasma* activity.

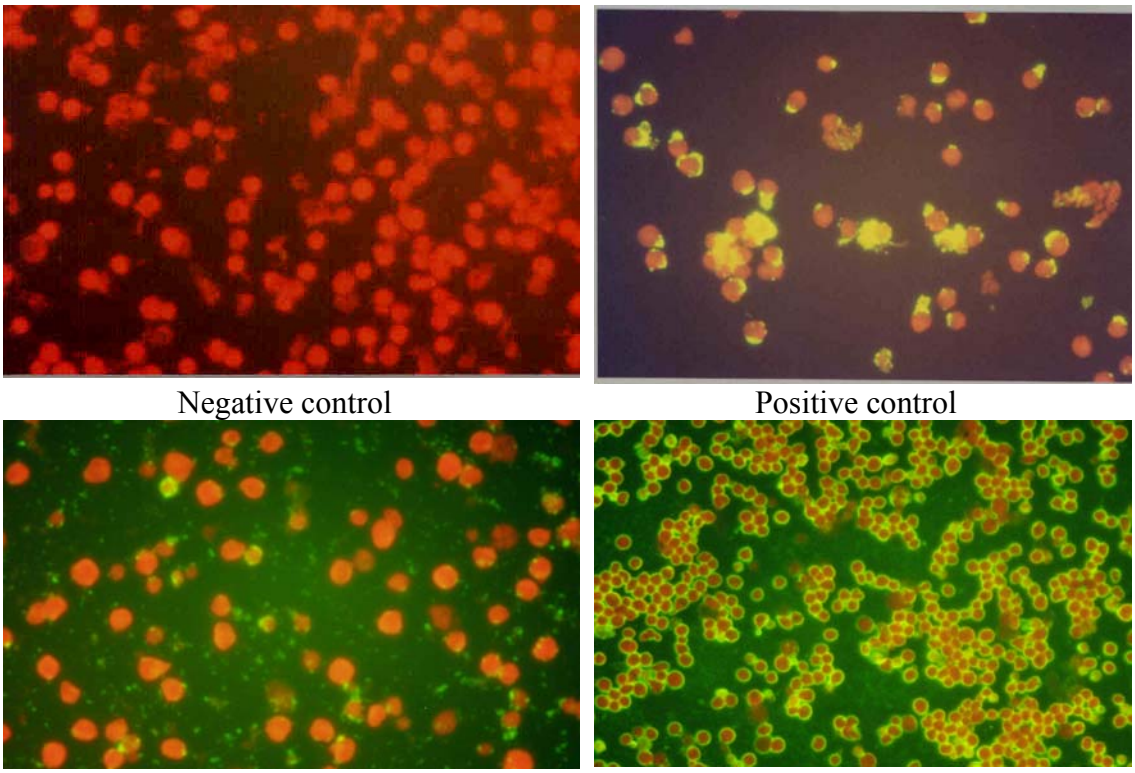
Work done during the year

74 primary and continuous cell lines were screened by immunofluorescent assay (Fig. 1) and Hoechst staining (Fig. 2) from 19 Tissue Culture laboratories in Delhi (Table 1). Out of 74 cell lines, only 4 cell lines were found contaminated with *Mycoplasma* by both the methods and all four cases were from a single laboratory. Our study suggests that the immunofluorescent method employed is as sensitive as the DNA (Hoechst) staining method and *Mycoplasma* contamination of the cell cultures in India is about 5 % which is at the minimum of what is reported in the West. However, it signifies the need for having *Mycoplasma* quarantine and regular screening in cell culture facilities including IVF

facilities across our country. Further analysis of *Mycoplasma* contamination is being undertaken by PCR.

Institution	**No.of cultures**	Positive		Negative	
		IFA	**Hoechst**	IFA	**Hoechst**
AIIMS	**58**	**4**	**4**	**54**	**54**
Lab 1	17	-	-	17	17
Lab 2	7	-	-	7	7
Lab 3	5	-	-	5	5
Lab 4	2	-	-	2	2
Lab 5	11	-	-	11	11
Lab 6	5	-	-	5	5
Lab 7	5	-	-	5	5
Lab 8	6	**4**	**4**	2	2
JNU	**8**	**-**	**-**	**8**	**8**
Lab 1	2	-	-	2	2
Lab 2	1	-	-	1	1
Lab 3	1	-	-	1	1
Lab 4	1	-	-	1	1
Lab 5	1	-	-	1	1
Lab 6	1	-	-	1	1
Lab 7	1	-	-	1	1
Lab 8	1	-	-	1	1
VP Chest	**1**	**-**	**-**	**1**	**1**
IGIB	**1**			**1**	**1**
Inst. of Path	**7**	**-**	**-**	**7**	**7**
Lab1	1	-	-	1	1
Lab2	6	-	-	6	6

