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4.1. STUDY SUBJECTS

A nested case control study design was employed. Study subjects were divided into three groups: group I represented the patients chiefly with delusional disorder, second group comprised three different diseases, like paranoid schizophrenia, mood congruent delusion and early Alzheimer's disease which represented delusion as one of the symptom and the third was age and sex matched healthy control group. All the subjects belonged to the India Born Bengalee population. All the subjects were typed for HLA-Class I antigens by serological method (i.e. two-stage microlymphocytotoxicity assay) as well as by molecular method (ARMS-PCR SSP typing). Besides HLA, short lymphocyte culture was performed to prepare karyotype from all the subjects.

4.1.1. GROUP I: Patients with Delusional Disorder

The subjects were recruited from the Psychiatric OPD, North Bengal Medical College and Hospital. On an average 1500 new patients with different psychiatric illnesses and about 4000 recurrent follow up cases attend the OPD every year. A total number of 150 unrelated patients with delusions were enrolled and studied for a period of five years. All the cases were screened independently by two psychiatrists using the Structured Clinical Interview (SCID) for DSM-IV for delusional disorder . Diagnostic criteria listed in **Table-13** were used for diagnosing the patients with delusional disorder. After longitudinal follow up, 100 patients represented the genuine cases of delusional disorder of various subtypes and remaining 50 patients turned out to have the cases of other psychiatric illnesses like paranoid schizophrenia, substance abuse disorder, dementia and mood congruent delusions and were excluded from the patient group.

Table 13: DSM-IV Diagnostic Criteria for Delusional Disorder

A. Nonbizarre delusions (i.e. involving situations that occur in real life, such as being followed, poisoned, infected, loved at a distance, or deceived by spouse or lover, or having a disease) of at least one month's duration.
B. Criteria A for schizophrenia have never been met. Note: Tactile and olfactory hallucinations may be present in delusional disorder if they are related to the delusional theme.
C. Apart from the impact of the delusion(s), or its ramifications, functioning is not markedly impaired and behavior is not obviously odd or bizarre.
D. If mood episodes have occurred concurrently with delusions, their total duration has been brief relative to the duration of the delusional periods.
E. The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

It was observed that maximum patients were clustered between 25 to 55 age group with average mean 39 ± 1.29 years with a male to female ratio of 1:1.44. Patients were mostly from middle class urban society belonging to a nuclear family. A method of routine enquiry was made to all persons attending Psychiatry OPD to find any case in the family who is suspicious and /or jealous. This resulted in attendance of at least 25% of total number of our cases who otherwise would not have consulted a psychiatrist.

A total number of 15 entire families with multiple cases of delusional disorder were also considered to study the incidence of delusional disorder/paranoid psychoses in the first-degree biological relatives of the probands. The pedigrees of those families have been given in **Annexure II**. Detailed record of the patients in a structured format **(Annexure I)** consisting of subtype and extent of delusion, routine assessment and history of treatment were completed for each patient and followed up carefully for 2-3 years. The SCID format used for screening of the patients is given in **Annexure IV**. The details of the subjects as well as clinical subgroups of the patients with delusional disorder included for HLA based studies are depicted in **Table 14** and **Table-15** respectively.

Table 14: Study subjects and clinical groups considered for HLA based studies

Subjects	HLA- Class I Typing
Group I: DELUSIONAL DISORDER	100
SEX	
MALE	41
FEMALE	59
Group II: OTHER DISORDERS WITH PARANOID SYMPTOMS	50
1) PARANOID SCHIZOPHRENIA	
SEX	37
MALE	13
FEMALE	
	30
2) EARLY ALZHEIMER'S DISEASE	
SEX	19
MALE	11
FEMALE	
	30
3) MOOD CONGRUENT DELUSION	
SEX	19
MALE	11
FEMALE	
Group III: HEALTHY CONTROLS	
For Delusional Disorder	100
SEX	
MALE	41
FEMALE	59
For Paranoid Schizophrenia	50
SEX	
MALE	37
FEMALE	13
For Early Alzheimer's Disease	30
SEX	
MALE	19
FEMALE	11
For Mood Congruent Delusion	30
SEX	
MALE	19
FEMALE	11

Table-15: Showing the clinical subgroups with basic delusional theme and the number of each group studied:

Delusional Disorder Subtype	Common Clinical Presentation	No. of patients
Erotomanic subtype	Patients present with the belief that some important person is secretly in love with them. Clinical samples are often female and forensic samples contain a preponderance of males. Patients may make efforts to contact this person, and some cases are associated with dangerous or assaultive behavior. Rescue fantasies regarding the secret admirer are common.	6
Grandiose subtype	Patients believe they fill some special role, have some special relationship, or possess some special ability(ies). They may be involved with social or religious organizations.	4
Jealous subtype	Patients possess the fixed belief that their spouse or partner has been unfaithful. Often, patients collect bits of evidence and attempt to restrict their partner's activities. This type of delusional disorder has been associated with forensic cases involving murder.	13
Persecutory subtype	This is the most common presentation of delusional disorder. Patients are convinced that others are attempting to do them harm. Often they attempt to obtain legal recourse, and they sometimes may resort to violence.	57
Somatic subtypes	Patients vary in presentation, from those who have repeat contact with physicians requesting various forms of medical or surgical treatment to patients who are delusionally concerned with bodily infestation, deformity, or odor.	10
Mixed subtype	Delusions characteristic of more than one of the above types but no one theme predominates.	8
Unspecified subtype		0

