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### DIAGNOSIS OF FRAGILE X SYNDROME - ANALYSIS OF EXPRESSION OF FMRP IN BLOOD SMEARS

The fragile X syndrome (FXS) is the most common, specific, monogenic X-linked cause of mental retardation in humans and has a prevalence of 1 in 4000 amongst mentally retarded boys and 1 in 6000 girls<sup>1</sup>. Fragile X males tend to have a long face with large anteverted ears and mandibular prognathism, macro-orchidism, speech defects along with lack of eye-to-eye contact and moderate to profound mental retardation. Affected girls mainly present with anxiety and ear defects<sup>2,3</sup>. The fragile X mental retardation 1 (FMR1) gene is located in the DNA on X chromosome. This gene contains the genetic information for how to synthesize FMRP (fragile X mental retardation protein). The fragile X syndrome occurs when FMRP is missing. The FMR1 gene contains a CGG repeat at the 5' region that is polymorphic in the normal population and varies from 6-54 repeats. Premutation alleles show the CGG repeats between 52 and 200 repeats while full mutation alleles show a number beyond 200<sup>4</sup>. The knowledge about this expansion of CGG repeats within exon 1 of the FMR1 gene has resulted in the development of reliable diagnostic methods by using direct DNA analysis<sup>5</sup>. This expansion of CGG repeats leads to hypermethylation, which leads to silencing of the FMR1 gene thus preventing protein production. The lack of expression

of FMR1 gene is responsible for the mental retardation. The FMR1 gene is expressed in almost every tissue with the highest expression being in brain and testes.

Since the discovery of the gene defect the molecular diagnosis of FXS has been carried out mainly by the use of southern blot hybridization or PCR analysis<sup>2,6</sup> which are time consuming and generally take more than seven days. The present write-up describes a non-invasive antibody test to identify FXS patients. This test requires only two drops of blood and the results of the test are available within 24 hours.

The female premutation carriers have a high risk of having affected sons and daughters. There is a strong correlation between the length of the premutation and the risk for expansion to full mutation in the next generation<sup>7,8</sup>. The likelihood of repeat instability increases with the repeat number. There is no expansion of the premutation allele to the full mutation if it is transmitted to the offspring by males. Thus male premutation carriers will never have affected children<sup>9</sup>. These men are called normal transmitting males because all their daughters will inherit their father's unchanged expansions and hence they become female premutation carriers "at risk" of having offsprings with full mutation.

This write-up reports the evaluation of antibody test by determination of FMRP expression in 500 blood smears from healthy individuals and subjects suspected to be afflicted with FXS. This simple test involves the use of a monoclonal antibody against FMR1 protein--FMRP in peripheral blood smear<sup>10</sup>. In normal individuals FMRP is detected as a pink precipitate in the cytoplasm of the lymphocytes while in lymphocytes of male fragile X patients, there is no precipitate (since protein is not expressed).

In blood smears from control individuals and carriers with prenatation, FMRP can be detected in cytoplasm of lymphocytes, whereas lymphocytes of male FXS patients are devoid of FMRP. However, it is observed that some lymphocytes of affected males expressed FMRP possibly due to a prenatation in the positively labeled cells<sup>10</sup>.

The study presented here was carried out at a school in Pune and 18 special schools of Mumbai Municipal Corporation. The antibody test was carried out in 25 normal individuals and 475 children with idiopathic mental retardation after ruling out all other causes.

The indirect alkaline phosphatase technique employed in the study was essentially similar to the one described by Willenson et.al. in 1995<sup>10</sup> with suitable modifications. Blood smears were made from one or two drops of blood directly after bleeding and subsequently stored at -20°C before processing within one week. The slides were air dried and fixed in 0.1M phosphate buffer pH 7.3 containing 3% paraformaldehyde for 15 min. at room temperature. Further, the cells were permeabilized by treatment with 100% methanol for 20 min. at room temperature. After washing with phosphate buffered saline containing 0.15% glycine and 0.5% bovine serum albumin, the lymphocytes were incubated with mouse monoclonal antibodies against FMR1 protein for 16h at 4°C. Visualization of antibody-antigen complex was achieved by an indirect alkaline phosphatase technique using goat antimouse immunoglobulin conjugated with biotin (60 Min) and streptavidin-biotinylated alkaline phosphatase complex (45 Min) as secondary and tertiary steps respectively. The new fuchsin substrate-chromogen system was used for 30m in the final staining step utilizing alkaline phosphatase activity. Lavamisole was added in the substrate solution (1g/40ml) to block endogenous alkaline phosphatase activity. Slides were counter stained with Gill's Haematoxylin and mounted with aqua mount.

