Chapter 3

Materials and Methods

3.1 Study Population:

Two human groups were considered for the present study, one consisting individuals with healthy immune system and the other one containing individuals with suppressed immune system. Some of the individuals included in the non-immunocompromised group belong to different tribal population residing in the northern part of West Bengal. Some non tribal samples were also collected in this group. The immunocompromised group comprised of individuals receiving steroid drugs, cancer patients receiving chemotherapy, organ transplant patients receiving immunosuppressive therapy and pregnant women in their 3rd trimesters. Samples from both these groups were collected from different areas of Darjeeling, Coochbehar and Alipurduar districts. Urine, blood or CSF samples were collected according to the availability.

The study methodology was approved by our Human Ethical Committee of Zoology Department, University of North Bengal (Dated 11.12.2009) and North Bengal Medical College and Hospital (Dated 25.3.2013) and the samples were taken from individuals only after their consent.

Tribal populations included in the study were Oraon, Munda, Rabha and Mech. Oraons are the second largest and the Mundas are the third largest tribal population after the Santhals, constituting about 14% and 7.8% respectively of total tribal population of West Bengal State of India (Census of India, 2001). Oraon are Dravidian linguistic group residing in Jharkhand, Odisha and Chattisgarh states of India and have migrated to tea gardens of Assam and West Bengal. They speak kurukh language. Mundas are Austroasiatic speaking ethnic group of India.They speak Mundari language and are found mainly in the Jharkhand, Odisha and West Bengal. Rabha or Rava are an indigenous Assamese community mostly residing in Assam, Meghalaya and West Bengal. They mainly speak Assamese language, but in some areas Rabha dialect is also used. In West Bengal, they are mostly found in Jalpaiguri and Coochbehar districts. Mech tribe (also known as Bodo-kachari tribe) belongs to the kachari tribal group and mainly speaks Bodo language which is a Tibeto-Burman dialect. Mech tribes have migrated to India through

Patkoi Hills between India and Burma and currently reside in parts of Assam and West Bengal (Sarkar, 2011).

Oraon and Munda tribal group samples were collected from Kiran Chandra tea garden, Naxalbari, Darjeeling district and Manjha tea garden, Naxalbari, Darjeeling district. Rabha or Rava tribe samples were collected from Poro Busty, Tufanganj of Coochbehar district and Mech tribe samples from Mendabari of Alipurduar district. Few samples were also collected from normal healthy general population of this region.

For collection of samples from immunocompromised individuals' permission from different hospitals of this region was taken. Samples collected from North Bengal Medical College and Hospital of Darjeeling district, Jalpaiguri Sadar Hospital of Jalpaiguri district, Siliguri Mahakuma Hospital of Darjeeling district, M. J. N. Hospital of Coochbehar district and North Bengal Oncology Centre of Darjeeling district were all part of the study.

3.2 Sample Collection:

For collection of urine sample, 3 ml of urine was taken from each individual in sterile vial and taken to the laboratory for DNA isolation. Whole of the sample was used during the DNA isolation if done by the boiling method, otherwise if done by kit, only 200 µl of sample was used and the remaining sample was stored in –20°C refrigerator.

About 1 ml of blood sample was collected from each individual in sterile vial containing EDTA, out of which 500 µl of sample was used to isolate DNA and the rest, was preserved in the -20°C refrigerator.

And in case of CSF sample collection 1 ml of sample was taken from each individual in sterile vials. About 500 µl of CSF was used during the viral DNA isolation and the remaining sample was then stored at –20°C refrigerator for future use. Details of the samples collected are given in the **Tables 5** and **6**.

Table 5: Details of Samples from each group included in the study			
S.No.	**Sample Groups**	**Specimen Types**	**Number of samples collected**
1	Pregnant women	Urine	83
		Blood	114
2	Steroid drug receiving patient	Urine	56
3	Normal healthy	Urine	24
4	Tribal people	Urine	160
		Blood	165
5	Chemotherapeutic patients	CSF	11
		Total	613

Table 6: Types of Tribal group included in the study			
S.No.	**Tribal Group**	**Specimen Type**	**Number of samples**
1	Oraon and Munda	Urine	74
		Blood	112
2	Rabha	Urine	50
		Blood	53
3	Mech	Urine	36
		Total	325

3.3 Designing of Oligonucleotides:

Oligonucleotides were designed to amplify NCCR, Large T-antigen and VP1 region of JCPyV by selecting published sequences and by searching their target sequences in available DNA sequence databases with the help of nucleotide Blast searches and also through a search in Primer Blast to check their authenticities. Six primers for Real Time PCR amplification were also designed following the same method. The sequences of the Primers designed, their names, the region of amplification of the JCPyV genome and references are listed in **Table 7** below.