4.1.2. GROUP II: OTHER DISORDERS WITH PARANOID SYMPTOMS

A total number of 50 unrelated patients with paranoid schizophrenia who attended the OPD of Psychiatry, North Bengal Medical College and Hospital were

considered for the present study. All patients belonged to the same ethnic background as the main subjects. Patients were diagnosed independently by two psychiatrists according to the standard diagnostic criteria of DSM-IV **(Table-16)** and were assessed by the Brief Psychiatric Rating Scale (BPRS) **(Annexure-V)**. The average age was 34.96 ± 1.40 and male to female ratio of 2.8:1 were studied.

Table-16: DSM-IV Diagnostic criteria of Paranoid Schizophrenia

A. Preoccupation with one or more delusions or frequent auditory hallucinations.
B. None of the following is prominent: disorganized speech, disorganized or

[redacted]

A total number of 30 unrelated patients with early Alzheimer's disease who attended the OPD of Psychiatry of North Bengal Medical College and Hospital were considered for the present investigation. All the patients met the standard diagnostic criteria of DSM-IV **(Table-17)** and belonged to the same ethnic group as the main subjects. The average age (in years) of the patients was 66.57± 1.35 and male to female ratio was 1.7:1.

Table-17: DSM IV and proposed criteria for psychosis of AD (Jeste & Finkel, 2000).

Patients with AD must have the following:
1. Characteristic delusions or hallucinations in the presence of the possible or probable AD
2. Onset of psychotic signs and symptoms, and which are present at least intermittently for at least 1 month; and
3. Symptoms severe enough to disrupt patients' or others' functioning,
4. Not better accounted for by another psychotic disorder, medical condition, or effects of a drug, and not occurring during the course of a delirium.

A total number of 30 unrelated patients with severe depression with psychotic features i.e. mood congruent delusion who attended the OPD of Psychiatry of North Bengal Medical College and Hospital were recruited for the present study. All patients belonged to the same ethnic background as the main subjects. All patients were diagnosed independently by two psychiatrists according to the standard diagnostic criteria of DSM-IV **(Table-18)**. The average age was 37 ±1.86 and male to female ratio was 1.7:1.

Table 18: DSM-IV diagnostic criteria of for Psychotic Major Depressive Episodes

With psychotic features: Delusions or hallucinations. If possible, specify whether the psychotic features are mood-congruent or mood incongruent.
A. Mood –congruent psychotic features: Delusions or hallucinations whose content is entirely consistent with the typical; depressive themes of personal adequacy, guilt, disease, death, nihilism, or deserved punishment.

4.1.3. GROUP III: Healthy Controls

Healthy donors were also divided into different subgroups corresponding the number of the patients in the group I and group II as all the control subjects are sex and age matched. A total number of 100 unrelated healthy individuals were considered as control for delusional disorder, 50 for paranoid schizophrenia, 30 for early Alzheimer's disease and 30 for mood congruent delusion. All the control subjects belonged to the same ethnic background. None of the controls had any family history of delusional disorder or any other conditions associated with delusions. All controls subjects were screened for a recent history of intercurrent infections and allergies. Those with a past history of autoimmune or psychiatric disorders were excluded. All the patients and controls gave informed consent to participate in the study.

4.2. SEROLOGICAL TYPING (MICROLYMPHOCYTOTOXICITY ASSAY) OF HLA CLASS-I ANTIGENS

Serological typing of HLA - Class I antigens was carried out in all the subjects (Delusional Disorder = 100 + 100 healthy controls, Paranoid Schizophrenia = 50 + 50 healthy controls, Early Alzheimer's = 30 + 30 healthy controls, Mood Congruent Delusion = 30 + 30 healthy controls) by complement mediated two-stage microlymphocytotoxicity assay (Terasaki & McClelland, 1964).

The principle behind this technique is the binding of anti HLA antibody to a specific HLA (class I or class II) antigen expressed on the lymphocytes. A suspension of lymphocytes mixed with a specific antiserum results in the formation of antigen- antibody complex. In the presence of complement, if the antibody used has the potential for complement activation, cells carrying the appropriate antigen are damaged resulting in increased permeability. The damaged cells are not completely lysed but suffer membrane damage to allow uptake of stain such as Eosin. Where an antigen on the cell surface does not react with antibody, the lymphocytes are not stained (Negative Reaction). Microscopic identification of the stained cells indicates the presence of a specific HLA antibody. Viable cells appear bright and refractory while the dead cells look dark and distinctly larger.