FMRP expression was seen in lymphocytes of normal subjects whereas no or very low expression was seen in

lymphocytes of fragile X patients. In every blood smear 100 lymphocytes were examined and scored for the presence of FMRP. The number of lymphocytes labeled for FMRP was expressed as a percentage of the total lymphocytes screened. The mean and SD of the percentage of FMRP expression in normal males and affected males was calculated.

The mentally retarded children suspected to have FXS by antibody test were subjected to direct analysis and Southern blot, which showed full mutation. The mentally retarded males with full mutation had 5-20% lymphocytes positive for FMRP while normal males showed 85-95% positive cells. Expression of FMRP in normal and retarded males showed a clear cut demarcation. Twenty samples negative for FXS by antibody test and processed for DNA analysis gave negative results. In clinical practice, a cut off point is used for diagnostic tests. Below the cut off point, the individual is suspected to be suffering from FXS. The cut off point has been determined to be 20% for affected males and 85% for normal. The antibody test showed 100% sensitivity, 97.5% specificity and accuracy when used only for males.

To date the identification of FXS patients have been carried out by direct DNA analysis and PCR analysis and Southern blots. For screening large number of patients these methods are time consuming and inconvenient. The antibody test is an efficient and reliable method to detect FXS in screening programmes. In males the statistical analysis of data confirms high sensitivity of the FMRP test. Data from affected males show high specificity, as there was no overlap between patients and controls. This implies that the FMRP test can discriminate between affected and normal males on the basis of FMRP expression in lymphocytes. However, prenatation carriers cannot be detected with this test as the percentage of expression was between 50-60%.

The antibody test identified male patients showing either no or a very low expression of FMRP positive lymphocytes. All the 20 negative controls were positive for FMRP in their lymphocytes. The test is easy to carry out, inexpensive, and can be used on several samples in a short time and the result can be obtained in a day. However, since this test is not an ELISA, the microscopic evaluation of lymphocytes is needed because of endogenous alkaline phosphatase activity.

The mentally challenged individuals evaluated in this study were not tested for FXS earlier by DNA analysis.

All the samples where the FMRP expression was <70% were studied by Southern blot analysis for confirmation. Maximum of 8% FMRP expression was observed in lymphocytes of affected group.

Not many reports are available from India revealing frequency for this disorder among institutionalized mentally retarded population except a very few in Delhi where a group of scientist reported it to be around 7.7% and another at the All India Institute of Medical Sciences, New Delhi reported the frequency of FXS to be around 2.2%<sup>11</sup>. The frequency in the present study was around 4.8%. This variation in frequency may be due to the small sample sizes in each group.

The rapid antibody test enables discrimination of normal individuals from affected one making it a suitable test for screening of mental retardates in special schools for children with learning disabilities. This test may also be used for screening neonates enabling counselling for parents and children.

FX carrier screening studies in Israel involving 14,334 women has shown that the cost of the test is much cheaper than the cost of life time care for a mentally challenged person due to the higher prevalence of the condition in general population<sup>12</sup>.

The antibody test employed in the present study is very efficient, rapid, simple and cost effective for screening large number of cases. This could be introduced into the school health programmes in developing countries, to identify affected individuals and subsequently carriers of the FX mutation. All identified patients will need to be referred to a clinical genetics centre to allow genetic counselling of family involved. Further evaluation will automatically include DNA analysis for carrier detection. For patients and families a correct diagnosis is essential and leads to availability of adequate support for the behavioural and physical problems. In addition, more female carriers can benefit from genetic counselling with respect to the risk for their offspring, with an option for prenatal diagnosis and for themselves about premature ovarian failure with the option of completing their families early.

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This write-up has been contributed by Dr. Z.M. Patel, Deputy Director and Dr. S.R. Menon, Technician, ICMR Genetic Research Centre, Mumbai.