Table 7: Sequences of the designed PCR primers with their respective names				
S. No.	**Region of Ampli-fication**	**Primer Names**	**Primers Sequence (Length)**	**References**
1	NCCR	Forward-SDJ1	5′-CCCTATTCAGCACTTTGTCC-3′	Monaco *et al.*, 1998
		Reverse-SDJ2	5′-CAAACCACTGTGTCTCTGTC-3′	
		JRR1	5′-CTTCTGAGTAAGCTTGGAGGCGG-3′	Ryschkewitsch *et al.*, 2013
2	Large T-Antigen	Forward-JCVT1	5′-GAATAGGGAGGAATCCATGG-3′	Monaco *et al.*, 1996
		Reverse-JCVT2	5′-GGAATGCATGCAGATCTACAGG-3′	
		JTP1	5′-GCAGCTTAGTGATTTTCTCAGG-3′	Ryschkewitsch *et al.*, 2004
3	VP1	Forward-JCVP1	5′-TTTTGGGACACTAACAGGAG-3′	Agostini *et al.*, 1996
		Reverse-JCVP2	5′-GTCAACGTATCTCATCATGT-3′	Boldorini *et al.*, 1998
		Forward-JCVPF	5′-CCTCAATGGATGTTGCCTTT-3’	Reid *et al.*, 2011
		Reverse-JCVPR	5′-AAAACCAAAGACCCCTC-3′	Reid *et al.*, 2011
		ECO-12	5′-TGGAATTCTGGCCACACTGTAACAAG-3′	Agostini *et al.*, 1996
4	VP1 (Real Time PCR)	Forward-JCVRVF	5'-TCAATGGATGTTGCCTTTACTTT-3'	Randhawa *et al.*, 2005
		Reverse-JCVRVR	5'-ACGGGGTCCTTCCTTTCTC-3'	
5	LTAg (Real Time PCR)	Forward-JCVRTI	5'-AAGAAATTAAACCTTTCAACTAAC-3'	Sehbani *et al.*, 2006
		Reverse-JCVRT2	5'-TATTATAATGCCCAAATTTTTGCAG-3'	
6	LTAg (Real Time PCR)	Forward-JCVRTF	5'-CTAAACACAGCTTGACTGAGGAATG-3'	Egli *et al.*, 2009
		Reverse-JCVRTR	5'-CATTTAATGAGAAGTGGGATGAAGAC-3'	

3.4 Viral DNA isolation from urine samples by boiling method:

3.4.1 Materials:

1. Oakridge centrifuge tubes (10 ml)
2. Microcentrifuge tubes (1.5 ml)
3. Waterbath
4. Tissue Paper
5. Ultracentrifuge Machine
6. PBS (Phosphate buffered saline)

   - 137 mM NaCl - 8 g
   - 2.7 mM KCl - 0.2 g
   - 10 mM Na_2HPO_4 - 1.44 g
   - 2mM KH_2PO_4 - 0.24 g

   Dissolved in 800 ml of distilled water and then pH was adjusted to 7.4 with HCl. Volume was then made upto 1000 ml and sterilized by autoclaving. The buffer was then stored at room temperature.

7. TE Buffer (10 mM Tris-Cl, 1mM EDTA)

   - 1M Tris-Cl – 1 ml (10 mM)
   - 0.5 M EDTA – 0.2 ml (1 mM)
   - Sterile distilled water – 98.8 ml

   All the things were mixed and stored at 4^oC refrigerator.

3.4.2 Methods:

JCPyV DNA was isolated from the urine samples that were collected from individual volunteers by boiling method (Jin *et al*., 1993).

