4. MATERIALS AND METHODS

4.1 Sampling

The study was approved by the institutional review board of Chettinad Academy of Research and Education and performed during April 2012 - August 2013 (Appendix I). Infertility patients were enrolled from the department of Reproductive medicine, Chettinad Hospital and Research Institute (CHRI) in Chennai, India. An informed consent was obtained from each volunteer after explaining the study in local language (Appendix II) prior to sampling. A questionnaire was constructed by the investigator and detailed information such as age, BMI, duration of marriage, type and duration of infertility, occupation, nativity, caste, medical history, lifestyle, family history of infertility etc. were recorded (Appendix III).

4.1.1 Subjects/Patients

A total of 506 infertile patients were enrolled for the study including 277 females and 229 males. The samples were collected routinely at the outpatient block of which a few samples were excluded based on the exclusion criteria. The selected candidate genes were known to influence the fertility directly or indirectly. The study has enrolled infertility patients with both known and unknown causes. Patients with known causes like Oligozoospermia, OAT, PCOS, Tubal blocks etc were enrolled to evaluate the polymorphism association with those causes. The selection of patients was as follows.

Inclusion criteria

- The married couples who failed to conceive after at least one year of unprotected intercourse were taken for the study.

- Both male and female infertility patients were included.

- Both the PI and SI patients were enrolled.
• Infertility patients within the reproductive age 18–45 years were selected.

• Patients who completed the routine diagnostic tests were recruited.

Exclusion criteria

• Infertility patients with the known cause such as anatomical abnormalities, abnormal karyotype, and hormonal imbalances were excluded.

• Patients without proper diagnosis were not included.

4.1.2 Controls

A total of 246 fertile population/subjects were enrolled as controls, of which 125 were women and 121 were men. Healthy population, who attended regular/routine health check up were recruited in the present study.

Inclusion

• Healthy fertile men and women from Chettinad Hospital and Research Institute, having at least one child without any aid of Assisted Reproductive Technologies were recruited as controls.

• Controls within the age group 18-45 years were selected.

Exclusion

• Population who were not willing to participate was excluded from the study.

4.2 Distribution of causes of infertility

The study population consisted of 181 couples, of those 48% females (n=86) are solely responsible for infertility. Combined factors infertility was found in one
fourth of the couples. The remaining couples had male factor (17%) and unexplained infertility (9%). The diagrammatic representation was given in Figure 3.

![Etiology of couples (n= 181)](chart)

**Figure 3. Distribution of Infertile couples**

### 4.2.1 Female infertility causes

The present study has enrolled 277 infertile women including both PI and SI. A high proportion of tubal block patients were reported followed by PCOS. Unexplained and/or male factor infertility patients were also present. Around 13% of the patients with various abnormalities such as DOR, endometriosis, uterine abnormalities were included in others category. The causes of female infertility are represented in Figure 4.

### 4.2.2 Male infertility causes

The study has recruited 229 male infertility patients attending the infertility clinic of which only 40% of the patients had abnormal semen parameters. A huge proportion of male patients had normal semen parameters and their partners have the cause of infertility. The cases were divided into a few categories based on the condition. The OAT patients represent a major group followed by Oligozoospermia. The remaining categories such as Asthenozoospermia, Azoospermia and OA were present in equal measure. The causes of male infertility are represented in Figure 4.
4.3 Diagnosis of infertility

- Physical examination: All the female infertile patients were initially physically examined for weight changes, BMI, Thyroid, Hirsutism, Pelvic examination in women and scrotal volume, testes size, Penile length, urethral opening in men.
- Blood tests to measure the sex hormone levels, diabetes, cholesterol in appropriate cases.
- Life style habits like smoking, alcohol, drug abuse were also recorded.

4.3.1 Female infertility

4.3.1.1 PCOS Diagnosis - USG

PCOS is commonest reproductive endocrinopathy in women in the reproductive age with varying prevalence in different ethnicities. According to Rotterdam criteria, the diagnosis of PCOS is made when at least two of three following features are present (ESHRE, 2003).

i) Oligomenorrhoea or Anovulation

ii) Clinical or Biochemical hyperandrogenism

iii) Poly cystic ovaries –on Ultra sound
Other signs and symptoms may include Hirsutism, obesity, metabolic syndrome and Infertility.

Feature of Poly cystic ovary include (Wild et al, 2010)

- Presence of ≥12 follicles.
- Each follicle with similar size and measuring 2-9 mm in diameter.
- Peripheral distribution of follicles.
- Increase in ovarian volume (>10cc).
- Central stromal brightness.

