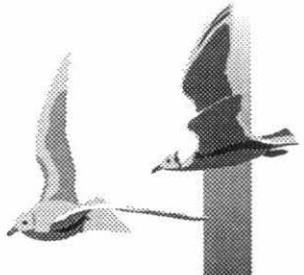
Section - 4

Materials and Methods



4. MATERIALS AND METHODS

4.1 SUBJECT

A total number of 136 schizophrenic patients were included in this study. Out of 136 schizophrenic patients, 118 were paranoid, 9 were disorganised, 1 was catatonic, 5 were undifferentiated and 3 were residual. All the subjects were India born schizophrenic patients of West Bengal referred to the outpatient department (OPD) of Psychiatry, North Bengal Medical College and Hospital, Siliguri. Three major selection criteria were considered for the screening of schizophrenic group: - (i) unrelatedness of individuals from each other, (ii) resident of the state of West Bengal and Bengalee by ethnicity, (iii) subjects satisfying DSM-IV-TR diagnostic criteria for schizophrenia (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, text revision, 2002). The exclusion criteria followed in the present study include:- (i) history of smoking or substance abuse, (ii) presence of personality disorder, (iii) presence of dementia and mental retardation (iv) general medical condition. The patients group comprise of 87 Bengali, 9 Nepali, 11 Bihari, 17 Tribal and 12 Rajbanshi individual ranging from 16-55 years of age. The patients were diagnosed by psychiatrist using Structured Clinical Interview (First, 1996) and according to the standard diagnostic criteria of DSM-IV-TR and assessed by the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham,1962). Some of the demographic variables are also considered for the study.

A total number of 150 unrelated, ethnically matched healthy individuals were considered as controls. The criteria for the selection of controls include :- (i) same ethnic group as the patients,(ii) sex and age matched with patients,(iii) absence of family history of autoimmune and psychiatric disorder,(iv) recent history of intercurrent infection and allergies,(v) no history of smoking or any substance abuse and (vi) no history of general medical condition. All the participants provided their written consent for giving the blood sample after the study procedures were explained. The study has been carried out according to the principles of the Declaration of Helsinki (1964) and approved by the ethical committee of the institution.

The schizophrenic and control subjects were divided into 4 different groups for various studies such as :- (i) HLA association (if any) (ii) serum IL-2 and IL-6, (iii) CRP and (iv) CD4+ and CD8+ percentage.

4.2 TYPING OF HLA CLASS-I

In the present study 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).

4.2.1 SEROLOGICAL TYPING OF HLA CLASS I ANTIGEN (Microlymphocytotoxicity assay)

Serological typing of HLA-ClassI antigens were carried out in all the subjects by complement mediated two-stage microlymphocytotoxicity assay (Terasaki & McClelland, 1964). In the two-stage test, the first step employs the sensitization step of HLA antigen with antibody. The second step is the 'specificity test', achieved by the addition of rabbit complement.

The principle behind this technique is the binding of anti-HLA antibody to a specific HLA Class I 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 the antigens on the cell surface do not react with antibody, the lymphocytes are not stained (Negative Reaction). Microscopic identification of the stained cells indicate the presence of a specific HLA antibody. Viable cells appear bright and refractory while the dead cells look dark and distinctly larger.

4.2.1.1 Isolation and preparation of lymphocyte cell suspension

1. Approximately 5ml 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 $2x10^6$ 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 min. Dead cells became dark blue. At least 85% viability was required.

4.2.1.2 Method of HLA class I typing

Two sets of well defined HLA antisera (one from BAG, Germany and another obtained from 12^{th} 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 polysterene material (NUNC, Denmark) containing 60-72 wells were used for testing of class I antigens. The typing trays were thawed before use at room temperature. 1µl of lymphocyte suspension ($2x10^6$ 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 30minutes. 5µl nontoxic rabbit complement was added to each well with Hamilton six-needle dispenser (250µl capacity). The trays were further incubated for a period of 60minutes. 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 12). Typing trays were scored on a 'subjective scale' taking into consideration the amount of 'background' (dead cells) in the negative control well.

