2.1 Study Population

The present study is a case-control study comprised of a RPL group, which is compared with that of a control group.

2.1.1 Control group

The control women were volunteers with at least two live births and without any history of abortion, endometriosis, or infertility. The control group comprised of eighty couples and an additional of forty women who fulfilled the criteria for control.

2.1.2 RPL group

Hundred and four couples with unexplained RPL were enrolled in the study from different hospitals in and around Chennai. Medical records of the patients were verified for the reports of the routine diagnostic procedures such as karyotyping of the couples, torch test, hormone levels, antiphospholipid antibodies and hysteroscopic examination. Only those patients who had normal medical reports for all the above tests were categorized to exhibit unexplained pregnancy loss and were included in the study. All of them were primary aborters and none of them had any pregnancy-related complications such as hypertension, thyroid abnormalities, diabetes, etc. All the patients were ethnic South Indians.

The cases and controls were similar in ethnicity (from South India). Occupationally neither the cases nor the controls were exposed to any specific toxic chemical. The study was approved by the Institutional Medical and Ethics Committee. About 4-5ml of blood sample was collected from the subjects with an informed written consent.

To determine the polymorphisms in HLA G and KIR genes, sample from both the partners was analysed in cases as well as controls. This involved 104 couples with RPL and 80 control couples. Whereas, to determine the polymorphism of genes involved in placental vascular network, xenobiotic metabolism and DNA repair only female samples
were analysed. This involved 104 women with RPL and 120 control women. The mean age of the study subjects and the number of pregnancy loss in RPL women have been tabulated in Table 2.1 and 2.2 respectively.

Table 2.1: Mean age of study subjects

<table>
<thead>
<tr>
<th>RPL patients</th>
<th>Mean Age (Years)</th>
<th>Controls</th>
<th>Mean Age (Years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>104 couples</td>
<td>Male – 34</td>
<td>80 couples</td>
<td>Male – 36</td>
</tr>
<tr>
<td></td>
<td>Female – 30</td>
<td></td>
<td>Female – 33</td>
</tr>
<tr>
<td>104 females</td>
<td>30</td>
<td>120 females</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 2.2 Details of pregnancy loss in RPL cases

<table>
<thead>
<tr>
<th>Number of pregnancy loss</th>
<th>N</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>55</td>
</tr>
<tr>
<td>≥ 4</td>
<td>17</td>
<td>16</td>
</tr>
</tbody>
</table>


2.2 Materials Required

2.2.1 Reagents and buffers for genomic DNA isolation from peripheral blood

2.2.1.1 1M Ammonium Chloride: 53.49g of ammonium chloride was added to 800ml of double distilled water, dissolved completely and the final volume was made up to 1000 ml.

2.2.1.2 1M Sodium bicarbonate: 84.0g of sodium bicarbonate was added to 800ml of double distilled water dissolved completely and the final volume was made up to 1000 ml.

2.2.1.3 Tris-HCl- 1M (pH 8.0): 121.1 g of Tris-base was dissolved in 900 ml of double distilled water and the pH was adjusted to 8.0 with concentrated HCl. The final volume was made up to 1000ml with double distilled water and sterilized by autoclaving.

2.2.1.4 EDTA di-sodium salt – 0.5M (pH 8.0): 18.6g of di-sodium EDTA was dissolved in 80 ml of double distilled water and 15.20g of sodium hydroxide pellets were added to increase the pH towards 8.0. When the sodium hydroxide pellets dissolved completely, 1N HCl was added to bring pH to 8.0. The final volume was made up to 100ml and sterilized by autoclaving.

2.2.1.5 Sodium chloride- 5M: 29.2g of sodium chloride was added to 80ml of double distilled water and warmed to assist dissolution. The final volume was made up to 100 ml with double distilled water.

2.2.1.6 Sodium dodecyl sulphate (SDS) - 10% (pH 7.0): 10g of SDS was dissolved in 80 ml of sterile double distilled water gently by slow mixing to avoid frothing. The solution was kept in a water bath at 65°C to assist complete dissolution. The final volume was made up to 100ml and the solution was filter sterilized.
2.2.1.7 **Proteinase K solution:** It is used at a final concentration of 100 µg/ml. 100mg proteinase K was dissolved in 10 ml of distilled water and stored at –20°C.