This is two-stage test that employs the sensitization step of HLA antigen with antibody. The second step is the 'specificity test', achieved by the addition of rabbit complement.

4.2.1. Isolation and preparation of lymphocyte cell suspension

1. Approximately 5 ml of blood sample was collected from each individual with the help of disposable syringes and was added 100-150 IU preservative free Lithium Heparin in a clean test tube and mixed well.
2. The blood was diluted with equal volume of PBS and mixed thoroughly by gentle shaking.

3. The diluted blood was layered over lymphoprep carefully with a Pasteur pipette in a ratio of 2:1 respectively.
4. The tubes were centrifuged at 2000 rpm for 20 minutes at room temperature.
5. Using a clean Pasteur pipette, the interface (white-foggy) layer of mononuclear cells was transferred into a clean test tube.
6. The cells were washed 2-3 times with PBS (pH 7.2) by centrifuging at 1000 rpm for 10 minutes at room temperature and lymphocyte cell suspension was prepared.
7. Finally, the lymphocyte cell suspension was counted in a Neubauer Haemocytometer and adjusted to a final concentration of 2×10^6 cells/ml using PBS.
8. Viability test was done using 1% trypan blue and observing under phase contrast microscope. A drop of cell suspension was mixed with a drop of trypan blue on a clean glass slide and allowed to stand for 5 minutes. Dead cells became dark blue. At least 85% viability was required.

4.2.2. HLA class I typing

Two sets of well defined HLA antisera (one from BAG, Germany and another obtained from 12th International Histocompatibility Workshop and Conference) were used. The HLA class I antisera used for the present study included 17 A-locus alleles and 25 B-locus alleles. Terasaki plates made up of nontoxic disposable polystyrene material (NUNC, Denmark) containing 60-72 wells was used for testing of class I antigens. The typing trays were thawed before use at room temperature. 1µl of lymphocyte suspension (2×10^6 cells/ml) was dispensed carefully into each well and the cells and sera were mixed well. The trays were incubated at 22-24°C for 30 minutes. 5 µl nontoxic rabbit complement was added to each well with Hamilton six-needle dispenser (250µl capacity). The trays were further incubated for a period of 60 minutes. 4µl of 5% Eosin-Y dye (centrifuged before use) was added to each well. After 5 minutes 4µl of 40% formalin

(centrifuged before use) was added to each well. The trays were capped tightly and kept in the refrigerator for reading the results after a gap of at least 2 hours.

Trays were read using inverted phase contrast Microscope (Leitz, West Germany) with 10x objective and 10x eyepiece. Live or viable cells that excluded dye were small, refractile and unstained, while dead cells looked dull, larger in size and stained red with Eosin. Scoring was done by estimating the percentage of cell lysis as adapted in the 8th International Histocompatibility Workshop **(Table-19)**. Typing trays were scored on a 'subjective scale' taking into consideration the amount of 'background' (dead cells) in the negative control well.

Table-19: Scoring system

SCORE	INTERPRETATION	INCREASE IN CELL DEATH OVER THE NEGATIVE CONTROL-%
1	Negative (-ve)	0-19% kill
2	Doubtful weak positive (+)	20-29% kill
4	Positive (++)	30-39 % Kill
6	Strong Positive (+++)	50-79% Kill
8	Very Strong Positive (++++)	80-100% Kill
0	Invalid	Not readable

4.3. ARMS-PCR SSP TYPING OF HLA CLASS I GENES

4.3.1. DNA Extraction (Phenol Chloroform Method)

DNA was isolated from frozen peripheral blood samples using phenol chloroform extraction method with slight modifications.

Sample Preparation and Digestion

1. Sample was thawed first.
2. 500µl blood sample was taken and washed with 1 ml of 1X SSC, mixed gently and centrifuged at 5000 rpm for 10 minutes.

3. The supernatant was removed and 1.2 ml of 50 mM KCL was added and mixed gently and then centrifuged.
4. Again the supernatant was removed and 375µl of High Salt Lysis Buffer was added.
5. 25µl of 10% SDS and 12.5µl of 8 mg/ml proteinase K stock was added and incubated for 1 hour at 56°C.

Extraction of DNA

1. The proteinase K digested suspension was transferred into a new microfuge tube and an equal amount of PCl was added and spinned at 12000 rpm for 20 minutes.
2. The aqueous phase was recovered and a second PCl extraction was made.
3. 13µl of 4M NaCl and 0.6 vol of 100% isopropanol were added. Mixed gently by inversion until the precipitate was formed.
4. The DNA was recovered and rinsed with 500µl of 70% C_2H_5OH, three times.
5. The DNA was dried and dissolved in 50µl of TE.