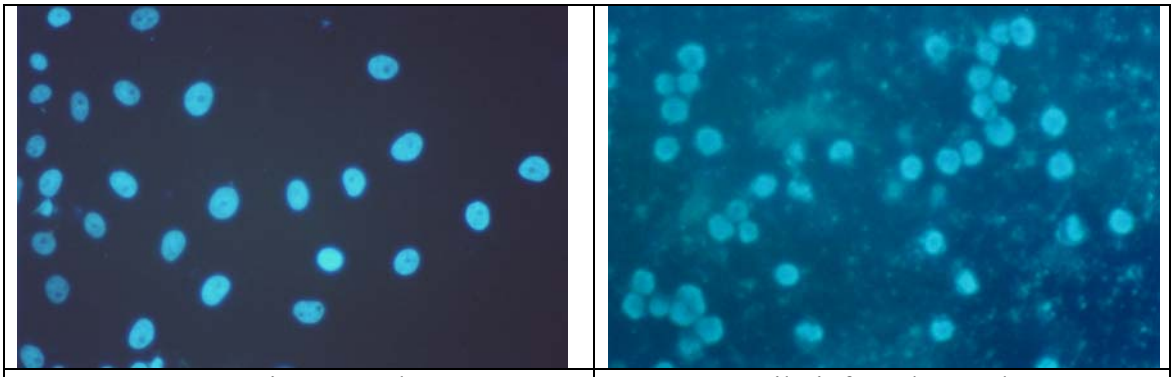
Table 1: The incidence of *Mycoplasma* contamination in cell cultures from various reputed Institutes in Delhi.



Negative control | Positive control

Moderately infected T3 culture | Heavily infected T8culture

Fig. 1: *Mycoplasma* contamination in cell culture as detected by Immunofluorescent assay



Negative control	Heavily infected T8 culture

Fig. 2: *Mycoplasma* contamination in cell culture as detected by Hoechst (33258) DNA staining.

Future Plan of Action

Application of human epidermal sheets cultured from autologous epidermal stem cells in burns patients. Submitted to DBT under the programme entitled "Cell Based Therapy". In this project, it was proposed to undertake a study on the evaluation of proliferative potential of human sera on human epidermal stem cells by growth curves and BrdU labeling for flow cytometric analysis in addition to undertaking clinical application of cultured epidermis on 50 patients in phased manner. It is proposed to collaborate with Burns Division, Safdarjung hospital, New Delhi and Research & Referral hospital.

3. Pathophysiological Role of Estrogen in males

I . Immunohistochemical Evaluation of Estrogen receptor and estrogen induced proteins in the ejaculated spermatozoa of infertile subjects

Scientific Staff : *Dr. S. Jayaraman, Mr. Varun Kapur, Mr. Shakaut, Mr.Sajad and Ms. Deepali Mathur*
In collaboration with : *Dr. MM Misro, NIHFW. New Delhi.*
Technical Staff : *Mr. Satya Pal Singh Kasana.*
Duration : *2005 (Till August).*

i) Immunohistochemical profiles of Estrogen Receptor α, Estrogen Receptor β, Andogen Receptor, Estrogen induced / related proteins like Progesterone Receptors, Cathepsin D, HSP-27 and Aromatase P 450 in the ejaculated spermatozoa from infertile subjects

Immunohistochemical evaluation carried out with ejaculated spermatozoa from the subjects attending the infertility clinics indicated that:

a) 23/73 (31.5%) samples demonstrated the presence of most of Estrogen Receptor α Estrogen Receptor β, Andogen Receptor, Estrogen induced / related proteins like Progesterone Receptors, HSP-27 in the ejaculated spermatozoa, however, these proteins were totally absent in 38/73 (52.05%) samples, while a variable percentage of spermatozoa expressed these receptors in the rest of the samples.

b) A number of subjects in the third group mentioned above showed only one or two of the above mentioned proteins in the spermatozoa

c) Cathepsin D was consistently absent in the spermatozoa in all the groups.

d) A significant correlation(P>0.01) between the percentage of spermatozoa showing forward motility and the spermatozoon presence of Estrogen Receptor α Estrogen Receptor β, Aromatase P 450- - indicating their diagnostic and prognostic significance with fertility potential of the spermatozoa. The observations also suggest that their inhibitors may act as useful vaginal contraceptives.

II) Flowcytometric evaluation of Estrogen Receptor (ER) and estrogen induced proteins in the rat testicular germ cells.

Scientific Staff : *Dr. S. Jayaraman, Mr.Varun Kapur, Mr.Shakaut, Mr.Sajad and Ms. Deepali Mathur.*
Technical Staff : *Mr.Satya Pal Singh Kasana.*
Duration : *2005 (Till August).*

Investigations were undertaken to confirm the earlier immunohistochemical observations on the presence of ER α in rat testicular cells using and to evaluate the presence of Estrogen induced proteins in the rat testicular germ cells using flow cytometry. . Rat testicular cells were collected and differential centrifugation procedures to separate the different germ cells and to carry out the flow cytometric evaluation of the estrogen receptors as well that of different estrogen induced proteins. How ever the studies could not be completed as the number of testicular germ cell types following differential centrifugation procedures using Percol gradients were insufficient following staining. Hence the results of immunohistochemical localization of the same were carried out in the isolated testicular germ cells.

