Introduction

Minor mutations like deletion, duplication and substitution cannot be detected through routine cytogenetic studies. These may affect largely on pregnancy loss (Ghorbian *et al.,* 2012). The association between Y chromosome microdeletion and RPL was first reported by Dewan and co-workers in 2006. The human Y chromosome accounts for only 2% to 3% of the haploid genome and in human genome it is one of the smallest chromosome. Y chromosome harbors genes which are not vital for life. It has been long viewed that sex determination which is regulated by the SRY gene is a sole function of Y chromosome but in recent years other function like control of spermatogenesis and several genes are mapped on Y chromosome (Foresta *et al.,* 2005). It encompasses MSY (male specific region of the Y) which do not recombine with X chromosome and pseudoautosomal regions PAR1 and PAR2 which exhibits meiotic exchange with X chromosome. The MSY region alone encompasses 23Mb containing 156 transcriptional units including 78 protein coding genes which codes for 27 distinct proteins (Skaletsky *et al*., 2003; Simoni *et al*., 2004). The association between Y chromosome microdeletion and male infertility was first hypothesized by Tiepolo and Zuffardi in the year 1976. They observed microdeletion in the euchromatic region of the long arm of Y chromosome (Yq11) through routine karyotyping and named as azoospermia factor (AZF) since they identified all the deletions in the azoospermic subjects (Tiepolo and Zuffardi, 1976). Later in the year 1996 AZF region has been sub grouped into 3 regions AZFa, AZFb and AZFc in the proximal, central and distal segment of Yq11 respectively by Vogt *et al.,* (1996). The palindromic map and the genes

situated on Yq region is depicted in Figure 4.1 and 4.2. Foresta with his colleagues mapped many genes within AZF regions which controls spermatogenesis (Foresta *et al.,* 2001).

The first region AZFa located in the deletion interval 5 spanning ~800kb (Sun *et al.,* 1999) and harbors several single copy genes like *USP9Y* (ubiquitin specific protease 9, Y chromosome) which was formerly known as *DFFRY* gene, *DBY* (dead box on the Y) (Lang and Page, 1997) and *UTY* (ubiquitous TPR motif on the Y) (Mazeyrat *et al.,* 1998). *USP9Y* and *DBY* are essential for the germ cell development by coding proteins which are vital for testis specificity and post translation regulators (Sun *et al.,* 1999). *UTY* is ubiquitously expressed. Complete deletion of AZFa deletion leads to SCO (Sertoli cell only) syndrome (Kamp *et al.,* 2001).

The AZFb region encompasses 32 genes and overlaps with AZFc region (Repping *et al.,* 2002). *RBMY* (RNA-binding motif on the Y) gene which was formerly known as *YRRM* (Y specific RNA recognition motif) the protein coding gene which is located in this region. *RBMY* plays a vital role in spermatogenesis process by coding germ cell-specific nuclear protein (Shinka and Nakahori, 1996; Chai *et al.,* 1998; Vogt 2005) and regulates alternate splicing by coding heterogeneous nuclear ribonucleoproteins (Heinrich *et al.,* 2009; Ostareck-Lederer and Ostareck, 2012). It is estimated to have 30-40 copies of *RBMY* gene family and majority are pseudogenes and only two *RBMY1* and *RBMY2* are transcribed (Ma *et al.,* 1993). Mutation in this gene results in germ cell arrest in the meiotic I there by leading to azoospermia or severe oligospermia (Reijo *et al.,* 1995; Belangero *et al.,* 2009; Foresta *et al.,* 2001).

AZFc region is located in the distal of Yq11 with molecular extension of 500kb (Reijo *et al.,* 1995). It contains a cluster of *DAZ* gene (which was formerly known as SPGY), two copies of *PRY* (PTP-BL Related Y), *BPY2* (Basic Protein Y2) and copies of *CDY* (Chromodomain Y) and *RBM* (RNA-binding motif). The function of *BPY2* and *PRY* has not been determined but they are Y specific since they are expressed in the testis alone (Reijo *et al.,* 1995; Lahn and Page, 1997). It is a strong candidate gene which codes for RNA binding protein (Mulhall *et al.,* 1997; Meschede and Horst, 1997; Ferlin *et al.,* 2007). Deletion of this region in few men results in hypospermatogenesis which causes azoospermia or severe oligospermia (Pryor *et al.,* 1997; Simoni *et al.,* 1997; Mulhall *et al.,* 1997; Ferlin *et al.,* 2005).