Quantification of DNA

10µl of dissolved DNA was diluted to 1.5 ml using deionized water and mixed properly. OD of the diluted DNA was measured at wavelengths of 260 nm and 280 nm using a UV spectrophotometer. Protein-free DNA samples gave 1.7-2.0 reading at 260/280. The concentration of DNA was calculated using the formula - OD at 260 X dilution factor X 50 (1 OD = 50µg of double stranded DNA)

4.3.2. PCR Amplification

PCR was carried out in thermostable PCR-tubes with 50µl of PCR reaction mixture consisting of PCR buffer, $MgCl_2$, primer set, deoxynucleotide triphosphate mix and Taq DNA polymerase along with the template DNA. The methodology and sequence information were taken from Bunce *et al* 1995 as well as from the 12th IHW protocols. The nucleotide sequences of primers used for amplification of

various HLA-A and HLA-B locus allele in this study are listed in **Table 20 & 21** respectively.

Table - 20: List of primers used for HLA -A locus typing.

A*01			
	P1	GGA CCA GGA GAC ACG GAA TA	20
	P2	AGG TAT CTG CGG AGC CCG	18
A*02			
	P1	TCC TCG TCC CCA GGC TCT	18
	P2	GTG GCC CCT GGT ACC CGT	18
A*03			
	P1	AGC GAC GCC GCG AGC CA	17
	P2	CAC TCC ACG CAC GTG CCA	18
A*23			
	P1	GGC CGG AGT ATT GGG ACG A	19
	P2	CCT CCA GGT AGG CTC TCA A	19
A*24			
	P1	GGC CGG AGT ATT GGG ACG A	19
	P2	CCT CCA GGT AGG CTC TCT G	19
A*25			
	P1	TCA CAG ACT GAC CGA GAG AG	20
	P2	ATG TAA TCC TTG CCG TCG TAA	21
A*2674301			
	P1	ACT CAC AGA CTG ACC GAG C	19
	P2	ATG TAA TCC TTG CCG TCG TAA	21
A*2572674766			
	P1	GGA GTA TTG GGA CCG GAA C	19
	P2	ATG TAA TCC TTG CCG TCG TAA	21
A*11			
	P1	ACG GAA TGT GAA GGC CCA G	19
	P2	CTC TCT GCT GCT CCG CCG	18
A*29			
	P1	AGG ATG GAG CCG CGG GCA	18
	P2	AGC GCA GGT CCT AGT TCA A	19
A*30			
	P1	CCC GGC CCG GCA GTG GA	17
	P2	CCG TCG TAG GCG TGC TGT	18
A*31			
	P1	GAT AGA GCA GGA GAG GCC T	19
	P2	AGC GCA GGT CCT AGT TCA A	19
A*32011			
	P1	TCA CAG ACT GAC CGA GAG AG	20
	P2	AGC GCA GGT CCT AGT TCA A	19
A*34766			
	P1	ACG GAA TGT GAA GGC CCA G	19
	P2	ATG TAA TCC TTG CCG TCG TAA	21

Table- 21: List of primers used for HLA -B locus typing.

B*07		
P1	GGA GTA TTG GGA CCG GAA C	19
P2	TAC CAG CGC GCT CCA GCT	18
B*08		
P1	GAC CGG AAC ACA CAG ATC TT	20
P2	CCG CGC GCT CCA GCG TG	17
B*13		
P1	TAC CGA GAG AAC CTG CGC	18
P2	GGG CCG CCT CCC ACT TGA	18
B*14		
P1	GAG CAG GAG GGG CCG GAA	18
P2	CGT CGC AGC CAT ACA TCC A	19
B*1501		
P1	ACC GGG AGA CAC AGA TCT C	19
P2	CCT TGC CGT CGT AGG CGG	18
B*1801		
P1	GGC GCC GTG GAT AGA GCA A	19
P2	GCC GCG GTC CAG GAG CT	17
B*27/7301		
P1	ACC GGG AGA CAC AGA TCT G	19
P2	GAG CCA CTC CAC GCA CTC	18
B*35/5301		
P1	GAC CGG AAC ACA CAG ATC TT	20
P2	GGA GGA GGC GCC CGT CG	17
B*3701		
P1	GCC GCG AGT CCG AGG AC	17
P2	CCT CCA GGT AGG CTC TGT C	19
B*4001		
P1	CCA CTC CAT GAG GTA TTT CC	20
P2	CCG CGC GCT CCA GCG TG	17
B*4201		
P1	GAC GAC ACC CAG TTC GTG A	19
P2	CCG CGC GCT CCA GCG TG	17
B*44		
P1	TAC CGA GAG AAC CTG CGC	18
P2	CCA GGT ATC TGC GGA GCG	18
B*44/4501/4514		
P1	ACC GGG AGA CAC AGA TCT C	19
P2	CCA GGT ATC TGC GGA GCG	18
B*4901/5001		
P1	CCA CTC CAT GAG GTA TTT CC	20
P2	ATC CTT GCC GTC GTA GGC T	19
B*5101/5105		
P1	GGA GTA TTG GGA CCG GAA C	19
P2	CGT TCA GGG CGA TGT AAT CT	20
B*52012/52012		
P1	ACC GGG AGA CAC AGA TCT C	19
P2	CGT TCA GGG GGA TGT AAT CT	20
Positive Internal Control		
P1	ATG ATG TTG ACC TTT CCA GGG	21
P2	ATT CTG TAA CTT TTC ATC AGT TGC	24