## ABSTRACTS

## Some Research Projects Completed Recently

Development and synthesis of UV lamp phosphorus for phototherapy

The study was planned with the aim to synthesize commercially used phosphorus for UV therapy lamps and standardize the synthesis method; synthesize new potential UV emitting phosphorus fulfilling narrow-band criterion especially required in UVB therapy and test reproducibility, cost effectiveness and use of indigenous raw materials in the synthesis.

Phosphorus  $\text{SrAlF}_5: \text{Ce}^{3+}, \text{Gd}^{3+}$  and  $\text{PrF}_3: \text{Ce}^{3+}$  were prepared by a novel low temperature soft chemistry method. The method was found to be the most effective in preparing fluorides because OH-ions cannot attach themselves with the lattice thereby reducing luminescence intensity. Very strong Ce emission in UV region was observed in all the samples including above.

As Gd has its excitation band below 305 nm, it has a good overlap emission spectrum of Ce. Codoping with Gd yielded phosphorus with very strong narrowband emission.

The study could produce a very good lamp phosphorus substitute in CO-504  $\text{Sr}_{0.8}\text{Ce}_{0.1}\text{Gd}_{0.1}\text{AlF}_5$  where intensity is very large (more than commercial UV lamp phosphorus) and the synthesis method is a single step low temperature soft chemistry route. It is therefore a strong candidate for UV lamp phosphorus in UVB region. Similarly CO-506  $\text{Pr}_{0.85}\text{Ce}_{0.1}\text{Gd}_{0.05}\text{F}_3$  is also a potential candidate as lamp phosphorus in UVB region.

Apart from being stable and reproducible, the lamp phosphorus was found highly cost effective.

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Carcinoma associated antigens (Tn, s-Tn and T): Efficacy in detecting and monitoring patients with squamous cell carcinoma of oesophagus

The study was undertaken with the main aim of analyzing the early glycobiological changes in the oesophageal squamous epithelial carcinoma (OSEC)

using specific lectin. The peanut agglutinin (PNA) binding TF antigen (Galb 1-3 GalNAc) was chosen as a marker in this study.

All patients were carefully staged according to the standard rules of TNM, FIGO and WHO classification. About 10 ml of blood sample was collected before and after treatment (surgery, and chemo/radiotherapy) of patients with different stages of OSEC. The analysis of TF-antigen expression in OSEC was carried out by quantitative assays (biochemical assays, protein assay, hexose assay, hexosamine assay, sialic acid assay and mucin assay) for the quantification of preliminary data for the study of mucin in the blood samples of the cancer patients.

The semi-qualitative analysis of serum glycoproteins was done by (i) SDS-PAGE, immunoblot analysis Western blot analysis of Tn, S-Tn and T antigen using WA, Limulin and PNA, dot blot analysis of Tn, S-Tn and T antigen using WA, Limulin, and PNA, doubleimmunodiffusion, counter immunoelectrophoresis and enzyme linked lectin assay (ELLA). The serological results were further confirmed with histochemical analysis using fluorescence labeled lectin studies.

Increased circulating levels of TF-antigen was observed in the sera of OSEC patients compared to healthy controls. Higher levels of simple sugars and mucin were found in OSEC patients. Blotting analysis of TF-antigen with lectin PNA reveals that the higher molecular weight binding, specifically to peanut agglutinin lectin was observed increasingly in the OSEC patients. Light microscopical analysis of membrane TF antigen level showed an increased expression in OSEC patients. Altered histological expression of TF antigen in OSEC patients didn't return to normal levels of healthy oesophageal tissue. The study clearly revealed a trend in the altered expression of TF antigen analyzed by PNA with tissue as well as sera of OSEC patients.

