CHAPTER 4

Materials & Methods

MATERIALS AND METHODS

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4.1. STUDY SUBJECTS

A nested case control study design was employed. Study subjects were divided into two groups: group I represented the patients chiefly with delusional disorder and the second group comprises of age and sex matched healthy controls.

All the subjects belonged to the India Born Bengalee population. All the subjects were typed by molecular method ARMS-PCR SSP typing) for dopamine receptor genes, tyrosine hydroxylase genes and dopamine transporter genes. Besides these, HPLC analysis of plasma homovanillic acid and latex agglutination test for c-reactive protein was also performed.

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^{TR} diagnostic criteria listed in Table 13 were used or diagnosing the patients with delusional disorder. After longitudinal follow up, 100 patients represented the genuine cases of delusional disorder of various subtypes. The patients having any one of the following conditions were excluded from the study like: 1) substance abuse during the past year; 2) any history of other general medical illness or treatment with anti-inflammatory or immunosuppressive medication; 3) any past history of psychotic illness and 4) any history of co morbidity.

Further, the delusional patients and the family members were made to answer a questionnaire included self reported age, sex, caste, medical history, age of onset, age of severity, pedigree, disease duration among patients.

Written informed consent was obtained from each subject after a complete description of the study. The study was conducted as per the norms of the "World

Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects".

Table-13: DSM-IV^{TR} Diagnostic Criteria for Delusional Disorder

A.	Nonbizarre delusions (ie, involving situations that occur in real life, such as being followed, poisoned, infected, loved at distance, or deceived by spouse or lover, or having a disease) of at least 1 month's duration .
B.	Criterion A for schizophrenia has never been met (ie, patients do not have simultaneous hallucinations, disorganized speech, negative symptoms such as affective flattening, or grossly disorganized behavior). 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 (eg, a drug of abuse, a medication) or a general medical condition.

It was observed that maximum patients were clustered between 25-55 age group with average mean 42.09 ± 1.29 years with a male to female ratio of 3:2. 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 100 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 completed for each patient and followed up carefully for 2-3 years. The BPRS scoring sheet and SCID format used for screening of the patients is given in **Annexure IV and V.** The details of the subjects with the clinical subgroups are depicted in Table 14 and Table 15 respectively.

Table 14: Study subjects and clinical groups considered for molecular typing

Subjects	PCR-SSP Analysis
Group I: DELUSIONAL DISORDER	100
SEX	
MALE	40
FEMALE	60
Group II: HEALTHY CONTROLS	100
SEX	
MALE	40
FEMALE	60

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 behaviour. Rescue fantasies regarding the secret admirer are common. Delusional love is usually intense in nature. Signs of denial of love by the object of the delusion are frequently falsely interpreted as affirmation of love	3
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.	6
Jealous subtype	This is the most common presentation of delusional disorder.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.	46
Persecutory subtype	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.	22
Somatic subtype	The core belief of this type of disorder is delusions around bodily functions and sensations. The most common are the belief that one is infested with insects or parasites, the belief of emitting a foul odor, the belief that parts of the body are not functioning, and the belief that their body or parts of the body are misshapen or ugly. Patients are totally convinced in physical nature of this disorder, which is contrary to patients with hypochondriasis who may admit that their fear of having a medical illness is groundless. Patients are usually first seen by dermatologists, cosmetic surgeons, urologists,	18

	gastroenterologists, and other medical specialists. Sensory experiences associated with this illness (eg, sensation of parasites crawling under the skin) are viewed as components of systemized delusions	
Mixed subtype	Patients exhibit more than one of the delusions simultaneously and no one delusional theme predominates.	4
Persistent	Delusional themes fall outside the specific categories or cannot be clearly determined. Misidentification syndromes such as Capgras syndrome (characterized by a belief that a familiar person has been replaced by an identical impostor) or Fregoli syndrome (a belief that a familiar person is disguised as someone else) fall into this category. Misidentification syndromes are rare and frequently are associated with other psychiatric conditions (eg, schizophrenia) or organic illnesses (eg, dementia, epilepsy). Another unusual syndrome is Cotard syndrome, in which patients believe that they have lost all their possessions, status, and strength as well as their entire being, including their organs.	1

4.1.2 GROUP II: Healthy controls

A total number of 100 unrelated healthy individuals were considered as controls. 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 control subjects were screened for a recent history of intercurrent infections and allergies. Those with a past history of autoimmune, neurological or psychiatric disorder were excluded. All the controls gave informed consent to participate in the study.