- 5ml of PBS (phosphate buffered saline) was added to 3ml of urine samples and were subjected to ultracentrifugation (Super Speed Vacuum Centrifuge, Model: VS-35SMTi, Vision Scientific Co. Ltd., Korea) at 1, 00,000 x g for 20 minutes at 4ºC.
- The pellets obtained were then resuspended in 8ml of PBS and centrifuged again at 1, 00,000 x g for 20 minutes at 4ºC. The resultant pellets were then resuspended in 100 µl of TE buffer and boiled for 10 minutes in microcentrifuge tubes followed by a centrifugation at 12,000 rpm for 10 minutes.
- The supernatant was taken in a fresh tube leaving the pellet undisturbed.

3.5 Viral DNA isolation from blood samples by Phenol-Chloroform extraction method:

3.5.1 Materials:

1. Microcentrifuge tubes (1.5 ml)
2. Tissue paper
3. Centrifuge machine
4. Water bath
5. Red Cell Lysis Buffer (RCLB):

    - 2 M Tris Base - 15.76 g
    - 1 M $MgCl_2$ – 10.165 g
    - 3M NaCl – 8.766 g

Dissolved in 30 ml of distilled water and volume was adjusted to 50 ml. Sterilized by autoclaving and stored in at 4^oC refrigerator.

6. 20X SSC:

    - NaCl – 175.3 g
    - Sodium citrate – 88.2 g

Dissolved in 800 ml of distilled water and pH was adjusted to 7.0 with few drops of 14N HCl. The volume was then adjusted and sterilized by autoclaving.

7. 4M KCl:

298g of KCl was dissolved in 800 ml of distilled water and then the volume was made upto 1000 ml. Sterilized by autoclaving and stored at room temperature.

8. 1M Tris-Cl (pH 7.4 and pH 8.0):

121.1 g of Tris base was dissolved in 800 ml of distilled water. The pH was then adjusted to the desired value by adding concentrated HCl.

pH	**HCl**
7.4	70 ml
7.0	42 ml

Volume was then adjusted to 1000 ml with distilled water and sterilized by autoclaving.

9. 5M NaCl:

292 g of NaCl was dissolved in 800 ml of distilled water. Volume was then adjusted to 1000 ml with distilled water and sterilized by autoclaving. The solution was then stored at room temperature for further use.

10. 1M $MgCl_2.6H_2O$:

203.3g of $MgCl_2.6H_2O$ was dissolved in 800 ml of distilled water. Volume was adjusted and then sterilized by autoclaving.

11. 5M EDTA (pH 8.0):

186.1g of EDTA was added to 800 ml of distilled water and stirred vigorously. The pH was adjusted to 8.0 with NaOH, volume adjusted to 1000 ml with distilled water and then sterilized by autoclaving.

12. High Salt Buffer (HSB):

- 1M Tris-Cl (pH 7.4) – 1 ml (10 mM)
- 4M KCl- 250 µl (10 mM)
- 5M NaCl – 8 ml (0.4 M)
- 1M $MgCl_2$ – 1 ml (10 mM)
- 0.5M EDTA – 400 µl (2 mM)

All the above constituents were mixed and volume adjusted to 100 ml with sterile distilled water. The buffer was then stored at 4^oC refrigerator.

13. 10% SDS:

10g of SDS was dissolved in 100 ml of distilled water and heated to 68^oC. The volume was then adjusted with distilled water and stored at room temperature.

14. Proteinase K:

Proteinase K was initially centrifuged at 12000rpmfor 30sec. The concentration of the solution adjusted to 10mg/ml by adding required amount of sterilized Milli-Q water. The solution was divided into small aliquots of 1 ml and stored at -20°C.

15. 3M Sodium Acetate (pH 5.2):

403.8g of sodium acetate.$3H_2O$ was dissolved in 800 ml of distilled water and pH was adjusted to 5.2 with glacial acetic acid. Volume was then adjusted up to 1000 ml and sterilized by autoclaving.

16. 70% Ethanol:

70 ml of dehydrated ethanol was added to 30 ml of distilled H_2O and stored at -20°C.

17. Water saturated Phenol
18. Chloroform

19. Isoamylalcohol
20. Isopropyl alcohol
21. TE buffer

3.5.2 Methods:

DNA extraction from blood samples was done using the Phenol-Chloroform extraction method (Sambrook *et al.*, 1989) which includes some modifications.