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Mutational Screening of rhodopsin gene in patients of autosomal dominant retinitis pigmentosa (RP) and RPE65 gene in patients of childhood onset autosomal recessive retinitis pigmentosa and lebers Congenital Amaurosis

The study was carried out to determine the frequency of rhodopsin gene (Rho) mutations in autosomal dominant retinitis pigmentosa (adRP) and retinal pigmentosa epithelium 65 (RPE65) gene mutations in autosomal recessive retinitis pigmentosa (arRP) and lebers congenital amaurosis LCA cases. Coding regions and intron exon boundaries of Rho (53 adRP & 50 isolated) were screened by allele specific PCR, restriction fragment length polymorphism (RFLP), single strand conformational polymorphism (SSCP) and RPE65 gene

in 50LCA, 15 arRP and 50 unrelated healthy controls by direct sequencing. Two pathogenic mutations, Gly106Arg in Rho and Pro471Leu, a novel missense mutation in RPE65 was detected in a LCA patient. The findings of the study suggested that Rho contribute to 2% of adRP and RPE 65 to 2% of LCA in our population. It was concluded that Rho and RPE65 do not seem to be the common causative pathogenic genes in RP and LCA in our population.

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#### ICMR NEWS

The following meetings of various technical groups/committees were held:

Scientific Advisory Committees (SACs):

SAC of National Institute of Malaria Research July 7, 2006

SAC of Regional Medical Research Centre for Tribals, Jabalpur August 3, 2006

Task Forces (TFs)/Advisory Groups (AGs)/Working Groups (WGs)/ and Other Meetings held at New Delhi:

TF on Epidemiology of Viral Hepatitis in Tribals of Orissa, Madhya Pradesh and Chattisgarh July 14, 2006

WG on Communicable and Non-communicable Diseases for 11th Plan July 25, 2006

EG of the TF on Mental Health Needs and Service Delivery Model on Disaster Affected Population in Gujarat July 28, 2006

Project Advisory Committee of ICMR Coordinated Project on Sub-Clinical Vitamin A Deficiency August 5, 2006

EG on Task Force Project on Relationship between Genetic Polymorphism in Drug Metabolizing Enzymes CYP1A1 and GSTM1 and Risk of Developing Oral Precancer and Cancer among Consumers of Tobacco and Chewing Products August 8, 2006

TF on Intervention Programme to Reduce the Risk Factors on CHD in Indian Population August 10, 2006

TF on Inborn Metabolic Disorders August 22, 2006

II Annual Review of TF Project on Multi-Site Epidemiological and Virological Monitoring of Human Influenza Surveillance Network in India-Phase-I August 25, 2006

Sub-Committee on Review of Management Guidelines on Buccal Mucosa Cancer and Chronic Myeloid Leukaemia August 30, 2006

EG on Indo-German Workshop on Cancer Research August 30, 2006

TF on Multicentric Study on Hospital-based Surveillance on Rotavirus Diarrhoea in India September 1, 2006

EG on Stem Cell Research and Therapy	September 5, 2006	PRC on Tribal Health	July 25, 2006
Indo-US Meeting on Emerging and Re-emerging Infectious Diseases and Disease Surveillance	September 6, 2006	PRC on Otorhinolaryngology	August 3, 2006
Technical Committee on Formulation of Guidelines for Use of Double Fortified Salt as a Measure to Reduce Prevalence of Anaemia	September 8, 2006	PRC on Pharmacology	August 17, 2006
EG and Training Workshop on ICMR Coordinated Project in North-East Region on Nutrition Profile Of Assam, Manipur, and Meghalaya	September 9-10, 2006	PRC on Traditional Medicine	August 18, 2006
TF on Remote Sensing and Geographical Information System	September 14, 2006	Special PRC on North-East Projects	August 22, 2006
TF on Immunology	September 14, 2006	PRC on Environmental Hygiene and Occupational Health	August 23, 2006
Expert Committee to Assess the Source of Infection in Outbreak of Avian Influenza in Maharashtra	September 20, 2006	PRC on Malaria, Filariasis and Leishmaniasis	September 9, 2006
Indo-US Joint WG on Maternal and Child Health And Human Development Research	September 21-22, 2006	PRC on Oncology and Pathology	September 9, 2006
TF on Camel Milk and Diabetes	September 26, 2006	PRG on Nutrition	September 15, 2006
EG on Multicentric Open Randomized Controlled Clinical Trial of Combination Therapy with Ribovirin and Oral Glycyrrhizin	September 27, 2006	PRC on Ophthalmology	September 25, 2006
Project Review Committees (PRCs)/Project Review Groups (PRGs) held at New Delhi:		Workshops/Training Programmes:	
PRC on other Microbial Diseases and Viral Diseases	July 5, 2006	A WHO Workshop on Road Traffic Injuries Surveillance was organized at New Delhi during July 11-12, 2006.	
PRC on Leprosy, Tuberculosis and Other Chest Diseases	July 14, 2006	A Training on Cancer Registration was organized at Shimla during July 25-27, 2006.	
		An ICMR/FERCAP/WHO Training Course on Standard Operating Procedures for Ethics Committees was organized at Mumbai during August 30 - September 1, 2006.	
		ICMR/AGBIOS, Canada and BCIL, Delhi Workshop on Safety Assessment of Foods Derived from Genetically Modified Crops were organized at Hyderabad and Lucknow during September 18-22 and 25-29, 2006 respectively.	
		Participation of ICMR Scientists in Scientific Events:	
		Dr. V.A. Arankalle, Deputy Director and Dr. M.S. Chadha, Assistant Director, National Institute of Virology (NIV), Pune, participated in the XII International Symposium on Viral Hepatitis and Liver Disease at Paris (July 1-5, 2006).	