4.2. ARMS-PCR SSP TYPING OF GENES INVOLVED IN THE DOPAMINERGIC SYSTEM

4.2.1. DNA Extraction (Phenol Chloroform Method)

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

Sample Preparation and Digestion

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

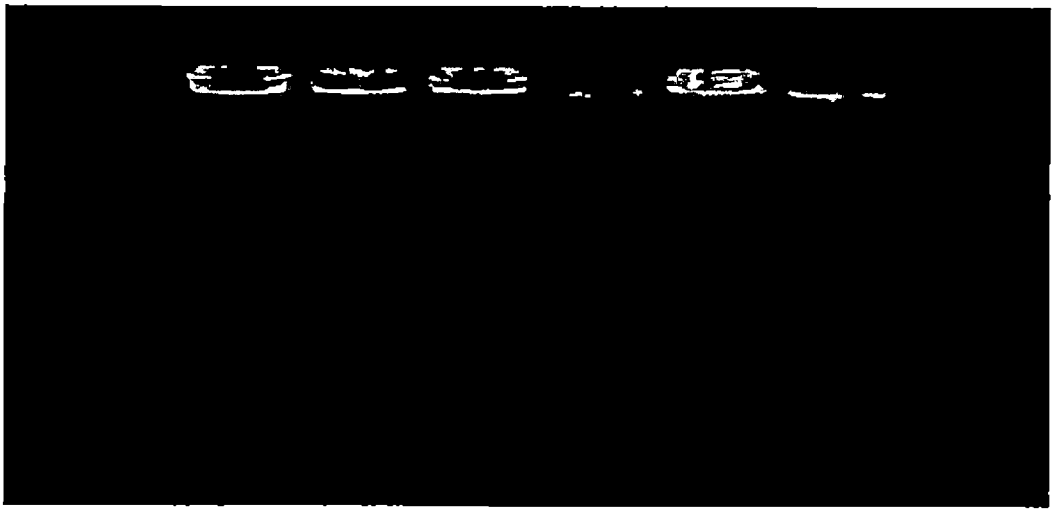
3. the supernatant was removed and 1.2ml of 50mM KCL was added and mixed gently and then centrifuged.
4. Again the supernatant was removed and 375µl of High Salt Lysis Buffer was added.
5. 25 µl of 10% SDS and 12.5 µl of 8mg/ml proteinase K stock was added and incubated for 1 hour at 56°C.

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 12000rpm for 20mins.
2. The aquous phase was recovered and a second PCl extraction was made.
3. 13µl of 4M NaCl and 0.6Vol 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% ethyl alcohol, three times.
5. The DNA was dried and dissolved in 50µl of TE.

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 260nm and 280nm 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 260X dilution factorX50 (1OD= 50µg of double stranded DNA).



4.2.2 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 was taken from Peter Sambrook (manual of Molecular genertics) and sequence information was taken from NCBI. The nucleotide sequence of primers used for amplification of dopamine receptor genes (DRD), tyrosine hydroxylase genes (TH) and dopamine transporter genes (DAT) used in this study and its gene accession number are listed in **Table 20.**

Preparation of PCR reaction mixture:

PCR amplification was performed in thermostable sterile PCR tubes on a DNA thermal cycler (Perkin Elmer, systems 2400, USA).containing 50µl reaction mixture of the following combination:

Reagents	Final conc. Of stock solution	Reaction mix for 1 test
dH_2O	----------	38.1 µl
10X PCR Buffer	1X	5µl
dNTP mix	10mM	1 µl
Primer (F)	12pmole	1.2 µl
Primer (R)	12pmole	1.2 µl
Template DNA	10ng	2 µl
Taq Polymerase	1.5 unit	1.5 µl