Table 12: Scoring system as adapted in the 8th International Histocompatibility Workshop.

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

4.2.2 MOLECULAR TYPING OF HLA CLASS I GENES (ARMS-PCR SSP TYPING) Amplification refractory mutation system-polymerase chain reaction- sequence specific primer

4.2.2.1 DNA Extraction (Phenol Chloroform Method)

DNA was isolated from frozen peripheral blood samples using phenol chloroform extraction method with slight modifications. The procedure is as follows :-

1. Blood samples were thawed first.
2. 500µl blood sample was taken and washed with 1ml of 1X SSC, mixed gently and centrifuged at 5000rpm for 5 minutes.

3. The supernatant was removed and 1.2 ml of 1X SSC was added and mixed gently and centrifuged at 5000rpm for 2 minutes.
4. The supernatant was removed and 1.2ml of 50 mM KCl was added and mixed gently and then centrifuged.
5. Again the supernatant was removed and 375µl of High Salt Lysis Buffer was added.
6. 25µl of 10% SDS and 12.5µl of 8mg/ml proteinase K stock was added and incubated for 1 hour at 56°C.
7. The proteinase K digested suspension was transferred into a new microfuge tube and an equal amount of Phenol-Chloroform (PCl) was added and spinned at 12000 rpm for 20 minutes.
8. The DNA was recovered and rinsed with 1ml of 100% C_2H_5OH .
9. The DNA was rinsed with 500µl of 70% C_2H_5OH, three times.
10. The DNA was dried and dissolved in 50µl of TE.

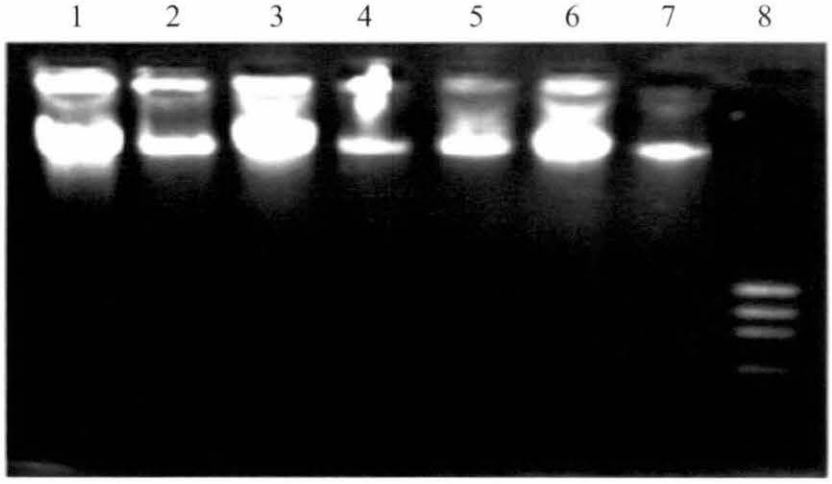


Figure 20: Electrophoregram showing the electrophoresis of DNA Lane 1-7 DNA, lane 8 ladder.

4.2.2.2 Quantification of DNA

10µl of dissolved DNA was diluted to 1.5ml 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

260Xdilution factorX50 (1 OD = 50µg of double stranded DNA). The DNA were also visualized in the agarose gel (Fig. 20).

4.2.2.3 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- and HLA-B locus alleles in this study are listed in table2 & 3

4.2.2.3.1 Preparation of PCR reaction mixture

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

Reagents		Reaction Mix (50µl)
Initial conc. Of stock solution	Final conc. Of stock solution	For 1 test
dH_2O	----	36.8µl
10X 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

Table 13: List of primers used for HLA-A locus typing.