Dr. Sunita Saxena, Director and Dr. Sujala Kapoor, Assistant Director, Institute of Pathology (IOP), New Delhi, participated in the UICC World Cancer Congress 2006 at Washington D.C. (July 8-12, 2006).

Dr. L.K. Yemeni, Senior Research Officer, IOP, New Delhi, participated in the XVI International Congress of International Organization for Mycoplasma at Cambridge (July 9-14, 2006).

Dr. P.K. Nag, Deputy Director (Senior Grade), National Institute of Occupational Health (NIOH), Ahmedabad, participated in the XVI World Congress on Ergonomics at Maastricht (July 10-14, 2006).

Dr. B.N. Murthy, Deputy Director, National Institute of Epidemiology, Chennai, participated in the XXIII International Biometric Conference at Montreal (July 16-21, 2006).

Dr. L.R. Yeolekar, Senior Research Officer, NIV, Pune, participated in the Laboratory Workshop on Avian Influenza at Nonthaburi (July 17-21, 2006).

Dr. Arun Lata Mittal, Deputy Director, IOP, New Delhi, participated in the XIV IUSTI - Asia Pacific Conference 2006 at Kuala Lumpur (July 27-30, 2006).

Dr. Aruna Dewan, Deputy Director (Senior Grade) and Shri A.B. Patel, Senior Research Officer, NIOH, Ahmedabad, participated in the V Asia Pacific Association Medical Technology (APAMT) Congress at Colombo (August 4-8, 2006).

Dr. Roshan B. Colah, Deputy Director, Institute of Immunohaematology (IIH), Mumbai, participated in the Asian Thalassaemia Network meeting and Novartis Symposium on Thalassaemia, at Brisbane (August 6-7, 2006).

Dr. S.K. Sharma, Assistant Director, Regional Medical Research Centre (RMRC), Dibrugarh and Dr. Malay B. Mukherjee and Dr. V. Babu Rao, Senior Research Officer, IIH, Mumbai, participated in the XI International Congress of Human Genetics at Brisbane (August 6-10, 2006).

Dr. J. Mahanta, Director, RMRC, Dibrugarh, Dr. S.K. Ghosh, Deputy Director, National Institute of Malaria Research (NIMR), Delhi and Dr. Rajeev Yadav and Dr. C.K. Dolla, Research Officers, RMRC for Tribals, Jabalpur, participated in the XI International Congress of Parasitology at Glasgow (August 6-11, 2006).

Dr. Nasreen Z. Ehtesham, Assistant Director, National Institute of Nutrition (NIN), Hyderabad, participated in the Biotechnology Asia 2006 Conference at Kuala Lumpur (August 9-11, 2006).

Dr. Soumya Swaminathan, Deputy Director (Senior Grade), Tuberculosis Research Centre (TRC), Chennai and Dr. S.P. Tripathy, Deputy Director and Dr. Seema Sahay, Senior Research Officer, National AIDS Research Institute (NARI), Pune, participated in the XVI International AIDS Conference at Toronto (August 13-18, 2006).