Y chromosomal deletions are caused due to homologous recombination between the identical parts of the palindromic sequences (Kuroda-Kawaguchi *et al.,* 2001; Repping *et al.,* 2003; Skaletsky *et al.,* 2003). Recently few studies have reported the partial deletions within the AZFc region which results in the smaller gene and transcription unit deletion (de Vries *et al.,* 2002; Ferlin *et al.,* 2002, 2004; Fernandes *et al.,* 2002, 2004; Repping *et al.,* 2003, 2004). The map of the AZFc region is depicted in Figure 4.3. Whole AZFc region which is also referred as b2/b4 deletion will be deleted when there is a recombination between b2 and b4 amplicons which spans about 3.5 Mb (Reijo *et al.,* 1995; Kuroda-Kawaguchi *et al.,* 2001). From the FISH, Southern blotting studies and SNV (single nucleotide variants) analysis, it has been reported that even partial *DAZ* deletions ends in spermatogenic impairment (Moro *et al.,* 2000; Bienvenu *et al.,* 2001; Ferlin *et al.,* 2002; Fernandes *et al.,* 2002; de Vries *et al.,* 2002 a, b; Ferlin *et al.,* 2004).

In the male partners of RPL couples, 82% had one microdeletion and 65% had three or more Y chromosomal microdeletion (Dewan *et al.,* 2006). But this study has criticized because of several pitfalls like experimental design, low sample size and result interpretation (Noordam *et al.,* 2006). The other study also reported microdeletions but it has been criticized because all these are not true deletion this result may be due to polymorphisms or methodological mistakes (Karaer *et al.,* 2008). Using STR markers 13.3% of the microdeletions were observed among RPL males (Soleimanian *et al.,* 2013). Couple of studies did not reported any association between RPL and Y microdeletions (Wettasinghe *et al.,* and Koc *et al.,* 2010). In all the studies the sample size was not more than 80, the STS used were not according to the guidelines and all the data have not revealed the details of the semen. Based on these reports to overcome the flaws and pitfalls the present study was undertaken.

This chapter deals with the investigation of classical AZF deletion along with the AZFc partial deletion with the sample size of 150, 100 subjects and 50 controls. The STS's were selected according to the EAA/ EMQN guidelines (European Academy of Andrology and European Molecular Genetics Quality Network). For AZFc sub deletions markers were selected according to Repping *et al.,* (2004). This result was co-related with the semen parameters and function test.

Materials and methods

Out of 200 male partners of RPL females 100 male partners with basic semen and sperm functional abnormalities were recruited as a subject group. As a control group, 50 males with proven fertility and normal semen profile were recruited. From both the group two milliliter of intra venous blood was anti-coagulated with EDTA and stored at 4^0C until DNA isolation was performed.

Isolation of genomic DNA:

QIAamp DNA blood mini kit (Qiagen, Netherlands) was used for genomic DNA extraction and the protocol is as follows

- Stored blood samples were equilibrated to room temperature.
- To 1.5ml microcentrifuge tube 20µl of QIAGEN protease was pipetted.
- 200 µl of equilibrated whole blood was added.
- 200 µl of Lysis buffer (AL) was added to the blood samples and mixed by vortexing for 15 seconds.
- Above mixture was incubated for 10 minutes at 56^0C using water bath.
- Briefly centrifuged to remove drops from the inside of the lid.
- 200 µl of ethanol was added to the sample and mixed by vortexing for 15 seconds then briefly centrifuged to remove drops from the inside of the lid.