Name of the allele	Sequence	nucleotide Length
A*01		
P1	5′-GGA CCA GGA GAC ACG GAA TA-3′	20
P2	5′-AGG TAT CTG CGG AGC CCG-3′	18
A*02		
P1	5′-TCC TCG TCC CCA GGC TCT-3′	18
P2	5′-GTG GCC CCT GGT ACC CGT-3′	18
A*03		
P1	5′-AGC GAC GCC GCG AGC CA-3′	17
P2	5′-CAC TCC ACG CAC GTG CCA-3′	18
A*23		
P1	5′-GGC CGG AGT ATT GGG ACG A-3′	19
P2	5′-CCT CCA GGT AGG CTC TCA A-3′	19
A*24		
P1	5′-GGC CGG AGT ATT GGG ACG A-3′	19
P2	5′-CCT CCA GGT AGG CTC TCT G-3′	19
A*25		
P1	5′-TCA CAG ACT GAC CGA GAG AG-3′	20
P2	5′-ATG TAA TCC TTG CCG TCG TAA-3′	21
A*26/4301		
P1	5′-ACT CAC AGA CTG ACC GAG C-3′	19
P2	5′-ATG TAA TCC TTG CCG TCG TAA-3′	21
A*11		
P1	5′-ACG GAA TGT GAA GGC CCA G-3′	19
P2	5-CTC TCT GCT GCT CCG CCG-3	18
A*29		
P1	5′-AGG ATG GAG CCG CGG GCA-3′	18
P2	5′-AGC GCA GGT CCT AGT TCA A-3′	19
A*30		
P1	5′-CCC GGC CCG GCA GTG GA-3′	17
P2	5′-CCG TCG TAG GCG TGC TGT-3′	18
A*31		
P1	5′-GAT AGA GCA GGA GAG GCC T-3′	19
P2	5′-AGC GCA GGT CCT AGT TCA A-3′	19

Table 14: List of primers used for HLA-B locus typing.

Name of the allele	Sequence	nucleotide Length
B*07		
P1	5′-GGA GTA TTG GGA CCG GAA C-3′	19
P2	5-TAC CAG CGC GCT CCA GCT-3	18
B*08		
P1	5′-GAC CGG AAC ACA CAG ATC TT-3′	20
P2	5′-CCG CGC GCT CCA GCG TG-3′	17
B*1801		
P1	5′-GGC GCC GTG GAT AGA GCA A-3′	19
P2	5′-GCC GCG GTC CAG GAG CT-3′	17
B*27/7301		
P1	5′-ACC GGG AGA CAC AGA TCT G-3′	19
P2	5′-GAG CCA CTC CAC GCA CTC-3′	18
B*3701		
P1	5′-GCC GCG AGT CCG AGG AC-3′	17
P2	5′-CCT CCA GGT AGG CTC TGT C-3′	19
B*4001		
P1	5′-CCA CTC CAT GAG GTA TTT CC-3′	20
P2	5′-CCG CGC GCT CCA GCG TG-3′	17
B*4201		
P1	5′-GAC GAC ACC CAG TTC GTG A-3′	19
P2	5′-CCG CGC GCT CCA GCG TG-3′	17
B*44		
P1	5′-TAC CGA GAG AAC CTG CGC-3′	18
P2	5′-CCA GGT ATC TGC GGA GCG-3′	18
B*44/4501/1514		
P1	5′-ACC GGG AGA CAC AGA TCT C-3′	19
P2	5′-CCA GGT ATC TGC GGA GCG-3′	18
B*5101-5105		
P1	5′-GGA GTA TTG GGA CCG GAA C-3′	19
P2	5′-CGT TCA GGG CGA TGT AAT CT-3′	20
Positive internal Control		
P1	5′-ATG ATG TTG ACC TTT CCA GGG-3′	21
P2	5′-ATT CTG TAA CTT TTC ATC AGT TGC-3′	24

4.2.2.3.2 Amplification Procedure

50 µl of reaction mix was dispensed into PCR tubes. The tubes were spun for 1min for mixing and arranged in the heat block of thermal cycler. The primer for the hemoglobin gene was used as positive internal control. We used touch down method and following conditions were adopted.