Table 20: List of the alleles considered for molecular typing

Name of the allele	Sequences 5'-3'	Length (bp)	Gene Bank accession no./ reference
DRD1			
F	CAGTCCACGCCAAGAATTGCC	21	Acc.no.X55760
R	ATTGCACTCCTTGGAGATGGAGCC	24	
DRD1A			
F	GGCTTTCTGGTGCCCAAGACAGTG	24	Dmitrzak et al., 2006
R	AGCACAGACCAGCGTGTTCCCCA	23	
DRD2			
F	GCAGCCGAGCTTTCAGGGCC	20	Acc.no S69899
R	GGGATGTTGCAGTCACAGTG	20	
DRD2S			
F	GAGGGCTCCACTAAAGGAGG	20	Acc.no S69899
R	GGGATGTTGCAGTCACAGTG	20	
DRD2L			
F	ACCAGCTGACTCTCCCCGACCGGT	24	Arinami et al., 1994
R	GGAAGGACATGGCAGGGAATGGGAC	25	
DRD3			
F	GCGTTACTACAGCATCTGCCAGGAC	25	Segal et.al,1997
R	AGACAGGATCTTGAGGAAGG	20	
DRD4			
F	TGCTGTGCTGGACGCCCTTCTTCG	24	Mulcrone and
R	CGTTGCGGAACTCGGCGTTGAAGA	24	Kerwin1996
DRD5			
F	GTCGCCGAGGTGGCCGGTTAC	21	Acc.no X58454
R	GCTGGAGTCACAGTTCTCTGCAT	23	
DRD5A			
F	GCCTTGGTCATGGTCGGCCTGGCA	24	Dmitrzak et al., 2006
R	CCAGCAGCTGGGCAAACACCTTCTGA	26	
TH1			
F	AGC TAT GCC TCA CGC ATC CA	20	
R	TAG CCA ATG GCA CTC AGC GC	20	Morimoto et al, 2002
TH2			
F	ACA GGG AAC ACA GAC TCC ATG	21	
R	CCT TAT TTC CCT CAT TCA TTC ATT C	25	Morimoto et al, 2002
DAT			
F	AGCAGAACGGAGTGCAGCT	19	
R	GTATGCTCTGATGCCGTCT	19	Acc.no L24178

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.

Programme for DRD1, DRD2S,DRD2L DRD5 and DRD5A genes

No. of cycle	Time	Temperature
Initial denaturation	5 mins	94°C
20 cycles of	1 min	94°C
	1 min	60°C
	1min 30 secs	72°C
20 cycles of	1 min	94°C
	1 min	66°C
	1min 30 secs	72°C
Final extension with 2 holds	7 mins	72°C

Programme for DRD3 and DAT gene

No. of cycle	Time	Temperature
Initial denaturation	5 mins	94°C
20 cycles of	1 min	94°C
	1 min	57°C
	1min 30 secs	72°C
20 cycles of	1 min	94°C
	1 min	66°C
	1min 30 secs	72°C
Final extension with 2 holds	7 mins	72°C

Programme for TH1, TH2 and DRD4 gene

No. of cycle	Time	Temperature
Initial denaturation	5 mins	94°C
20 cycles of	1 min	94°C
	1 min	65°C
	1min 30 secs	72°C
20 cycles of	1 min	94°C
	1 min	68°C
	1min 30 secs	72°C
Final extension with 2 holds	7 mins	72°C

4.2.3. Amplification Check by Agarose Gel Electrophoresis

We used mini gel electrophoresis apparatus (Bangalore Genei, India) for rapid separation of amplified PCR products. PCR products were separated on 2% submerging agarose (Bangalore Genei, India) gel in TBE buffer containing 0.5µl /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, 872bp, 603bp, 310bp, 281/271bp, 234bp,194bp,118bp, and 72bp) was loaded in the first well.

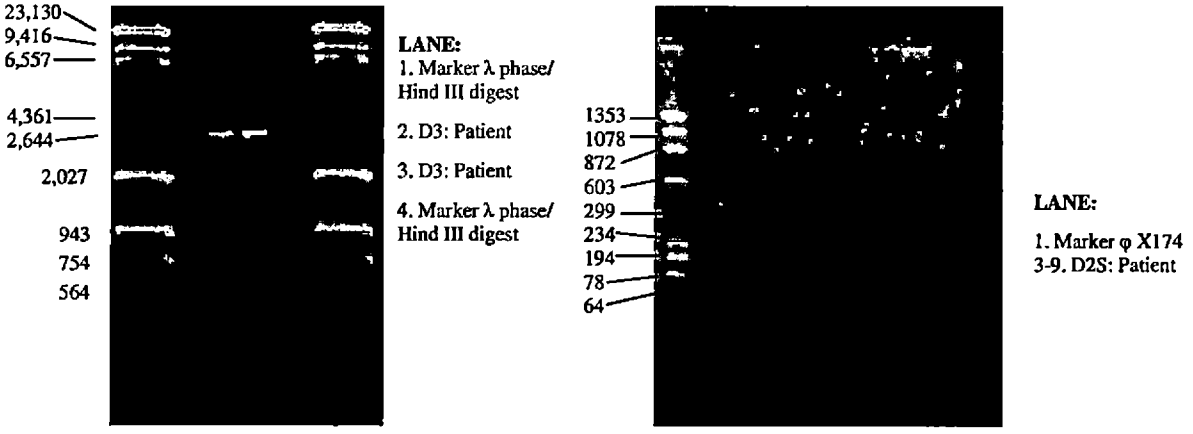
Procedure:

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 0.5mg/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 then left for 30 mins 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 80 volt and a current of 37mA. The gel was removed when the tracking dye traveled 2/3rd of the gel.