- Mixture was carefully transferred to the QIAamp Mini spin column without wetting the rim. Centrifuged at 8000rpm for a minute, tube containing filtrate was discarded and QIAamp Mini spin column was placed on to a fresh 2ml collection tube.
- 500 µl of wash buffer (AW1) was added to the QIAamp Mini spin column carefully without wetting the rim. Centrifuged at 8000 rpm for 1 minute, column was placed on to a clean 2ml collection tube and the tube containing filtrate was discarded.
- 500 µl of wash buffer (AW2) was added to the QIAamp Mini spin column carefully without wetting the rim. Centrifuged at 14000 rpm for 3 minutes.
- Column was placed on to a clean 1.5ml centrifuge tube and 200µl of Elusion buffer (AE) was added and incubated at room temperature for 1 minute and then centrifuged at 8000rpm for a minute. The tubes are stored at -20^{0}C for further use.

Quantification of DNA:

The isolated DNA was quantified using spectrophotometer. The absorbance of each sample was read at 260nm. The purity of the DNA was measured using absorption 260:280 ratio where the purity of the sample was around 1.8-2.0. Based on the concentration, all DNA samples were diluted to 100-200ng/µl using Tris-EDTA buffer for further PCR analysis.

PCR:

Analysis of microdeletion in the Yq AZF region of Y chromosome was performed employing STS markers. The markers were selected to target the candidate genes of AZFa, b and c region. The STS markers were selected according the guidelines of European Academy of Andrology (EAA) and European Molecular Genetics Quality Network (EMQN) (Simoni *et al.,* 2004). For *USP9Y* gene of AZFa region the STS employed were sY84 and sY86; sY127 and sY134 for AZFb targeting *RBMY* gene; sY254 and sY255 for *DAZ* gene of AZFc region. In addition to these markers AZFc sub deletion analysis was performed by selecting the STS like sY1291, sY1191 and sY1197 which has been recommended by Repping *et al.,* (2002). The STSs which are employed in the present study has been listed in Table 4.1.

Table 4.1: Sequence of different sets of primers which are employed in the present study

STS	Region/ Subregions	PCR product size (bp)	Primer's	Accession no.	Reference
sY84-F sY84-R	AZFa	326	AGAAGGGTCTGAAAGCAGGT GCCTACTACCTGGAGGCTTC	G12019	Simoni *et al.,* (2004)
sY86-F sY86-R	AZFa	320	GTGACACACAGACTATGCTTC ACACACAGAGGGACAACCCT	G49207	Simoni *et al.,* (2004)
sY127-F sY127-R	AZFb	274	GGCTCACAAACGAAAAGAAA CTGCAGGCAGTAATAAGGGA	G11998	Simoni *et al.,* (2004)
sY134-F sY134-R	AZFb	301	GTCTGCCTCACCATAAAACG ACCACTGCCAAAACTTTCAA	G12001	Simoni *et al.,* (2004)
sY254-F sY254-R	AZFc	380	GGGTGTTACCAGAAGGCAAA GAACCGTATCTACCAAAGCAGC	G38349	Simoni *et al.,* (2004)
sY255-F sY255-R	AZFc	120	GTTACAGGATTCGGCGTGAT CTCGTCATGTGCAGCCAC	G65827	Simoni *et al.,* (2004)
sY1191-F sY1191-R	b2/b3	385	CCAGACGTTCTACCCTTTCG GAGCCGAGATCCAGTTACCA	G73809	Repping *et al.,* (2002)
sY1197-F sY1197-R	b1/b3	453	TCATTTGTGTCCTTCTCTTGGA CTAAGCCAGGAACTTGCCAC	G67168	Repping *et al.,* (2002)
sY1291-F sY1291-R	gr/gr	527	TAAAAGGCAGAACTGCCAGG GGGAGAAAAGTTCTGCAACG	G72340	Repping *et al.,* (2002)

PCR was performed in a monoplex fashion for each primer set in the 25µl reaction volume which contains primers, dNTPs, assay buffer, genomic DNA and Taq polymerase. The composition and the volume of components which are used for single PCR mixture are provided in Table 4.2.