No. of Cycle	Time	Temperature
1 Cycle of 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 Hold at 15°C	10 mins	72°C

4.2.2.3.3 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 gel (low EEO)(Sisco Research Laboratories, India) containing 0.5 µg/ml ethidium bromide (Boehringer Mannheim, Germany) in TBE buffer to check efficiency and specificity of the reaction. ϕX174 DNA marker (Bangalore Genei, India) providing even banding patterns of uniform intensity (1353bp, 1078bp, 872bp, 603bp, 310bp, 281/271bp, 234bp, 194bp,118bp and 72bp) was loaded in one of the well. Figure 21 shows the electrophoregram of the HLA gene.

Procedure

1) The agarose gel was prepared in 1X 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 2mm 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 5µl gel loading buffer and 15µl amplified product was loaded in the well of the gel with the help of micropipette.

8) ϕX174 DNA was used as size marker.

9) The electrophoresis was then carried out at 80volt and a current of 40mAmp. The gel was removed when the tracking dye traveled 2/3rd of the gel.

4.2.2.3.4 Documentation and Interpretation

The gel was visualized under the UV-transilluminator (Vilber Laurmat, 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.

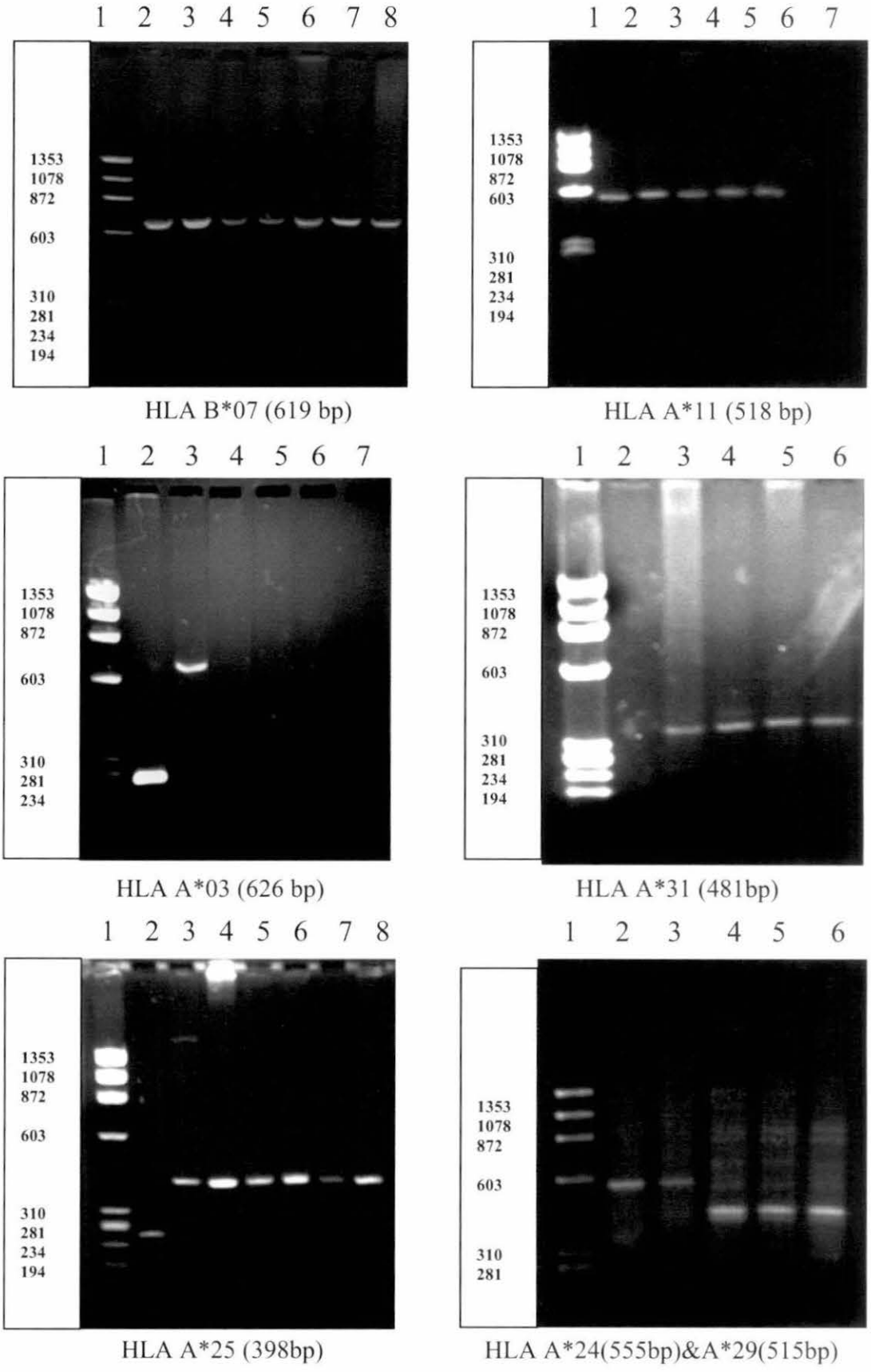


Figure 21: Electrophoregram showing the result of PCR-SSP typing of various HLA genes.

Lane 1, ϕX174 marker

4.2.3 STATISTICAL ANALYSIS

4.2.3.1 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:

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

Where, f_A = frequency of allele
n =number of subjects studied
nA=number of times an allele is present

4.2.3.2 Estimation of significance of difference

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

	Antigens	
	+	-
Patient	a	b
Control	c	d

$a + b = n1$
$c + d = n2$
$a + c = n3$
$b + d = n4$
$n1+n2+n3+n4 = N$

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

4.2.3.3 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 done.

4.2.3.4 Estimation of Relative Risk (RR)

Relative risk was estimated by using Haldane's method (1956) using the following formula:

$$\text{Relative Risk (RR)} = \frac{(2a+1)(2d+1)}{(2b+1)(2c+1)}$$

4.2.3.5 Calculation of Haplotype frequencies and linkage disequilibrium