2.2.1.8 **4M Ammonium acetate:** 154.16g of ammonium acetate was dissolved in 500 ml of distilled water and store in refrigerator at -20°C.

2.2.1.9 **Chloroform:** Chloroform was procured commercially from Qualigens.

2.2.1.10 **Isopropyl Alcohol:** It was commercially procured and stored under refrigerated condition.

2.2.1.11 **Ethanol-70%:** 70ml of absolute ethanol was mixed with 30ml of sterile double distilled water and stored at 4°C.

2.2.1.12 **RBC Lysis Buffer (for 500 ml):**

- 72ml of 1M NH₄Cl solution
- 0.5ml of 1M NaHCO₃ solution

The components were mixed with sterile double distilled water and made upto 500 mL.

2.2.1.13 **WBC Lysis Buffer (for 50 ml):**

- 4ml of 5M NaCl solution
- 0.2 ml of 0.5m of EDTA solution
- 0.25ml of Tris HCl (pH 8)

The contents were mixed and made to a final volume of 50ml with sterile water.

2.2.1.14 **TE buffer (Tris-EDTA buffer) – (pH 8.0)**

- Tris HCl (pH 8.0) - 10mM
- Na₂EDTA.2 H₂O (pH 8.0) - 1mM

The components were dissolved in sterile double distilled water.

All the chemicals were from Hi Media laboratories and the solvents were from Qualigens.
2.2.2 Reagents and buffers for qualitative and quantitative analysis of DNA

2.2.2.1 TAE buffer (Tris- acetate EDTA buffer) - 50X (pH 7.2)

Tris base – 2M
Glacial acetic acid - 1N
Na$_2$ EDTA.2 H$_2$O - 0.05M

Tris base and disodium EDTA were dissolved in sterile double distilled water. Using glacial acetic acid, the pH was adjusted to 7.2. The final volume was made up to 1000 ml and sterilized by autoclaving. The solution was stored in a clean sterile reagent bottle at room temperature (25°C)

2.2.2.2 Ethidium bromide- 10 mg/ ml: To 1 ml of sterile double distilled water, 10mg of ethidium bromide was added and mixed well for complete dissolution of the dye. The stock solution was stored in aliquots in air tight containers wrapped with aluminum foil.

2.2.2.3 DNA sample loading dye- 6X

Ficoll 400 - 6%
Bromophenol blue - 0.12%
Xylene cyanol FF - 0.12%
Tris- HCl (pH 7.5) - 12mM
Na2 EDTA.2 H2O - 120mM

All the components were dissolved in sterile double distilled water and stored at 25°C

2.2.2.4 Agarose Low EEO: Commercially available agarose was used (Bangalore Genei/Sigma)
2.2.3 Reagents for Polymerase Chain Reaction (PCR)

All the PCR fine chemicals were commercially procured from Bangalore Genei Pvt.Ltd.

2.2.3.1 10 X PCR Taq DNA Polymerase buffer
2.2.3.2 Taq DNA Polymerase (3Units/µl)
2.2.3.3 d NTP mix (10mM)
2.2.3.4 Primers: Primers were obtained as lyophilized powder of different OD values and were reconstituted with TE buffer.

2.2.4 Reagents for Restriction Fragment Length Polymorphism

Restriction enzymes and Buffers were procured from New England Biolabs and Bangalore Genei Pvt.Ltd.

2.2.5 Reagents for DNA Polyacrylamide Gel Electrophoresis

2.2.5.1 Acrylamide: Bisacrylamide—30%

29.2g of acrylamide and 0.8g of bisacrylamide were mixed and the volume was made up to 100ml with sterile double distilled water. The solution was filter sterilized with 0.45µm sterile filter and stored in clean airtight dark bottle.

2.2.5.2 TBE Buffer (Tris - borate EDTA) - 10X (pH 8.3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>0.89M</td>
</tr>
<tr>
<td>Boric acid</td>
<td>0.89M</td>
</tr>
<tr>
<td>Na₂EDTA. 2H₂O</td>
<td>0.04M</td>
</tr>
</tbody>
</table>

Tris base and EDTA di sodium salt were dissolved in 800ml of sterile double distilled water. Boric acid was added to adjust the pH to 8.3 and the volume was made up to 1000ml with distilled water. The solution was sterilized by autoclaving and was stored in clean sterile glass bottle at room temperature.
2.2.5.3 Tetraethylene methylene diamine tetra acetic acid (TEMED) - 100% commercially available TEMED.