4.3.1.2 Tubal patency test – HSG

Fallopian tubal assessment was carried out by Hysterosalpingogram (HSG). In recent times HSG, hystero salpingo contrast sonography and laparoscopy dye test are conventional methods for detecting tubal patency, each has its own advantages and disadvantages. HSG is easy, requires less time, safe and inexpensive. The sensitivity, specificity, positive and negative predictive values cannot be predicted in the present study due to the lack of tubal findings comparison with other tests in the present study. It is reliable for the detection of tubal blockage since it has 83% specificity and 65% sensitivity (Sutton et al, 1992; Chapman et al, 2001). HSG was done by the experienced gynecologist from the department of Reproductive medicine, Chettinad Hospital and Research Institute.

The test was performed on 7-10th day of menstruation and before ovulation to avoid the risk of radiation exposure to pregnancy. The procedure can visualize the size and shape of the uterine cavity, tubes, spillage of medium on both sides and tubal blocks with its sites (Shrivastava et al, 2009). The test can diagnose both uterine and tubal abnormalities. Several complications that may rarely associate with HSG are uterotubal distension, Infections in patients with history of pelvic inflammatory disease, Vasovagal reaction, venous intravasation and allergy to
contrast medium; which can be minimized with proper care and handling (Sutton et al, 1992; Chapman et al, 2001).

4.3.2 Male infertility

There are many factors associated with male infertility, most of them influence or impair the spermatogenesis directly or indirectly. The infertile male patients were originally recruited based on non conception and confirmed by semen analysis/parameters. No histological tests were performed, since these patients were all attending the IVF clinic and no invasive methods, biopsy can be adopted on ethical grounds. The WHO had altered the reference values of semen parameters five times thus far in the last 3 decades. The first four reports have similar semen parameters where as the last report in 2010 has many modifications which are listed below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Previous values</th>
<th>Present values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen volume (ml)</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>Sperm concentration (10^6/ml)</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Sperm motility (% progressive)</td>
<td>50</td>
<td>32</td>
</tr>
<tr>
<td>Sperm morphology (%)</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

4.3.2.1 Semen analysis

The diagnosis of male infertility was carried out by semen analysis. WHO, 2010 guidelines were used as standard for assessing male infertility. The quality of semen depends on mode of collection of semen sample, completeness of sample and abstinence period. A few critical steps involved in routine semen analysis are mentioned below.

- Keeping the semen container at room temperature for liquefaction
- Appearance of semen was recorded (Pale grey colour)
- Measuring the semen volume (1.5 ml)
- $p^H$ measurement (7.2)
- Sperm concentration (15 million/ml)
- Sperm motility (32% progressive motility)
- Sperm morphology (4% normal)
- At least two semen analyses are required to confirm male infertility.

4.4 Caste distribution among the study population

As mentioned earlier Tamilnadu has many castes isolating each other in breeding/marriages. It’s interesting to carry out the genetic studies in different castes where each caste has its own genetic pool (Pitchappan et al, 2002). In the present study, more than 70 castes were recorded among 752 subjects including both cases and controls. The major caste groups were presented in the Figure 5. As vanniyar population is high in and around Chennai, they represented a major portion in this study. Apart from that Mudaliyar and Adi dravidar communities were also enrolled in a considerable portion. The total list of castes is given in Appendix IX.

![Figure 5. Caste distribution (%) of study population](image-url)
4.5 Geographical distribution of Study population

Patients and controls were enrolled from the chettinad health city, Kelambakkam, Kanchipuram district, Tamilnadu. Hence it is located very close to Chennai city nearly 50% of the study population was from Chennai and Kanchipuram districts. Around 25% population was enrolled from Vellore, Thiruvannamalai, Thirunelveli, Cuddalore and Villupuram districts. Existence of many IT companies around the hospital helped in recruiting 5% of study population from the surrounding states Andhra Pradesh & Kerala. The remaining 20% population are recruited from different districts each sharing 1-2%.