- 500 µl of red cell lysis buffer (RCLB) was added to the EDTA containing blood and centrifuged (Eppendorf Centrifuge, model: 5424R, Germany) at 5,000 rpm for 5 minutes.
- The pellets obtained were resuspended in 500 µl of distilled water and centrifuged again at 5,000 rpm for 5 minutes.
- Supernatant was discarded and the pellets were resuspended in 1.2 ml of 1X SSC and centrifuged at 5,000 rpm for 5 minutes.
- 1.2 ml of 50 mM KCl was added to the pellet, mixed properly and then centrifuged at 5,000 rpm for 5 minutes.
- Supernatant was discarded and to it 375 µl of high salt buffer, 25 µl of 10% SDS, and 12.5 µl of Proteinase K (10 mg/ml concentration) was added and incubated at 56ºC for 1 hour.
- After the incubation, equal volume of phenol, chloroform and isoamylalcohol (25:24:1 v/v/v) and 3M sodium acetate, pH 5.2 ($1/10^{th}$ the volume of phenol/chloroform/isoamylalcohol) was added and incubated on ice for 15 minutes and centrifuged at 12,000 rpm for 10 minutes.
- Aqueous layer was transferred to a tube containing 500 µl of isopropanol and incubated at -20ºC for 30 minutes.
- It was then centrifuged at 12,000 rpm for 10 minutes at 4ºC in order to precipitate the DNA.
- The resultant pellet was resuspended in ice cold 70% ethanol and centrifuged at 13,000 rpm for 10 minutes at 4ºC. Pellets were then dried and resuspended in 100 µl of TE buffer (pH 8).

3.6 Viral DNA isolation from urine, blood and CSF samples using high pure viral nucleic acid kit (Roche, Switzerland):

3.6.1 Materials:

1. Microcentrifuge tube (1.5 ml)
2. Centrifuge machine
3. Waterbath
4. Tissue paper
5. Ethanol (96-100%)
6. High pure viral nucleic acid kit (Roche, Switzerland)

3.6.2 Methods:

DNA isolation from urine and blood samples was done using the High Pure Viral Nucleic Acid Kit from Roche as per the manufacturer's instruction.

- To 200 µl of samples, 200 µl of freshly prepared working solution (carrier RNA-supplemented Binding buffer) and 50 µl Proteinase K solution was added and incubated at 72°C for 10 minutes.
- Then to it 100 µl of binding buffer was added and centrifuged at 8000 rpm for 1min.
- The flow-through liquid was discarded and 500 µl inhibitor removal buffer was added and centrifuged again at 8000 rpm for 1 min.
- The flow-through was discarded and washed twice with 450 µl wash buffer.
- After washing, 50 µl elution buffer was added and centrifuged again at at 8,000 rpm for 1 min.
- The isolated DNA were then stored at -20°C refrigerator until use.

3.7 Viral DNAisolation from urine, blood and CSF samples using QIAmp DNA mini kit (Qiagen, Germany):

3.7.1 Materials:

1. Microcentrifuge tube (1.5 ml)

2. Centrifuge machine

3. Tissue paper

4. Ethanol (96-100%)

5. QIAmp DNA Mini kit (Qiagen, Germany)

3.7.2 Methods:

DNA isolation from urine and blood samples was done using the QIAmp DNA Mini Kit from Qiagen as per the manufacturer's instruction.

- To 20 µl of Proteinase K, 200 µl of sample was added and mixed.
- 200 µl of Buffer AL was added to it and mixed thoroughly and incubated at 56ºC for 10 minutes.
- 200 µl of ethanol (96-100%) was added and mixed properly and the whole mixture was then transferred to a QIAmp mini spin column.
- The samples were centrifuged at 8000 rpm for 1 min and the flowthrough was discarded.
- 500 µl of Buffer AW1 was added and centrifuged at 8000 rpm for 1 min and flowthrough was discarded.
- 500 µl of Buffer AW2 was added and centrifuged at 14000 rpm for 3 minutes.
- Flowthrough was discarded and 200 µl of Buffer AE was added to it and incubated for 1 minute.
- The samples were then centrifuged at 8000 rpm for 1 min and the isolated DNA was stored at -20º C refrigerator.