2.2.5.4 Ammonium per sulphate APS - 10%: 1g of APS was dissolved in 10 ml of sterile double distilled water and stored at 4°C.

2.2.5.5 Polyacrylamide gel mix: The gel mix was prepared as shown in Table 2.3

Table 2.3: Polyacrylamide gel mix

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Working Concentration</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>10%</td>
<td>5</td>
</tr>
<tr>
<td>TBE buffer</td>
<td>0.5 X</td>
<td>0.750</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.2%</td>
<td>0.030</td>
</tr>
<tr>
<td>APS</td>
<td>0.05%</td>
<td>0.075</td>
</tr>
</tbody>
</table>

10µl of the sample, 2µl of 6X BPB dye were mixed and loaded onto the gel. Electrophoresis was done at 4V/cm. Silver staining was done to observe the DNA.

2.2.6 Reagents for silver staining

2.2.6.1 Acetone: 50% acetone was prepared by mixing 30ml of acetone with 30 ml of distilled water.

2.2.6.2 Tricholoro Acetic Acid (TCA): 50%TCA was prepared by dissolving 12.5 g TCA in 25 ml of sterile distilled water.

2.2.6.3 Sodium thiosulphate (Na$_2$S$_2$O$_3$): 10% Na$_2$ S$_2$ O$_3$ was prepared by dissolving 2.5 g Na$_2$S$_2$O$_3$ in 25 ml of sterile distilled water.
2.2.6.4 **Silver nitrate**: 20% silver nitrate was prepared by dissolving 5g silver nitrate in 25 ml of sterile distilled water

2.2.6.5 **Formaldehyde**: Commercially available 37% formaldehyde was used.

2.2.6.6 **Sodium carbonate**: 1.4g was used for preparing the developer.

2.2.6.7 **Glacial Acetic acid**: 1% prepared from commercially available 100% glacial acetic acid (Merck) by diluting 1m acetic acid with 99 ml sterile distilled water

2.2.7 **Reagents for DNA sequencing and processing**

Big Dye™, 50% Hi-Di Formamide, 70% ethanol, 3M Sodium acetate pH 5.2

The genotypes of representative samples were confirmed by DNA sequencing. This was carried out in ABI 3130 DNA analyzer at the Tuberculosis Research Centre, Indian council of Medical Research (TRC, ICMR), Chennai.

2.2.8 **Reagents and buffers for antipaternal cytotoxic antibody analysis**

Ficol histopaque –Commercially available (Sigma)

Phosphate buffered saline - Commercially available (Sigma)

Rabbit Complement - Commercially available (Sigma)

Eosin – (Qualigens)

Formaldehyde - Commercially available (qualigens)
2.3 Methodology

2.3.1 Methodology for isolation of high molecular weight genomic DNA from blood sample by Miller’s method

Blood samples were collected from RPL patients and controls in sterile EDTA vacutainers. High molecular weight genomic DNA was isolated from the peripheral blood by Miller’s method (1)

2.3.1.1 RBC Lysis (Non-nucleated cell lysis buffer)

To 5ml peripheral blood, 40ml of RBC lysis buffer was added, mixed thoroughly and incubated at room temperature for one hour. Samples were then centrifuged at 2000rpm for 20 minutes and supernatant was discarded. The above step was repeated again till a white pellet free of heme was obtained.

2.3.1.2 WBC lysis (Nucleated cell lysis buffer)

The white pellet was resuspended in 3ml of NLB buffer, 200 μl of 10% SDS and 50 μl of proteinase K (10mg/ml), mixed well and incubated at 42 °C overnight. The next day, 4ml of 4M ammonium acetate was added and mixed thoroughly. Then 3ml of chloroform was added and mixed by slight vortexing. Following this, the sample was spun at 3000 rpm for 30 minutes at 4°C.