4.2.4. Documentation and interpretation

The visualization and the photographic documentation of the gel was done by using Gel documentation system (Vilber Lourmat, France). The gel picture was analyzed by using Bio 1D Analysis Software (Vilber Lourmat, France). Allele assignment and interpretation were done manually.



4.3. HPLC ANALYSIS OF PLASMA HOMOVANILLIC ACID

HPLC analysis of homovanillic acid was carried out from the plasma of all the subjects(Delusional Disorder patients 100+ 100 healthy controls). Homovanillic acid was measured using HPLC method.

Isolation of plasma

Blood samples were obtained collected in heparinized tube from all 40 individuals. None of the participating subjects took psychotropic medication. Each sample was obtained by vein puncture. The blood was centrifuged for 10mins at 4000xg, within 30 mins and the plasma was collected and stored at -80°C until use.

Sample preparation

Plasma (0.5ml) was mixed with 50µmol of EDTA, 1.82mmol of heptanesulfonic acid, 0.02mol/lit citric acid and 0.02mol/lit disodium phosphate (pH 3.1). Further, plasma proteins were precipitated by adding 20µl of concentrated perchloric acid and vortex for several seconds. Then the samples were centrifuged for 10mins at 42000xg. From 1.2 ml of supernatants HVA was extracted three times with 1.5ml of ethyl acetate. The combined organic phases were evaporated to dryness under a stream of nitrogen and the residue was dissolved in 0.4ml of 1mmol Na_2-EDTA. The extracts were stable for upto 3 days when frozen in liquid nitrogen and stored at -80°C.

Chromatographic system

HPLC analysis was carried out in a liquid chromatographic system consisted of a Model 6000A solvent delivery system (Waters Associates, Milford, MA); a Waters WISP 7101 automatic injector, and Waters 464 pulsed ECD with glassy carbon electrode. The detector was operated at 0.6V potential between the working electrode and the Ag/AgCl reference electrode at a sensitivity of 2nA full scale deflection. The column was a 150x3.9mm Novapack C_{18}, 5-µm (Waters, USA).Chromatograms were recorded with Model 023 recorder(Perkin-Emer Corp.,Norwalk,CT operated at 10mV full scale. A Waters guard column containing C_{18} Corasil was used. All separations were achieved isocratically at room temperature. Flow rate 1 ml/min. and injection volume was 20µl.

Quantification of HVA in plasma

The linearity of the detector response was estimated by injecting increasing amounts of standard HVA. Known amount of authentic HVA (Sigma-Aldrich Pvt. Ltd., USA) were added to study the linearity of the purification procedure and the analytical

recovery. The standard addition calibration curve was constructed for each plasma sample. The calibration curve was extrapolated to zero concentration of added HVA. The samples were assayed on the basis of peak heights relative to those of external standards. Detailed data graphs are shown in **Annexure VI.**

4.4. ESTIMATION OF C-REACTIVE PROTEIN

C-reactive protein was measured from the serum of all the subjects (Delusional Disorder patients 100+ 100 healthy controls). The CRP level in the serum was measured by latex agglutination slide test (Ranbaxy Fine Chemicals Ltd., HP, India).

Preparation of serum

5 ml of blood samples were collected from each patient. The samples were allowed to coagulate at 4°C for 2-3 hours. Blood clot was cut and centrifuged for separating the serum.

Measurement of C-reactive protein by latex agglutination test

The serum CRP level less than 0.6mg/dl was treated as categorical variable; undetectable or normal (<0.6mg/dl) and detectable or elevated level was considered to be greater than or equal to 0.6mg/dl (≥0.6mg/dl). The assay was performed blind to the subject's identity.

4.4. STATISTICAL ANALYSIS

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

4.5.1. 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:

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

4.4.2. Estimation of significance of difference

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

$$\chi^2 = \frac{(ad-bc)^2(a+b+c+d)}{(a+b)(c+d)(b+d)(a+c)}$$

4.4.3. One way analysis of variance

Analysis of variance (ANOVA) is a collection of statistical models, and their associated procedures, in which the observed variance is partitioned into components due to different explanatory variables.

$$F = \frac{\text{Variance of the group means}}{\text{mean of the within - group variances}}$$

4.4.4. Probability (P) value

The level of significance is expressed in terms of probability (p) value.

4.4.5. Estimation of Relative Risk (RR)

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

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