Preparation of PCR reaction mixture: PCR amplification was performed in thermostable sterile PCR tubes on a DNA thermal cycler (Perkin Elmer, USA) containing 50µl reaction mixture of the following combination:

Reagents		Reaction Mix (50 µl)
Initial conc. of stock solution	Final conc. of stock solution	For 1 test
dH_2O	--------	36.8 µl
10 X PCR Buffer	1X	5 µl
dNTPs	2.5 mM	3.2 µl
P1	10 pm	1 µl
P2	10 pm	1 µl
Target DNA	50µg	2 µl
Taq polymerase	3 unit	1 µl

Amplification Procedure

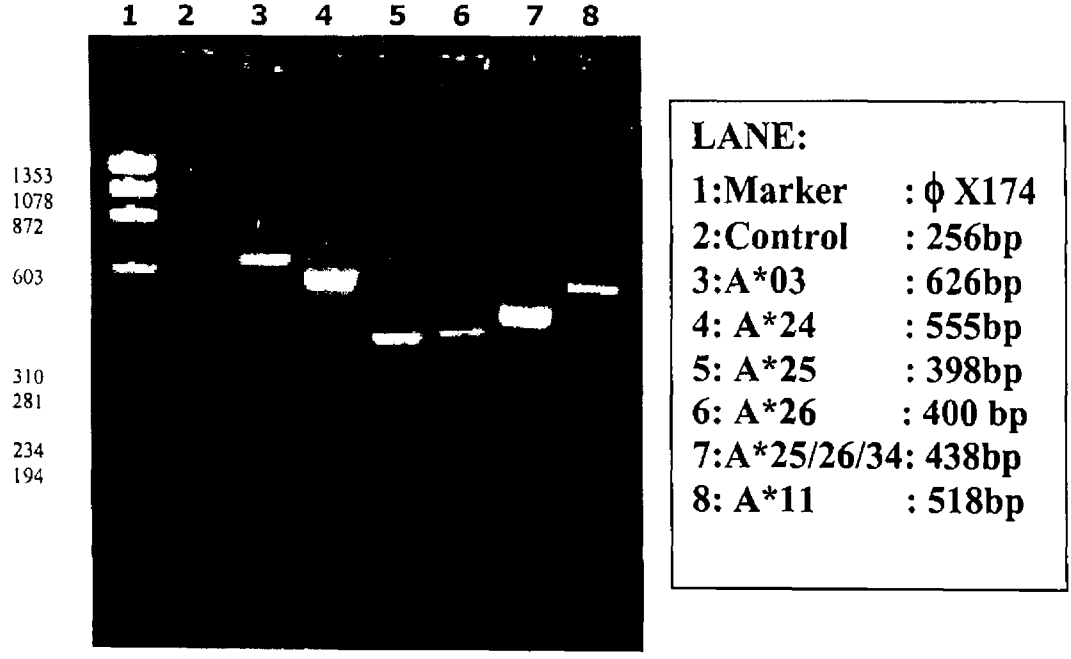
50 µl of reaction mix was dispensed into PCR tubes. Sterile distilled H_2O and standard DNA were used as positive internal controls. The mix was vortexed and the tubes were spun down briefly for 10-15 seconds and arranged in the heat block of thermal cycler. We used touch down method and following conditions were adopted.

No. of Cycle	Time	Temperature
1 cycle denaturation	1 min	96°C
5 cycles of	25 secs	96°C
	45 secs	70°C
	30 secs	72°C
21 cycles of	25 secs	96°C
	45 secs	65°C
	30 secs	72°C
4 cycles of	25 secs	96°C
	60 secs	55°C
	120 secs	72°C
1 cycle of	10 mins	
Hold at 15°C		72°C

4.3.3. Amplification Check by Agarose Gel Electrophoresis

We used mini gel electrophoresis apparatus (BIOTECH, India) for rapid separation of amplified PCR products. PCR products were separated on 2% submerging agarose (Bangalore Genei, India) gel in TBE buffer containing 0.5 μg/ml ethidium bromide (Boehringer Mannheim, Germany) to check efficiency and specificity of the reaction. ϕX174 DNA marker (Bangalore Genei, India) providing even banding patterns of uniform intensity (1353bp, 1078bp, 872 bp, 603 bp, 310 bp, 281/271bp, 234bp, 194bp, 118bp, and 72bp) was loaded in the first well. Fig 6. shows the electrophoregram containing PCR amplified products of different HLA class-I genes.

Fig.6: Electrophoregram showing the results of HLA- A*03, A*11, A*24, A*25, A*26, A*25/26/34 typing along with control and ϕ X 174 marker.



Procedure

1. The Agarose Gel was prepared in 1 X TBE buffer. The mixture was mixed properly and gently till the agarose was dissolved by using the microwave oven.