2.3.1.3 DNA Precipitation

The resulting supernatant was transferred into a centrifuge tube and 7ml of isopropyl alcohol was added. The tube was mixed gently until the DNA precipitated. The precipitated DNA was then transferred to a 1.5 ml sterile microcentrifuge tube and 500μl of cold 70% ethanol was added and spun at 2000rpm for 5min at 4°C. Ethanol was discarded, DNA was air dried and then dissolved in 150 µL of TE buffer. The sample was then refrigerated at 4°C for complete dissolution and stored at -20°C till further use.
2.3.2 Methodology for qualitative and quantitative analysis of DNA

2.3.2.1 Agarose gel electrophoresis

The quality of the DNA samples was checked in a 0.8% agarose gel. 0.8g of agarose was dissolved in 100ml of 1X TAE buffer by boiling. The solution was allowed to become lukewarm followed by which ethidium bromide was added to a final concentration of 0.1mg/ml. The gel was then poured on a gel-casting tray and allowed to solidify. The gel was placed in an electrophoresis tank with 1X TAE buffer. The DNA samples were mixed with bromophenol blue dye and loaded on the gel. The gel was electrophoresed at 2 volts/cm and visualized in a gel documentation system (Bio Rad) (Fig 2.1)

2.3.2.2 Spectrophotometric analysis

The quality and quantity of the DNA samples were assessed by spectrophotometer. 10 µl of the DNA was diluted with 990 µl of TE buffer, and the OD was read at 260 nm and 280 nm. The concentration of the samples was determined based on the OD value at 260 nm, where 1 OD corresponds to 50µg/ml of double stranded DNA. Samples with 260/280 OD ratio between 1.7-1.9 were selected for PCR. Samples with lesser than 1.7 OD were reprecipitated and then used for PCR.

2.3.3 Protocol for Polymerase Chain Reaction

Amplification of the gene of interest was performed using specific primers under appropriate cycling conditions of denaturation, annealing and extension in a thermal cycler (Master Cycler gradient- Eppendorf). A 20µl reaction was set up in the following concentration (Table 2.4)
Materials and Methods

A study on genetic polymorphisms associated with unexplained recurrent pregnancy loss in South Indian population

Table 2.4: PCR Mix

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Working Concentration</th>
<th>Working Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Taq DNA Polymerase Buffer</td>
<td>1 X</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>d NTP mix</td>
<td>200µM</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>Primer Forward Primer Reverse</td>
<td>50 pM 50 pM</td>
<td>0.5 0.5</td>
</tr>
<tr>
<td>4</td>
<td>Taq DNA polymerase</td>
<td>1.5 Units</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>Water (nuclease free)</td>
<td>-</td>
<td>15.1</td>
</tr>
<tr>
<td>6</td>
<td>Template</td>
<td>100ng</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

A master mix comprising of all components except the template DNA was prepared and aliquoted into separate tubes. The template DNA was then added; the tubes were placed in the thermal cycler and subjected to the standardized PCR conditions. The PCR conditions were standardized for each gene by gradient PCR. Except for the annealing temperature and time which differed for the different genes, the other PCR conditions are given in Table 2.5

Table 2.5: PCR conditions

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45sec -1min</td>
</tr>
</tbody>
</table>

Repeated for 30 cycles.

Final extension   | 72               | 5 min      |
| Hold at 4        |                  |            |

* different for different genes
2.3.4 Confirmation of PCR amplification

PCR amplification was confirmed by 2% agarose gel electrophoresis. 100bp DNA molecular weight marker was used confirm the amplicon size. Electrophoresis was carried out at 4V/cm and the gel was visualized in the gel documentation system.

2.3.5 Purification of PCR amplicons

To the PCR product, thrice the volume TE buffer was added. This was followed by the addition of one tenth the volume 3M sodium acetate pH 5.2. The product was then precipitated by the addition of double the volume ice-cold absolute ethanol and incubation at 0°C overnight or -70°C for 1 to 2 hours and centrifuged at 12000rpm for 30 minutes at 4°C. The precipitated product was washed once with 70% ethanol at 12000rpm for 30 minutes at 4°C. The pellet was then air dried and resuspended in 20 µl of TE buffer and stored at 0°C until further use.