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.

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

n = total no. of sample

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

		B	
		+	–
A	+	a	b
	–	c	d

4.3 ENZYME LINKED IMMUNO SORBENT ASSAY (ELISA) FOR INTERLEUKIN-2 AND INTERLEUKIN-6 ESTIMATION

The serum level of interleukins (ILs) was measured on the 50 of the schizophrenic patients and control. They were further classified into two groups, 20 schizophrenic patients who had stopped taking antipsychotic drugs for at least 6 weeks were considered as psychotropic medication free, rest 30 schizophrenic group were under antipsychotic medication. A total number of 30 unrelated, age, sex and ethnically matched healthy individuals were considered as controls.

4.3.1 PREPARATION OF SERUM

The blood samples from the patients and controls were collected between 10 am-12 noon by vein puncture method and allowed to clot at room temperature for 2-3 hrs. Blood clot was cut and centrifuged for separating the serum. The separated serum was aliquoted and stored at – 70^0C before use.

4.3.2 MEASUREMENT OF INTERLEUKIN-2 AND INTERLEUKIN-6 CONCENTRATION

Serum IL-2 and IL-6 levels were measured by enzyme linked immunosorbent assay kit (Endogen Human IL kit, Pierce Biotechnology, Inc. Rockford). The sensitivities for IL-2 and IL-6 were <6pg/ml and <1pg/ml,respectively, with inter and intra assay coefficient of variation of <10% in both the cases. Absorbance was measured by a microtiter plate reader set at 450 nm. Each assay was carried out by the same investigator.

4.3.3 ASSAY PROCEDURE

(i) 50µl of reconstituted standards and samples were added to each microtiter well.

(ii) 50µl of Biotinylated Antibody Reagent was added to each well. It is mixed by gently tapping the plate several times.

(iii) The plate was covered with an adhesive microtiter plate cover, ensuring all edges and strips were sealed and incubated for three hours at room temperature, 20-25°C.

(iv) The adhesive plate cover was removed and the plate was washed.

(v) 100µl of prepared streptavidin-HRP solution was added to each well.

(vi) Again a new adhesive plate cover was attached, ensuring all edges and strips were sealed tightly. The plate was incubated for 30minutes at room temperature, 20-25°C.