2. The content was cooled to 45°C and ethidium bromide to a final concentration of 0.5 mg/ml was added in the dissolved gel and mixed it by swirling.
3. The gel was then poured on the gel tray to make a 2 mm thick gel and comb was placed.
4. The gel tray was left for 30 minute to solidify the gel.
5. The comb was then removed carefully from the solidified gel.
6. The gel was then placed in the electrophoresis tank filled with TBE buffer.
7. A mixture of 5 µl gel loading buffer and 15µl amplified product was loaded in the well of the gel with the help of micropipette.
8. ϕ X 174 DNA was used as size marker.
9. The electrophoresis was then carried out at 80 volt and a current of 40 mAmp. The gel was removed when the tracking dye traveled 2/3rd of the gel.

4.3.4. Documentation and Interpretation

The gel visualized under the UV - transilluminator (Vilber Lourmat, France) and the photographic documentation of the gel was done by using Polaroid camera and analyzed by using Bio1D Analysis Software (Vilber Lourmat, France). Allele assignment and interpretation were done manually by referring the protocols of 12th IHW.

4.4. STATISTICAL ANALYSIS

All statistical tests were done through some computer programs developed in High level language C.

4.4.1. Estimation of phenotype frequency

Phenotype frequencies of antigens/alleles at various loci of MHC region in patients and controls were estimated by direct counting. The percent phenotype frequencies were calculated by using the following formula:

$$f_A = \frac{n_A}{n}$$

4.4.2. Estimation of significance of difference

Significance of difference in the frequency of different MHC alleles between patients and controls was calculated using X^2 analysis:

	Antigens	
	+	-
Patient	a	b
Control	c	d

$$a + b = n1$$

$$c + d = n2$$

$$a + c = n3$$

$$b + d = n4$$

$$n1 + n2 + n3 + n4 = N$$

$$\text{Chi-Square}(X^2) = \frac{(ad-bc)^2 \times N}{n1 \times n2 \times n3 \times n4}$$

4.4.3. Probability (P) Value and Bonferroni Correction

The level of significance is expressed in terms of probability (p) value. Since each individual is tested for several HLA alleles and the same data used to compare the frequency of all the detected alleles, it is probable that one of these alleles will by chance deviate significantly. To overcome this, Fisher's exact test was carried out. Testing for a large number of antigens can reveal at least one positive association where none really exists, the *p* values from each Fisher's exact test had to be less than the Bonferroni *p* (0.05 divided by the number of antigens tested minus two degrees of freedom [one for each of the two loci examined], which equals to 0.0014).

4.4.4. Estimation of Relative Risk (RR)

RR was ascertained by a modified method of Woolf (Haldane, 1956) using the following formula:

$$\text{Relative Risk (RR)} = \frac{\text{No. of positive patients x No. of negative controls}}{\text{No. of negative patients x No. of positive controls}}$$

4.4.5. Calculation of Haplotype frequencies and linkage disequilibrium (delta values)

The haplotype frequency was estimated by the equation derived from Cavalli-Sforza and Bodmer (1971) and linkage disequilibrium (delta values, D) for two locus model was calculated by the Chi Square test.

$$Delta = \sqrt{\frac{d}{n}} - \sqrt{\frac{(b+d)}{n}} x \frac{(c+d)}{n}$$

n = Total no. of sample

$$HF = 1 - \sqrt{\frac{b+d}{n}} - \sqrt{\frac{c+d}{n}} + \sqrt{\frac{d}{n}}$$

		B	
		+	–
A	+	a	b
	–	c	d

4.5. SHORT TERM *in vitro* LYMPHOCYTE CULTURE

The clinical investigation of chromosomal status on an individual depends on the brief period of cell division in the cell cycle - the mitosis. It is only during this short term (about 30 minutes) that the metaphase chromosomes - the primary requirement for cytogenetic analysis can be obtained. Therefore, the *in vitro* cultivation of cells has become the essence of clinical and investigative cytogenetics to obtain a high population of analyzable metaphase spreads.

4.5.1. Test Samples

A total number of 30 patients with delusional disorder were recruited into this cytological study. An equivalent number of healthy donors were considered as controls. Patients suffering from paranoid schizophrenia, early Alzheimer's disease

and mood congruent delusion were also considered and the number of patient in each category was 20 and then compared to the equivalent number of healthy controls against each category. Records were reviewed independently by two psychiatrists and diagnoses were made according to DSM-IV criteria. All the subjects were screened for any physical abnormalities and the history of substance abuse. The detail profile of study subjects included for cytological investigation is given in **Table-22.**

Table 22: Study Subjects and clinical groups considered for cytological studies.