![Geographical distribution of Study population](image)

Figure 6. Geographical distribution of Study population

4.6 Sample collection

4.6.1 Mouthwash collection

The participation rate in genetic association studies can be maximized by using non invasive methods for DNA extraction. Non invasive method like buccal
cell collection is preferred over blood for genetic testing. There are several methods for collecting buccal cells like cotton swabs, cyto brushes, mouthwashes etc. Different mouthwash solutions like saline, sucrose, water and a few commercial mouthwashes are available (Heath et al, 2001). DNA extracted from saliva may have high protein contamination and high fragmentation compared to blood but the genotyping results were comparable between both types of DNA (Abraham et al, 2012). Mouthwash samples were collected from each volunteer. Volunteers are advised not to drink tea, coffee or eat anything before one hour of sampling to decrease the cross contamination and increase the buccal epithelial cells. At room temperature, cells are stable at least for a week in mouthwash sample (Figelson et al, 2001). A 30 ml of reverse osmosis (RO) treated water was given in a plastic cup, asked to swish orally for one minute and spit it back to the same cup. Particles in the samples were allowed to settle down, 50 µl of 30% sodium azide was added to prevent the bacterial growth and transferred to 50 ml centrifuge tube.

4.6.2 DNA extraction from Mouthwash sample

The duration of oral swishing, tooth brushing and delay in DNA extraction after sample collection would change the quantity or quality of human DNA. The DNA yield could be optimized by collecting the samples before tooth brushing, swishing for one minute and processing for extraction within five days (Figelson et al, 2001). Though the DNA yield is relatively low, it is reliable for taqman assays, SNP testing and produced concordant results with blood (Abraham et al, 2012). Genomic DNA was extracted from all the mouthwash samples by salting out technique (Ausbel et al, 2002). Composition of the Lysis buffer and Tris EDTA buffers used for DNA extraction is given in (Appendix IV).

- The mouthwash samples collected in 50 ml tubes were spun at 2500 rpm for 10 min Supernatant was discarded and 2 ml of WCLB was added to the pellet.
- The pellet was gently tapped, suspended in WCLB and kept for overnight incubation at 42°C.
• One ml of 6M NaCl was added to the overnight incubated tube
• Vortexed for 10 sec and incubated on ice for 10 min
• After incubation, the tube was spun at 4000 rpm for 10 min.
• The supernatant was transferred to another 15 ml tube
• Add equal volume of absolute (100%) cold ethanol.
• Precipitate the DNA at 4000 rpm for 10 minutes.
• The supernatant was discarded and 1 ml of 70% ethanol was added to the DNA pellet,
• The suspension was transferred to a 1.5 ml tube and spun at 8000 rpm for 3 min.
• The pellet was washed twice with 1 ml of 70% ethanol at 8000 rpm for 3 min.
• Finally, the DNA pellet was allowed to air dry for 20 min.
• A 150 μl of Tris EDTA buffer was added and incubated at 42°C overnight.
• The dissolved DNA was stored at -20°C until further use.

4.6.3 DNA quantification

The purity and concentration of genomic DNA was quantified using UV-visible spectrophotometer (Shimadzu UV-1800, cat No.A11454805492). The Optical Density (OD) at 260 nm and 280 nm was measured from DNA (i.e., 1:10 times diluted from the stock). The DNA concentration was calculated as:

\[
\text{DNA concentration (µg/µl)} = \text{OD } 260 \times (\text{dilution factor} \times 50)
\]

The purity of the DNA was calculated as the ratio between OD 260 and OD 280. All the stock DNA was diluted to a concentration of 10 ng/µl and used for all the genotyping assays.
4.7 SNP Genotyping

4.7.1 Tetra Amplification-Refractory Mutation System PCR (ARMS PCR)

Extensive research was carried out in the last decade to develop the new technologies for SNP discovery and genotyping based on the need of studies. Nowadays there are many methods available ranging from low to high throughput SNP genotyping. Restriction Fragment Length Polymorphism (RFLP), Taqman assay, molecular beacons, Single Strand Conformation Polymorphism (SSCP) and Allele specific PCR are best available methods for low to moderate throughput studies (Kwok et al., 2003). Tetra Amplification refractory mutation system (ARMS) PCR, a new SNP genotyping method with low cost, less time and work has drawn attention in recent times (Ye et al., 2001), which is developed with minor adoptions to the previous technique (Newton et al., 1981). The genotype results obtained by ARMS PCR are 100% concordant to PCR-RFLP (Baris et al., 2010; Lajin et al., 2012) and sequencing (Ahlawat et al., 2014). These reports show the reliability of ARMS PCR in SNP genotyping.

The present study selected ARMS PCR method to assess the frequency of the genotype and allele frequencies. It is relatively easier to find the allele and genotype frequencies compared to other techniques such as taqman assay or RFLP where additional reagents, time and work is required after PCR. This method simply relies on the use of sequence-specific primers that allow the amplification of test DNA (Ye et al., 2001).