Dr. Dipika Sur, National Institute of Cholera & Enteric Diseases (NICED), Kolkata, participated in the Investigators meeting for the Multi Centre Severe Diarrhoeal Disease Burden and Etiology Study and Microbiology Training Session at Baltimore (August 21-23, 2006). Dr. T. Ramamurthy, Deputy Director, NICED, Kolkata, participated in the meeting and Training Session from August 21- September 2, 2006. Dr. R.K. Nandy, Senior Research Officer, NICED, Kolkata, participated in the meeting and Training Session during August 24 - September 2, 2006.

Dr. Surendra Kumar and Shri Dinesh Kumar, Senior Research Officers, RMRC for Tribals, Jabalpur, participated in the XI World Congress on Collective Health at Rio de Janeiro (August 21-25, 2006).

Dr. R.S. Paranjape, Director; Dr. S.M. Mehendale, Deputy Director (Senior Grade); and Dr. Seema Sahay and Dr. Madhuri Thakar, Senior Research Officers, NARI, Pune and Dr. Shekhar Chakraborty, Deputy Director (Senior Grade), NICED, Kolkata, participated in V AIDS Vaccine International Conference at Amsterdam (August 29 - September 1, 2006). Dr. Paranjape also participated in the International Conference on HIV/AIDS and Business: Challenges for Sustainable Development, at London (September 7-8, 2006).

Dr. Kamlesh Sarkar, Assistant Director, NICED, Kolkata, participated in the International Conference on Hepatitis C at Stockholm (September 7-11, 2006).

Dr. P. Paul Kumaran, Senior Research Officer, TRC, Chennai, participated in the Annual Training Seminar and Annual Board Meeting of Western Institutional Review Board at Seattle (September 8-9, 2006).

Dr. P. Raghu, Research Officer, NIN, Hyderabad, participated in the workshop on Standardization of In Vitro Digestion/Caco-2 Cell Method for Micronutrient Bioavailability at Columbus (September 12-13, 2006).

Dr. A.P. Dash, Director, NIMR, Delhi, participated in a Discussion for Collaborative Activities between NIMR and CDC at Atlanta (September 18-23, 2006).

Dr. A. Roy Chowdhury, Deputy Director (Senior Grade), NIOH, Ahmedabad, participated in the XLIII Congress of the European Society of Toxicology and VI Congress of Toxicology in Developing Countries at Dubrovnik/Cavtat, Croatia (September 20-24, 2006).

Dr. V.N. Gokani, Deputy Director, NIOH, Ahmedabad, participated in a meeting of International Task Force on Pesticide Sampling and Detection in Soft Drinks at Minneapolis (September 21-22, 2006).

Dr. Sudershan Rao, Senior Research Officer, NIN, Hyderabad, participated in the 2006 Food Safety Education Conference: Reaching at Risk Audiences and Today's Other Food Safety Challenges at Denver (September 25-29, 2006).

Dr. M.B. Singh, Assistant Director, Desert Medicine Research Centre, Jodhpur and Dr. N. Arlappa, Senior Research Officer, NIN, Hyderabad, participated in the

IWorld Congress of Public Health Nutrition at Barcelona (September 28-30, 2006).

Training/Course

Dr. M. V. Ghate, Senior Research Officer, NARI, Pune, availed training on Neuropsychological Test Battery and Neuropsychiatric Assessments at California (August 7-8, 2006).

Dr. Smita S. Kulkarni, Senior Research Officer, NARI, Pune, availed training in TZM-bI Assay at Duke University Medical Centre, Durham for 40 days w.e.f. August 7, 2006.

Dr. Jayanta Bhattacharya, Assistant Director, NARI, Pune, availed training in Molecular Cloning of Functional HIV-1 Envelop Protein and Application of Assays to Measure Neutralizing Antibody Response to HIV at Duke University Medical Centre, Durham for 40 days w.e.f. August 7, 2006.

Dr. M. Thomas, National Institute of Medical Statistics, New Delhi, participated in a Course on Epidemiology and Control of Infectious Diseases at London (September 3-15, 2006).

Dr. Sujala Kapoor, IOP, New Delhi, availed Technical Training for Automatic Slide Processor at Michigan (September 4-7, 2008).

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