The studies indicated that Estrogen α receptors were presented in primary spermatogonia, primary spermatocytes, round and elongated spermatids. The ER β in the spermatogonia round and elongated spermatids. The most important observation was that Cathepsin D could be localized only in the round spermatids,

while the Progesterone receptors, IGF2, HSP 27, PS2 were present in one or more of the rat germ cells.

The studies confirm the important role of Estrogen in proliferation, differentiation, maturation and perhaps in apoptosis as well. The localization of Cathapsin D is the first report in the literature as per our knowledge.

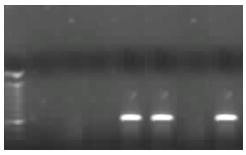
III) Detection of mRNA transcripts of Estrogen Receptor α in the ejaculated spermatozoa from fertile individuals.

Dr .S. Jayaraman and Mr. Pankaj Sharma.

Our earlier results indicated the presence splice variants of ERα and Progesterone receptor from the ejaculated spermatozoa from infertile individuals. However presence of wild type of ERα could not be demonstrated in the ejaculated spermatozoa from subjects attending infertile clinic but the spermatozoan characteristics were of 'fertile range'. Hence studies were undertaken in the mRNA isolated from the spermatozoa from known fertile individuals. The results are presented in Fig.1.

The results confirm our earlier hypothesis that presence or absence of some spermatozoan proteins may be playing a role , perhaps in the causation of male infertility.

The presence of wild type ER alpha mRNA transcripts fro human ejaculated spermatozoa from fertile individuals



4. Evaluation of Human T-cell Lymphotropic Virus Type-1 (HTLV-1) In Blood Donors and Patients With Leukemia/Lymphoma from Delhi

Scientific Staff : *Dr. Ranvir Singh, Dr. S Jayaraman*
In collaboration with : *Dr. Sumita Saluja, SJ Hospital, New Delhi*
Duration : *2004-2005*

Aims, Objectives and Background

A number of evidences indicate the association of HTLV-1 with various diseases including adult T-cell leukemia / non-Hodgkin's lymphoma, HTLV-1 associated myelopathy (HAM) / TROPICAL spastic paraparesis (TSP), development of inflammatory diseases in various organs such as eyes, lungs and joints, inflammatory ocular diseases such as endogenous uveitis, episcleritis, retinitis pigmentosa and degenerative choroiditis, etc. In these conditions, the presence of HTLV-1 can be associated as an accompaniment of repeated blood transfusions. However, recently in a pilot study, Ramalingam et al (2001) have reported a strong association of HTLV-1 with haematological malignancies (8/86 patients) and evidence for both horizontal and vertical transmission of the infection in South-Indian population.

In view of the reported association between HTLV-1 and haematological malignancies in a comparatively smaller number of subjects (n=86), the current investigations were planned to study the association between HTLV-1 and haematologic malignancies. Such a study carried out in another population namely the North-Indian subjects will confirm the above mentioned serological evidences and if confirmed may lead to the possible introduction of HTLV-1 monitoring policy in the National Blood Transfusion Services.

Work done during the year

A total of 186 blood samples, comprising 157 sera from blood donors and 29 sera from patients with haematological malignancies, were collected and tested for anti-HTLV-1 antibodies through Particle Agglutination test (PAT). No sample was found to be having anti-HTLV-1 antibodies. Blood samples from patients with haematological malignancies

and of blood donors were received from Safdarjang Hospital, New Delhi and Institute of Pathology, New Delhi, respectively.

Future plan of action

Nearly 160 blood donors and 30 patients with hematologic malignancies will be screened for detection of anti-HTLV-1 antibodies through PAT. Sera found positive by PAT will be confirmed through Line Immunoassay (LIA).

5. Quantitation of Immunohistochemical staining

Scientific Staff : *Dr. S Jayaraman, Dr. Usha Agarwal*
In collaboration with : *Dr. S L Kapoor*
Duration : *2004-2006*

Aims, Objectives and Background

Immunohistochemistry is routinely employed for diagnosis/prognosis as well as for therapeutic monitoring. It encompasses a wide variety of hormone receptors, apoptotic markers, tumour and proliferation markers, etc. The staining intensity obtained is quantified by a scoring system into grades of mild, moderate and severe. The staining is directly related to the presence of antigen. The direct relationship between the intensity of staining and the number of hormonal receptors in histological specimens has been accepted. Similarly, the use of DNA content in defining the prognosis of lung cancer patients has been recognized. However, the stoichiometric relationship between the antigen concentration and intensity of immunohistochemical staining is yet to be demonstrated. The diagnostic, prognostic as well as therapeutic management of the patient may vary if there is a relationship between the intensity of immunostaining and the concentration of antigen. Investigations were planned with an objective of developing an image analysis system for specifically recognizing and quantitating the area as well as the intensity of immunostain.

Work done during the year

The results achieved so far include identifying the Area of Interest (AOI), count of number of cells, giving area% of five different grades of brown present in the image. However, the quantification of the DAB product by analyzing the pixel values was observed to be affected by the haematoxylin. So attempts to develop a defined colour palette, which can be used as a universal standard, are being undertaken. The results of the collaborative studies suggest that we may be in a position to achieve the objective in the near future.