(vii) The plate cover was removed and the plate contents were discarded. Then the plate was washed again.

(viii) 100µl of TMB substrate solution was added into each well.

(ix) The plate was allowed to develop enzymatic color reaction at room temperature in the dark for 30 minutes.

(x) After 30 minutes, the reaction was stopped by adding 100µl of stop solution to each well. The substrate reaction yielded a blue solution that turned yellow when stop solution was added.

(xi) The absorbance was measured on an ELISA plate reader set at 450nm.

(xii) The obtained OD was converted to actual value (pg/ml) with the help of the software ORIGIN lab 6.1.

4.3.4 STATISTICAL ANALYSIS

For variables:- (i) mean and standard deviations were calculated and (ii) t-tests was done to test the equality of means of patient and control subjects. For attributes: (i) percentages were calculated and (ii) χ^2- tests were done to test the equality of percentages. The p-values of <0.05 were considered statistically significant.

4.4 ESTIMATION OF SERUM C-REACTIVE PROTEIN

After diagnosis, 64 schizophrenic patients were included for CRP study. The CRP level in the serum was measured by latex agglutination slide test (Ranbaxy Fine Chemicals Ltd., HP, India).

4.4.1 PREPARATION OF SERUM

About 5ml. of blood samples were collected from each patient by vein puncture method. The samples were allowed to coagulate at room temperature for 2-3 hours. Blood clot was cut and centrifuged for separating the serum.

4.4.2 MEASUREMENT OF C-REACTIVE PROTEIN BY LATEX AGGLUTINATION TEST

The freshly separated serum sample was used for the latex agglutination slide test (Fig. 22). The limitation of detection of serum CRP level was less than 6mg/L. CRP was treated as categorical variable: undetectable or normal(<6mg/L) and detectable or elevated (≥6mg/L).

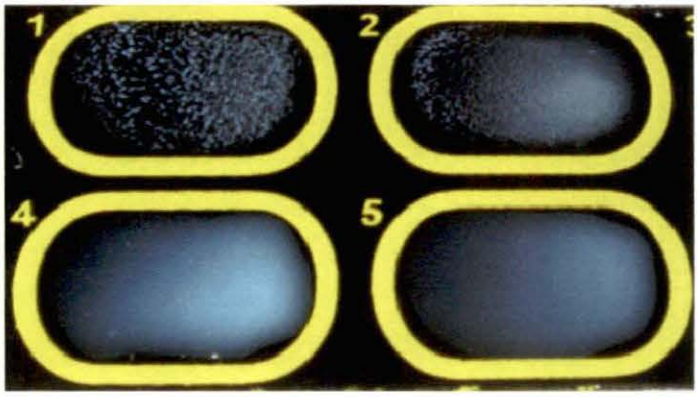


Figure 22: Latex agglutination test for C-reactive protein. 1 and 2 positive reaction from the patients and 4 and 5 negative reaction from the patients.

4.4.3 STATISTICAL ANALYSIS

Statistical analysis was performed for the bivariate associations between elevated CRP groups versus normal group by employing one-way analysis of variance. The association between CRP groups and deficit was examined by Chi-square analysis. The association between CRP group and other clinical/demographic variables were also examined by utilizing one way analysis for continuous variables and Chi-square tests for dichotomous variables.

4.5 ESTIMATION OF CD4+ AND CD8+ CELLS

4.5.1 COLLECTION OF BLOOD

The whole blood was collected by vein puncture method in heparinized and EDTA tubes between 10 am to12 noon.

4.5.2 METHOD

The collected blood samples were transported immediately to Suraksha Diagnostics, The Siliguri Clinic, Sevoke Road, Siliguri for the estimation of the lymphocyte subsets. The lymphocyte subsets were evaluated using a flow cytometer (Becton Dicknson) by the technician.

4.5.3 STATISTICAL ANALYSIS

Significance of difference in the percentage of CD4 and CD8 cells between the patients and controls were calculated using χ^2 analysis.