Subjects	Lymphocyte Culture
Group I: DELUSIONAL DISORDER	30
SEX	
MALE	17
FEMALE	13
Group II:	
1. PARANOID SCHIZOPHRENIA	20
SEX	
MALE	12
FEMALE	8
2. EARLY ALZHEIMER'S DISEASE	20
SEX	
MALE	14
FEMALE	6
3. MOOD CONGRUENT DELUSION	20
SEX	
MALE	11
FEMALE	9
Group III: HEALTHY CONTROLS	
For Delusional Disorder	30
SEX	
MALE	17
FEMALE	13
For Paranoid Schizophrenia	20
SEX	
MALE	12
FEMALE	8
For Early Alzheimer's Disease	20
SEX	
MALE	14
FEMALE	6
For Mood Congruent Delusion	20
SEX	
MALE	11
FEMALE	9

4.5.2. Principle of Lymphocyte Culture

The purpose of this process is to obtain an adequate number of good quality metaphases for chromosomal analysis. The mononuclear leucocytes called lymphocytes are the cells used for routine cytogenetic studies. Lymphocyte amount to about 35% of the total leucocytes in the normal circulatory blood of which more than 2% are large lymphocytes, 55-75% of the small lymphocytes are T cells and 15-30% are B cells.

T lymphocytes from peripheral blood are differentiated cells, which do not undergo cell division. They are induced to enter mitosis using phytohemagglutinin (PHA), which acts as a mitogen. At 72 hours of culture optimum mitotic index is reached. At this stage, the mitotic inhibitor (colchicine) is added. It prevents the formation of the mitotic spindle thus arresting mitosis in metaphase. The cells were harvested using standard hypotonic treatment and fixation.

4.5.3. Basic components used for lymphocyte culture

There are five major components required for peripheral blood culture for accumulating dividing cells, obtaining metaphase chromosome spreads for cytogenetic analysis:

1. Use of complete culture medium

RPMI-1640 (Gibco BRL) was used as a complete medium which is a chemically defined medium with the necessary supplements such as serum, L-glutamine and antibiotics.

2. Use of lymphocyte mitogen

Phytohemagglutinin (PHA), a plant lectin from *Phaseolus vulgaris* was used as mitogen. Mitogen induces small lymphocytes to undergo a transformation so that the cells enlarge, thus making them capable of cell division. In the present study, PHA-P (Bangalore Genei, Bangalore) was used.

3. Use of mitotic arrestant to arrest cells at metaphase

Colchicine - a natural alkaloid found in *Autumn crocus* was used as mitotic arrestant. It prevents microtubule formation and blocks the cell at metaphase stage within a few minutes.

4. Pretreatment of cells with hypotonic solutions

Hypotonic solution is a low salt solution than the physiological tonicity. It is used to swell the cells and avoid chromosome clumps before harvesting. 0.56% KCl was used as hypotonic solution.

5. Fixation of cells for the analysis of chromosomes

Up to hypotonic stage, the cells are alive, metabolically active and continuing to be under the influence of mitotic arrestant. The fixative preserves the cell by stopping all functions of the cell and prevents further swelling of the cells. Some proteins are removed from the cell and chromosomes during fixation, which is believed to be essential to obtain banding patterns. We have used 3 : 1 methanol: acetic acid as fixative for chromosome preparations.

4.5.4. Methodology of short term Lymphocyte Culture

The lymphocyte microculture method of Arakaki & Sparkes (1963) was used with slight modification.

4.5.4.a. Preparation of Culture Media

1. One packet of medium powder (RPMI-1640, Gibco BRL) meant for 1 litre was dissolved in 800 ml of autoclaved double distilled water by gentle stirring.
2. The original packet was rinsed with a small amount of distilled water to remove all traces of powder and added to the solution.
3. Sodium bi-carbonate ($NaHCO_3$) was added to 0.12% to the solution and stirred well until it dissolved.
4. Following this, antibiotic solution of penicillin (10000 IU/ml), streptomycin (100 µg/ml) and gentamycin (2 µl/ml) were added.

5. While stirring pH of the medium was adjusted to 0.1-0.3 pH units below the required pH of 7.2, since it may rise during filtration. 1 (N) HCl and 1 (N) NaOH were used to adjust the final pH.

6. Autoclaved double distilled water was added to make up the volume upto 1 litre and was sterilized immediately by filtration using 0.22μ filters. The medium was dispensed aseptically in the refrigerator at 2-6°C in dark.

4.5.4.b. Preparation of AB Serum

1. AB positive serum was obtained after centrifugation of the clotted blood collected aseptically in a sterilized tube.

2. The bottle was kept slanting overnight at an angle of 45° in the refrigerator allowing all the cells to settle down.

3. Next morning the serum was poured off and centrifuged at 3000 rpm twice for half an hour to remove all remaining cells. Serum was inactivated at 56°C for 30 minutes in a water bath and filtered using positive pressure pump of 0.5μ pore cellulose filter (Millipore). The serum was then aliquoted in 2 ml vial and stored at -20°C.

4.5.4.c. Sample Collection

1. 2 ml blood was collected in a sterile disposable tube contained 0.1ml (20 units) preservative free sodium heparin.

2. The identification and date of collection of blood sample along with information of the patients was recorded on the tube.

4.5.4.d. Setting up of Culture

1. Culture was set up under the laminar hood by adding 0.3ml whole blood in 5 ml culture medium along with serum (locally prepared AB serum as well as Fetal Bovine Serum, Biowest) and PHA. In each case multiple tubes were set using following components and quantity.