3.8 Amplification of NCCR:

Oligonucleotide primers SDJ1 and SDJ2 were used to amplify the noncoding control region (NCCR) of JCPyV genome. The expected product amplified from archetypal JCPyV genome has a size of about 586 bp. Amplification reactions were carried out in an Applied Biosystems Thermal Cycler (Model: 2720 Thermal Cycler, Life Technologies, USA). A total of 50 µl volume reaction mixture was prepared which contains 20 µl of viral DNA and the other constituents were mixed in the proportion given in **Table 8**. Sterile MilliQ water was used for the volume adjustment. The PCR cycling conditions for the amplification of the NCCR region are provided in **Table 9**.

Table 8: PCR reaction mixture for amplification of NCCR region of JCPyV genome.

Constituents	**Stock Concentration**	**Volume**	**Final Concentration**
Forward Primer (SDJ1)	20 pM	1 µl	0.4 pM
Reverse Primer (SDJ2)	20 pM	1 µl	0.4 pM
dATP	10 mM	1 µl	200 µM
dTTP	10 mM	1 µl	200 µM
dCTP	10 mM	1 µl	200 µM
dGTP	10 mM	1 µl	200 µM
PCR Buffer containing 1.5mM $MgCl_2$ (NEB, USA)	10X	5 µl	1X
Taq DNA polymerase (NEB, USA)	5 Unit	0.3 µl	1.5 Unit

Table 9: PCR cycling conditions for the amplification of NCCR region

Cycling Conditions	**Temperature**	**Time**
Initial Heating	94ºC	10 min
Denaturation	94ºC	1 min
Annealing	56ºC	1 min
Extension	72ºC	1 min
Final Extension	72ºC	5 min
Number of cycles: 30		

3.9 Amplification of T-antigen:

Positive samples were used to amplify large T-antigen region of the viral genome. Therefore, another set of primers namely, JCVT1 and JCVT2 were taken that amplified a fragment of approximately 770 bp size. Total reaction mixture volume was 50 µl that contained 10 µl of viral DNA and remaining constituents were added according to standardized PCR protocol. Details about the constituents of the PCR reaction mixture and PCR cycling conditions are given in **Table 10** and **11** respectively.

Table 10: PCR reaction mixture for amplification of T-antigen region of JC viral genome.

Constituents	**Stock Concentration**	**Volume**	**Final Concentration**
Forward Primer (JCVT1)	20 pM	1 µl	0.4 pM
Reverse Primer (JCVT2)	20 pM	1 µl	0.4 pM
dATP	10 mM	1 µl	200 µM
dTTP	10 mM	1 µl	200 µM
dCTP	10 mM	1 µl	200 µM
dGTP	10 mM	1 µl	200 µM
PCR Buffer [containing 1.5mM $MgCl_2$ (NEB, USA)]	10X	5 µl	1X
Taq DNA polymerase (NEB, USA)	5 Unit	0.3 µl	1.5 Unit

Table 11: PCR cycling conditions for the amplification of T-antigen region

Cycling Conditions	**Temperature**	**Time**
Initial Heating	94ºC	10 mins
Denaturation	94ºC	1 min
Annealing	55ºC	1 min
Extension	72ºC	1 min
Final Extension	72ºC	5 mins
Number of cycles: 35		

3.10 Amplification of VP1:

For the amplification of VP1 region of viral genome JCVP1 and JCVPR primers were used. JCVP1 and JCVPR amplified a fragment of about 500 bp. Reaction mixture is described in Table **12.** The total volume for the reaction mixture was 50 µl. Cycling conditions for the amplification of this region are mentioned in **Table 13**.