The method requires two set of primers; one set of outer primers and another set of inner primers, hence it is called Tetra ARMS PCR. Each set of primers are specific to each allele (wild and mutant). A second mismatch was given deliberately in each allele specific primer to increase its specificity (Ye et al., 2001). Primers used in this study were designed by using the bioinformatics tool like http://primer1.soton.ac.uk/primer1.html and evaluated for their specificity with NCBI blast. The primers were obtained from the Sigma Alhdrich Company and diluted according to the manufacturer’s instructions. The primers used in this study are listed in Table 8.
Table. 8. List of primers used for ARMS PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation site</th>
<th>Type of primer</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR β</td>
<td>+1730 G/A</td>
<td>IF</td>
<td>ACTGGCCCACAGAGGTCAAAA</td>
<td>A allele: 188</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IR</td>
<td>ACACTGGAGTTTCACGCTTCATCC</td>
<td>G allele: 110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OF</td>
<td>AGAGGACAGTAAAAGCAAGAGGGGC</td>
<td>Two outer primers: 254</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OR</td>
<td>GCCCTCTGCTAACAAGGAAACTAT</td>
<td></td>
</tr>
<tr>
<td>IL 1β</td>
<td>+3954 C/T</td>
<td>IF</td>
<td>AGCCTCGTTATCCCATGTGGCA</td>
<td>A allele: 183</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IR</td>
<td>GCTCCACATTCAGAAGCTATCTCTGTC</td>
<td>G allele: 118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OF</td>
<td>CACCAAGCTTTTGTGCTGTAGTCC</td>
<td>Two outer primers: 251</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OR</td>
<td>ACTCCAGCTTCATCCCTACTGGTG</td>
<td></td>
</tr>
</tbody>
</table>

All the amplicons were electrophoresed in 1% agarose gel prepared by 1X TAE buffer. The alleles are differentiated and assigned based on different amplicon sizes. The method was performed to genotype the ESR β +1730 G/A (rs4986938) and IL 1β+3953 C/T (rs1143634) polymorphisms.

Figure 7. Schematic representation of ARMS PCR method. The single nucleotide polymorphism used in this example is G to A substitution. (Adapted from Ye et al, 2001)
The figure illustrates “G” to “A” substitution where one inner primer corresponding to ancestral “G” allele and another to derived “A” allele. If the individual genotype is GG or AA (homozygous) only two bands are visualized, however three bands can be observed in case of “GA” (Heterozygous) (Ye et al, 2001).

4.7.2 Standardization of ARMS PCR

The better annealing temperature for each set of primers/ specific allele was determined by Gradient PCR. The annealing temperature for each allele varied making difficult to genotype in a single PCR. The ancestral and derived alleles were genotyped in separate reactions with allele specific primers i.e., Inner forward and outer reverse primers for ancestral allele; Inner reverse and outer forward primers for derived allele. However the conditions were optimized by primer balance and adjusting MgCl₂.

The PCR reaction was carried out in a final volume of 10 µl containing 50 ng of DNA, 1X PCR buffer, 0.3 µM each Primer, 0.2 mM dNTP and 0.5 U Taq DNA polymerase by using the applied bio systems thermal cycler.

Figure 8. ARMS PCR conditions
4.7.3 Agarose gel electrophoresis

It is one of the effective ways of separating DNA molecules of different sizes. To separate the amplicons, they were loaded into pre cast wells of 1.5% agarose gel placed in the 1X TBE buffer and power is applied. DNA is negatively charged and migrates from cathode to anode. Amplicons were separated based on their size and visualized with different banding pattern when the gel is placed on the UV lamp. The visibility is due to EtBr, an intercalating agent that stains DNA (Lee et al., 2012). The homozygous and heterozygous genotypes were validated with 100 kb ladder, positive and negative controls.

G allele – 188 bp  
A allele – 110 bp

Figure 9. Gel image of ESR β polymorphism

In ESR β +1730 G/A typing the ancestral A allele band was observed at 147 bp and derived allele band was observed in 294 bp respectively (Figure 9).
Similarly, for IL 1β +3953 C/T the ancestral ‘C’ allele band was observed in 118 bp and 183 bp for derived T allele respectively (Figure 10).