5. While stirring pH of the medium was adjusted to 0.1-0.3 pH units below the required pH of 7.2, since it may rise during filtration. 1 (N) HCl and 1 (N) NaOH were used to adjust the final pH.

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Components	Quantity
1. Medium RPMI -1640	5 ml
2. AB serum / FBS	0.6 ml
3. PHA	20 µl
4. Blood	0.3 ml

2. The whole blood and the medium were mixed properly.
3. Then it was incubated in a preset 37°C incubator for 70 hours.

4.5.4.e. Harvesting of Cultures

1. After 70 hours of incubation colchicine was added to the final concentration of 0.05 µg/ml. The vials were gently shaken and incubated for two more hours.
2. At the end of the incubation period, the vials were gently shaken and transferred to 15 ml centrifuge tubes for the centrifugation at 1000 rpm for 8 minutes.
3. The supernatant was discarded carefully leaving about 0.5 ml in the tube.
4. Cells were resuspended gently in the remaining 0.5 ml of supernatant.
5. About 10 ml of prewarmed (37°C) hypotonic solution (0.56% KCl) was added and mixed gently with a Pasteur pipette.
6. Tubes were then incubated at 37°C for 20 minutes and centrifuged at 1500 rpm for 5 minutes
7. Supernatant was discarded carefully leaving 0.5 ml of it in the tube.
8. Pellet was resuspended carefully in the remaining 0.5ml of supernatant by gentle tapping.
9. Chilled fixative (3:1 Methonal & Acetic Acid) was added drop wise along the wall of the tube.
10. Suspension was mixed properly and incubated for 15 minutes to overnight at 4°C.
11. At the end of the incubation, the tubes were centrifuged at 1500 rpm for 5 minutes.

12. The pellet was washed twice with chilled fixative.
13. The cell pellet was resuspended in a small volume (0.2-0.5ml) of fixative.

4.5.5. Preparation of Slides

1. The cells were dropped (1-4 drops) with a Pasteur pipette onto the chilled slide, starting from a distance of $^1/_4$" from the slide edge.
2. The slide was held at an angle while dropping the cell suspension and then air-dried.

The test slide was checked under the microscope for the concentration of cell suspension, spreading of chromosomes and mitotic index.

4.6. STAINING OF CHROMOSOMES

4.6.1 Conventional Staining

Slides were stained in 5% Giemsa buffered with phosphate buffer (pH 6.8) for 5 min and then rinsed briefly in distilled water. The slides were then air-dried and observed under the microscope.

4.6.2. Trypsin digestion & G-banding of the Chromosome:

Chromosome banding technique produces a series of consistent landmarks along the length of metaphase chromosomes within a genome and identification of specific segments of individual chromosomes. According to the Paris Conference (1971) a chromosome band is part of a chromosome that can be distinguished from adjacent segments by appearing darker or lighter by one or more banding techniques. These landmarks facilitate assessment of chromosome normalcy, identification of sites of chromosome breaks and alterations, and location of specific genes. G-banding is the most frequently used technique for routine chromosome analysis because of the permanence of the bands produced and the ease with which they can be photographed. The Giemsa bands (G-bands) are obtained by digesting the chromosomes with proteolytic enzyme trypsin. This technique is described as GTG-banding (G-bands by trypsin using Giemsa) (ISCN, 1995).

There are numerous G-banding techniques, however, we adopted GTG-banding technique of Seabright (1971) with slight modifications.

Procedure

1. A series of coplin jars containing HBSS, trypsin solution (1:250), HBSS, 70% ethanol, 90% ethanol, 2% Giemsa staining solution and distilled water was prepared.
2. The slides of metaphase chromosomes aged for 2-3 days were immersed briefly for ~ 10 seconds in HBSS.
3. Slides were then transferred to a jar containing trypsin (0.025%) for optimal trypsinization time (15-60 sec.)
4. Washed in HBSS and dehydrated through 70% and 90% ethanol and then air dried.
5. Stained in 5% giemsa solution and washed in distilled water and dried and mounted with DPX.

4.6.3. Screening of Metaphases:

The G-banded slides were scanned for metaphase spreads under 10X using WILL, Wetzlar photomicroscope. Position of well spread metaphases was recorded using Vernier scale on the microscope stage. The metaphases were analyzed in detail under 100 X oil immersion objective. A minimum of 20-30 banded metaphases were examined for all the subjects and considered for karyotyping.

4.7. Photography and Karyotyping

Well spread banded prometaphases and metaphases were photographed under 100 X oil immersion objective using OLYMPUS PM-6 photographic equipment attached with WILL microscope. Kodak colour film was used for the photographic purposes. Prints were developed and fixed using standard print developer and fixer. Individual chromosomes were cut from the photographic prints and karyotypes were prepared according to the ideograms of the International System for Cytogenetic Nomenclature (ISCN, 1995) and analyzed.

The chromosomes were categorized by size and morphology into 7 groups (A to G). Further with banding techniques they were identified individually.