Table 12: PCR reaction mixture for amplification of VP1 region of JC viral genome.			
Constituents	**Stock Concentration**	**Volume**	**Final Concentration**
Forward Primer (JCVP1)	20 pM	1 µl	0.4 pM
Reverse Primer (JCVPR)	20 pM	1 µl	0.4 pM
dATP	10 mM	1 µl	200 µM
dTTP	10 mM	1 µl	200 µM
dCTP	10 mM	1 µl	200 µM
dGTP	10 mM	1 µl	200 µM
PCR Buffer containing 1.5mM $MgCl_2$ (NEB, USA)	10X	5 µl	1X
Taq DNA polymerase (NEB, USA	5 Unit	0.3 µl	1.5 Unit

Table 13: PCR cycling conditions for the amplification of VP1 region.		
Cycling Conditions	**Temperature**	**Time**
Initial Heating	94ºC	10 mins
Denaturation	94ºC	1 min
Annealing	56ºC	1 min
Extension	72ºC	1 min
Final Extension	72ºC	5 mins
Number of cycles: 30		

3.11 Gel Electrophoresis:

3.11.1 Materials:

1. TAE Buffer (50X):

   - Tris Base – 242 g
   - Glacial acetic acid – 57.1 ml
   - 0.5M EDTA (pH 8.0) – 100 ml

   Volume was adjusted to 1000 ml with sterilized distilled water and stored at room temperature.

2. Gel Loading Dye (6X):

   - 0.25% (w/v) bromophenol blue
   - 0.25% (w/v) xylene cyanol FF
   - 40% (w/v) sucrose in H_2O

3. Ethidium Bromide (10mg/ml):

   1gm of Ethidium bromide was added to 100 ml of Milli-Q H2O. The solution was stirred on a magnetic stirrer for several hours. The solution was transferred to a dark bottle and stored at room temperature.

4. Agarose Powder (Lonza, Switzerland)
5. 100 bp ladder (NEB, USA)
6. Bench Top Lab systems electrophoretic apparatus (Model: BT-MS-300, Taiwan)
7. UV transilluminator (Spectroline BI-O-VisionUV/White Light Transilluminator NY, USA).

3.11.2 Methods:

All the PCR products were subjected to electrophoresis using 1% agarose gel containing 0.5 µg/ml of ethidium bromide after mixing with 6X gel loading dye. The gel

was prepared by dissolving agarose powder in 1X TAE Buffer. The solution was then boiled for few minutes and to it ethidium bromide was added. Slightly warm gel was poured in to the casting tray and left still for few minutes. The electrophoretic tank was also filled with 1X TAE buffer and gel was kept in the tank after it has been solidified. One-hundred (100) bp ladder, a marker used to determine the size of the PCR product, was also load in one of the lanes preferably in the first lane. The gel was run at 80 V for 1 hour approximately in the Bench Top Lab Systems (Model: BT-MS-300, Taiwan) electrophoretic apparatus and visualized in a UV transilluminator (Spectroline BI-O-VisionUV/White Light Transilluminator, NY, USA).

3.12 Purification of PCR products:

3.12.1 Materials:

1. Clean sharp razor
2. Microcentrifuge tube (1.5 ml)
3. Weighing machine (Afcoset Electronic balance, Model-FX-200, India)
4. Centrifuge machine
5. Waterbath
6. Isopropanol
7. Tissue paper
8. Purelink gel extraction and PCR purification kit (Invitrogen, USA)

3.12.2 Methods:

Successfully amplified and scored PCR products were then purified from agarose gel using Purelink Gel Extraction and PCR purification kit (Invitrogen, USA) for sequencing.

- A clean, sharp razor blade was used to excise the area of gel containing the DNA fragment of interest. The gel slice was weighed and taken in sterile microcentrifuge tube.

- Gel solubilization buffer (L3) was added to the excised gel in a 3:1 ratio. The tube was incubated at 50ºCfor 15 minutes.
- 1 gel volume of isopropanol was added to the dissolved gel slice and mixed properly. The dissolved gel was then pipetted onto a column inside a wash tube.
- The column was centrifuged at 12000 rpm for 1 minute and flowthrough was discarded.
- Column was placed again into the wash tube and 500 µl of wash buffer (W1) containing ethanol was added to the column.
- The sample was centrifuged at 12000 rpm for 1 minute and flowthrough discarded.
- The column was placed into the same wash tube and centrifuged again at maximum speed for 2-3 minutes.
- Remaining flowthrough was discarded and placed onto a fresh recovery tube.
- 50 µl of Elution buffer (E1) was added to the column and incubated for 1 minute at room temperature.
- The tube was centrifuged at 12000 rpm for 1 minute and the eluted DNA was stored at -20ºC refrigerator until use.