### 4.8 HLA DRB1 Genotyping

PCR - RFLP, PCR - sequence specific oligonucleotide (SSO) and PCR with sequence specific primers (PCR-SSP) are the conventional methods for HLA DRB1 genotyping. PCR SSP can be performed in two ways; low resolution typing does not require any specificity test since it is part of the amplification process and high resolution method requires microtitre plate hybridization following the previous typing. Low resolution typing can be performed within two hours including amplification and post amplification processing like gel documentation and data interpretation (Kawai et al., 1996). The results obtained from RFLP and SSO methods are completely concordant to low resolution SSP genotyping. The technique is rapid, inexpensive and reproducible. The accuracy of the method makes it more reliable for the disease association studies. The present study followed low resolution PCR SSP method and primers were adopted from the previous study (Olerup and Zetterquist, 1992). The sequence of the primers used for this assay is listed in Appendix V.

Each allele or a group of alleles have specific primer. A mismatch at the 3’ end of primer makes it more specific to detect the specific allele. An individual’s genotyping requires 20 PCR reactions of which 17 for HLA DRB1* alleles, 2 for HLA DRB3* and HLA DRB4* and the last is a negative control (ddH2O). Each reaction additionally contains internal control primers to validate the amplification process. A few alleles’ require more than two primers for their genotyping (Olerup and Zetterquist, 1992). The allele reaction pattern for the primer sets is described (Appendix VI). To make the process easy and reduce the assay set up time, allele specific primer mix were dotted in the 96 well plate i.e., 4 samples/plate (Figure 11).
Figure 11. Dotting of primers to a 96 well plate

PCR was carried out in Gene Amp 9700 thermo cycler (Applied Bio systems) using the different concentrations of reagents and temperature conditions presented in Table 9 and Figure 12 respectively.

Table 9. PCR reagents concentration for PCR- SSP

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock</th>
<th>Working</th>
<th>Vol/rxn (15 alleles)</th>
<th>Vol/rxn (3 alleles)</th>
<th>Vol/rxn (1 allele)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>50</td>
<td>10</td>
<td>Ng</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>PCR Buffer</td>
<td>10</td>
<td>1</td>
<td>X</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>dNTP</td>
<td>10</td>
<td>0.2</td>
<td>mM</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>5</td>
<td>0.5</td>
<td>U</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>CPrimer1</td>
<td>10</td>
<td>0.2</td>
<td>µM</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CPrimer2</td>
<td>10</td>
<td>0.2</td>
<td>µM</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Primer 1</td>
<td>10</td>
<td>1</td>
<td>µM</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Primer 2</td>
<td>10</td>
<td>1</td>
<td>µM</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Primer 3</td>
<td>10</td>
<td>1</td>
<td>µM</td>
<td>0</td>
<td>1.3</td>
</tr>
<tr>
<td>Primer 4</td>
<td>10</td>
<td>1</td>
<td>µM</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Final volume made up to 13 µL using double distilled water per reaction
4.9 Gel Documentation

A 1.5% agarose gel was prepared with 6 combs making 48 wells. At a time only two samples can be documented. To identify the different size bands, low electric charge was applied to run slowly. 5µl of each amplicon added to 3µl loading dye was loaded in each well. After 20-30 minutes, the gel was visualized and documented in UV gel documentation system. The gel picture of 19 HLA-DRB1* alleles tested for one individual is represented in Figure 13.
4.10 Interpretation of DRB SSP-PCR results

As mentioned earlier internal control primers were additionally added to allele specific primers in all reactions. The appearance of internal control band in all the wells ensures the amplification success. If both internal control and allele specific bands are visible, it is considered as the sample is positive for that specific allele. In spite of control band appearance, absence of allele specific band indicates the negativity of the sample for that particular allele. Finally, absence of both internal control band and allele specific band indicate PCR failure.

4.11 Quality control for HLA DRB typing

- Numerous studies have already validated the sensitivity and specificity of the primers used for HLA DRB1* genotyping.
- To validate/measure the quality control of PCR, 12 specific allele samples of the study were compared with a standard reference set of 12 specific allele samples developed using various International Histocompatibility Workshops.
- Alleles were assigned based on specific bands in each reaction and also considering the DRB3 and DRB4 allele status.
- A negative control was used where DNA is replaced with water.

4.12 Statistical Analysis

- Odds ratio (OR), 95% Confidence Interval for OR, Pearson Chi square, one tailed p value for alleles association was calculated using IBM Statistical Package for Social Sciences (IBM SPSS -Version 21). Yates correction was given wherever it was necessary.
- Meta analysis was performed using Comprehensive Meta Analysis (Version 3.3.070) accessed on line through www.meta-analysis.com.
- Hardy Weinberg Equilibrium (HWE) was calculated using online tool OEGE (Online Encyclopedia for Genetic Epidemiology studies).