2.3.6 Protocol for PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

The purified PCR amplicon was subjected to restriction digestion with the appropriate restriction enzyme and incubated for 1-2 hours at the optimum temperature for the enzyme. A 20µl restriction digestion reaction was set up as shown in Table 2.6.
2.3.7 Analysis of PCR-RFLP products

2.3.7.1 Agarose gel electrophoresis

Analysis of PCR-RFLP products was done by a 2% or 4% agarose gel electrophoresis based on the restriction fragment size. The gel was electrophoresed at 4V/cm and visualized in the gel documentation system. Genotypes were assigned based on the restriction pattern.

2.3.7.2 Polyacrylamide gel electrophoresis

To resolve the restriction fragments which differ in size by a few base pairs, a polyacrylamide gel electrophoresis was performed. The acrylamide gel mix for 15 ml was prepared and casted (Table 2.3).

2.3.7.3 Silver staining

After electrophoresis gel was carefully removed from the gel plates, transferred into a staining tray. It was first fixed by adding 60ml of 50% acetone, 1.5ml of TCA solution and 25µl of 37% formaldehyde, rocked gently for 5 min. The solution was discarded and the gel was rinsed with distilled water for 5-10 sec. Then 60 ml of 50% acetone was
added and gently rocked for 5 min. The solution was then discarded and 100µl of sodium thiosulphate in 60ml distilled water was added to the gel and rocked for 1 min. The gel was rinsed with distilled water and then stained with the solution containing 0.8ml of 20% silver nitrate; 0.6ml of 37% formaldehyde in 60ml of distilled water. The gel was rocked gently for 8min. The solution was then discarded and the gel was rinsed with distilled water. The gel was developed by adding the developer (1.4g of sodium carbonate, 25µl formaldehyde and 12.5µl sodium thiosulphate made up to 60 ml with distilled water). Staining was arrested by adding 1% glacial acetic acid. The stained gel was viewed on a white light transilluminator and documented in the gel documentation system.

2.3.7.4 Protocol for Sequencing

Sequencing of the PCR amplicon was carried out in 2 steps: The PCR amplicon to be sequenced was first subjected to a sequencing PCR followed by post PCR processing and sequencing.

2.3.7.4.1 Sequencing PCR was performed with the PCR amplicon as the template, with one of the respective forward or reverse primers. A master mix of BigDye™, primer and PCR water were prepared as given in Table 2.7 and then dispensed equally into MicroAmp 96well plate. The PCR products were then added to the wells and subjected to sequencing PCR reaction

2.3.7.4.2 Sequencing PCR was carried out in the GeneAmp 9600 thermalcycler (Perkin-Elmer). The reaction conditions are as follows: 95°C for 10sec, 50°C for 5sec, 60°C for
4 min, the conditions are repeated for 30 cycles, following which they were subjected to post PCR processing. To 3 ml absolute alcohol, 120 µl of 3 M Sodium acetate (pH 5.2) was added and 25 µl of this was added into each well of the plate. The plate was centrifuged at 4000 rpm for 20 min at 25°C. The plate was inverted to remove the supernatant. 100 µl of 80% ethanol was added to each well and again centrifuged at 4000 rpm for 10 min. The plate was once again inverted and subjected to a pop spin for few seconds at 750 rpm to remove the alcohol and covered with fresh aluminium foil. At the time of sequencing, 10 µl of 50% HiDye™ formamide was added to all the wells. The sample plates were run in the ABI Prism® 3130 DNA Analyzer.

2.4 Methodology for analyzing polymorphisms in Immunomodulatory genes and antipaternal cytotoxic antibodies

*HLA G* Polymorphisms in exon 2 (codon 31, 57 and 69), exon 3 (93, 107, 110 and 130) and exon 8 (I/D) of *HLA G* were analysed by PCR based methods (2-4). The primer sequence, PCR amplicon size, annealing temperature and restriction enzymes are given in Table 2.8. The PCR and PCR- RFLP products were run on agarose gel. Sequencing of representative samples was done to confirm the PCR-RFLP results.

Ten *KIR* genes were identified by allele specific PCR (5). Primer sequences, annealing temperature and amplicon size are given in Table 2.9.

The presence of circulating anti-paternal antibodies was determined by cross matching between maternal undiluted fresh serum with paternal lymphocytes.
2.9 References