3.13 Sequencing of positively amplified NCCR, VP1 AND T-Antigen and submission of sequences in NCBI:

Sequencing of positively amplified PCR products of NCCR, T-antigen and VP1 were done each at least twice by dideoxy chain termination method (Agrigenome Labs Pvt Ltd., Kerala). The sequence results obtained were then aligned in Bioedit to get the entire sequence. Nucleotide BLAST was done to verify the results. And then the curated sequences were deposited in NCBI public domain for GenBank accession number.

3.14 Quantification of Viral Load by Real Time PCR:

Viral load for JCPyV positive samples from both urine and blood was estimated by real time PCR quantification. Two oligonucleotide primers namely, JCVRVF and JCVRVR specific for VP1 region of the virus were used. The product size amplified by the primers was of about 109 bp. The amplification reaction was standardized using the

cloned JCPyV Mad1 DNA and standard curve was also prepared using this standard DNA in 10-fold dilution series.4 µl of DNA from each sample was added to the 20µl reaction mixture. Each sample was prepared in duplicate copies to prevent any error. Primers and SYBR green reagents were mixed in the proportion depicted in **Table 14**. Amplification reactions were performed in Roche Lightcycler 96 (Roche, Switzerland) and the cycling conditions for the amplification are mentioned in **Table 15**.

Table 14: Reaction mixture for real time PCR of VP1 region of JC viral genome.			
Constituents	**Stock Concentration**	**Volume**	**Final Concentration**
Forward Primer (JCVRVF)	10 pM	0.6 µl	0.3 pM
Reverse Primer (JCVRVR)	10 pM	0.6 µl	0.3 pM
SYBR Green Reagent (Faststart Universal SYBR Green Master (Rox), Roche, Germany)	2X	10 µl	1X

Table 15: Cycling conditions for real time PCR of VP1 region of JC viral genome		
Cycling Conditions	**Temperature**	**Time**
Initial Heating	95ºC	10 min
Denaturation	95ºC	30 secs
Annealing	63ºC	30 secs
Extension	72ºC	45 secs
Number of cycles: 30		

3.15 Statistical analysis by SPSS:

The Chi-square test was employed using SPSS version 21 (IBM Corporation, New York, USA) to compare between the JCPyV positivity status with that with the gender of the subjects studied. For the analyses, the $P < 0.05$ was considered significant.

3.16 Multiple sequence alignment using CLUSTAL X:

All NCCR sequences (region ranging from T-antigen coding start site to Agnogene coding start site) were aligned in Clustal X ver. 2.1 (Thompson *et al*., 1997) using default parameters, curated in BioEdit ver. 7.0.9.0 (Hall, 1999) to generate an alignment showing deletion/mutation and common transcription factor binding sites derived from experimental data of other research groups. Similarly, all the VP1 and T antigen sequences were also aligned Clustal X ver. 2.1 (Thompson *et al*., 1997) using default parameters, curated in BioEdit ver. 7.0.9.0 (Hall, 1999) to generate an alignment showing deletion or mutation in the sequences.

3.17 Pairwise alignment using Genomatix:

Nucleotide sequences of the endemic JCPyV NCCRs were aligned with the known NCCR sequences of JCPyV strains CY, LH3, Tai3, IN8 and Mad-1 using DiAlign alignment program of Genomatix suite v2.5 GmbH (Cartharius *et al*., 2005) to compare pairwise similarities (relative to the maximum similarity) among these sequences.

Nucleotide sequences of JCPyV VP1 region were aligned with known VP1 sequences of JCV Type 1A, Type 1B, Type 2A, Type 2B, Type 2C, Type 2D, Type 3, Type 4 and Type 6 using DiAlign alignment program of Genomatix suite v2.5 GmbH (Cartharius *et al*., 2005) to check pairwise similarities among these sequences.

In a similar way, nucleotide sequences of JCPyV T-antigen region were aligned with known T-antigen sequences of JCPyV Type 1A, Type 1B, Type 2A, Type 2B, Type 2C, Type 2D, Type 3, Type 4 and Type 6 using DiAlign alignment program of Genomatix suite v2.5 GmbH (Cartharius *et al*., 2005) to compare pairwise similarities among these sequences.

3.18 Transcription factor binding site prediction in NCCR by Genomatix:

The MatInspector program of Genomatix Software suite v2.5 GmbH (Cartharius *et al*., 2005) was used to search for the predicted transcription factor binding sites (TFBS) in the aligned portion of endemic as well as other mentioned JCPyV strains using a large library of weight matrices based on general core promoter and vertebrate promoter matrix families. Matches for transcription factors that are reported to be active in cells/tissues such as antibody-producing cells, antigen-presenting cells, bone marrow cells, hematopoietic and immune system, leukocytes, lymphocytes, monocytes, myeloid cells, phagocytes, brain, CNS, endocrine system, kidney, nervous system, urinogenital system were considered.

3.19 Phylogenetic analysis based on VP1 region of JC virus:

Phylogenetic tree was constructed using maximum likely method in MEGA version 10.0.4 based on our sequenced VP1 sequence against VP1 sequences of different JCPyV types obtained from NCBI.

The best nucleotide substitution model for phylogenetic analyses for VP1 region of JCPyV was selected using Model Testing programme, inbuilt in MEGA version 10.0.4 (Kumar *et al*., 2018). The best model obtained was Tamura 3 parameter according to the following scores: Bayesian Information Criterion (BIC) = 3049.787, Akaike Information Criterion corrected (AICc) = 2568.366, Maximum likelihood value (lnL) = 1220.922, Assumed or estimated values of transition or transversion (R) = 1.77, nucleotide frequencies (f): f(A) = 0.297, f(T)= 0.297, f(C) = 0.203, f(G) 0.203, rates of base substitution (r): r(AT) = 0.052, r(AC) = 0.036, r(AG) =0.131, r(TA) = 0.052, r(TC) = 0.131, r(TG) = 0.036, r(CA) = 0.052, r(CT) = 0.193, r(CG) = 0.036, , r(GA) = 0.193, r(GC) = 0.052, r(GT) = 0.036.

Tamura-3 parameter model was selected and 100 bootstrap replications were carried out during phylogenetic tree construction. The following default parameters were

selected: uniform rates, 1^{st}+ 2^{nd} + 3^{rd} + noncoding positions, Gap/Missing Data treatment: complete deletion, ML heuristic method: Subtree pruning regrafting extensive (SPR level 5), Initial tree for ML= Make Initial tree automatically (Default-NJ/BioNJ), strong branch swap filter and 4 number of threads.

3.20 Phylogenetic analysis based on T-antigen region of JC virus:

Using Maximum Likelyhood (ML) method phylogenetic trees were constructed in MEGA version 10.0.4 (Kumar *et al*., 2018) based on the sequenced T-antigen sequence of positive samples against sequences of the same region from different JCPyV types downloaded from NCBI.

The best nucleotide substitution model for phylogenetic analyses for T Antigen region of JCPyV was selected using Model Testing programme inbuilt in MEGA version 10.0.4. The best model obtained was Tamura 3 parameter according to the following scores: Bayesian Information Criterion (BIC) = 2850.691, Akaike Information Criterion corrected (AICc) = 2422.653, Maximum likelihood value (lnL) = 1156.153, Assumed or estimated values of transition or transversion (R) = 3.11, nucleotide frequencies (f): f(A) = 0.309, f(T)= 0.309, f(C) = 0.191, f(G) 0.191, rates of base substitution (r): r(AT) = 0.036, r(AC) = 0.022, r(AG) =0.147, r(TA) = 0.036, r(TC) = 0.147, r(TG) = 0.022, r(CA) = 0.036, r(CT) = 0.237, r(CG) = 0.022, , r(GA) = 0.237, r(GC) = 0.022, r(GT) = 0.036.

Tamura-3 parameter model was selected and 100 bootstrap replications were carried out during phylogenetic tree construction. The following default parameters were selected: uniform rates, 1^{st}+ 2^{nd} + 3^{rd} + noncoding positions, Gap/Missing Data treatment: complete deletion, ML heuristic method: Subtree pruning regrafting extensive (SPR level 5), Initial tree for ML= Make Initial tree automatically (Default-NJ/BioNJ), strong branch swap filter and 4 number of threads.