Table 4.2: PCR reaction mixture used in the present study

Reagents	Volume
Master mix	12µl
Forward primer	0.50 µl
Reverse primer	0.50 µl
Genomic DNA	2.0 µl
Triple distilled water	9.71 µl
BSA solution	0.30 µl

Amplification conditions were started with an initial activation step at 94^0C for 4 minutes followed by 30 cycles of denaturation at 94^0C for 30s, annealing at 58^0C (sY86, sY127), 58.2^0C for (sY254, sY255 and sY1197), 57^0C for (sY84 and sY134) and 60.4^0C (sY1291 and sY1191) for 30 seconds, extension at 72^0C for 30 seconds and final elongation for 5 minutes at 72^0C. The PCR was carried out using Corbett thermo cycler.

PCR product confirmation:

The PCR products are confirmed through ethidium bromide stained agarose gel electrophoresis and photographed under ultraviolet light. Two percent of agarose gel was prepared by dissolving 2g of agarose dissolving in 1X TAE buffer (Promega USA). This mixture was heated in microwave till the appearance of clear solution. After cooling ethidium bromide (SRL India) solution was added in the concentration of 0.5µg/ml and transferred into the casting tray. The gel was allowed to polymerize at room temperature for 20-30 minutes. One µl of gel loading dye bromo phenol blue was added to the 25 µl PCR

amplified product and centrifuged at 1000rpm for 10 seconds to get a uniform mixture. These mixtures were loaded into the wells of the gel. 100bp DNA ladder (Merk Millipore GeNeI, India) of known concentration was used as a marker to confirm the specific amplification. Positive and negative controls were run simultaneously with samples. Genomic DNA samples of proven fertile male and female were used as positive and negative controls. Blank was run by using distilled water instead of genomic DNA. The samples were electrophoresed at 75V for 40 minutes the results were documented using UV trans illuminator.

Deletion analysis:

The deletion of complete AZFa region was observed by the absence for the amplification of markers sY84 and sY86, absence of marker sY127 and sY134 indicates the deletion of AZFb region and the AZFc region deletion was observed by the absence of marker sY254 and sY255. The gr/gr deletion was screened by the absence of marker sY1291. The b2/b3 deletion was detected by absence of sY1191 and the absence of sY1197 indicates only b1/b3 deletion in the subjects.

Statistical analysis:

The obtained data was subjected to statistical analysis using the software SPSS version 20. Chi-square test was performed between subjects and control and considered as significant when p value < 0.05.

Results:

Out of 200 hundred male partners of the female with RPL 100 males were selected for the deletion analysis based on the abnormal semen profile. Of 100 males, deletions were reported in 7 males of RPL couples. None of the control group had deletions for any of the selected markers. Table 4.3 depicts the frequency of the deletions observed in both RPL and control group. No individual showed deletion in AZFa region for the selected markers sY84 and sY86 (Figure 4.4 and 4.5). For AZFb region no deletions were reported for the markers sY127 and sY134 (Figure 4.6 and 4.7). The markers of AZFc region sY245 and sY255 did not show any deletions in none of the individual in any of the group (Figure 4.8 and 4.9).

AZFc subdeletions were reported in 7 males of 100 RPL couples. The frequency of b2/b3 and gr/gr deletion was equally observed. gr/gr deletion alone was identified among two individuals and in another two cases it was associated with b1/b3 and b2/b3;b1/b3 (Figure 4.10). Similarly isolated b2/b3 deletion was demonstrated among three subjects and in another case, it was associated with gr/gr; b1/b3 (Figure 4.11). Isolated b1/b3 deletion was not observed but it was associated with b2/b3 and gr/gr (Figure 4.12). Details of the semen profile of the individuals observed with deletions are depicted in Table 4.4. The phenotype of case 17 with deletion in all sub regions sY1191 (b2/b3), sY1291 (gr/gr) and sY1197(b1/b3) was oligospermic and had lesser scores for AIT, while the case 65 and 78 with deletion for sY1291(gr/gr) had lesser score for both the sperm function tests NCD and HOS, where as the case 9, 54 and 56 with deletion in sY1191(b3/b3) had lesser scores for HOS test, sperm count and motility respectively, case 13 with deletion for sY1291 (gr/gr)

and sY1197 (b1/b3) has abnormal score for NCD test and AIT. No significant association between sub normal scores for semen parameters and AZFc subdeletions.

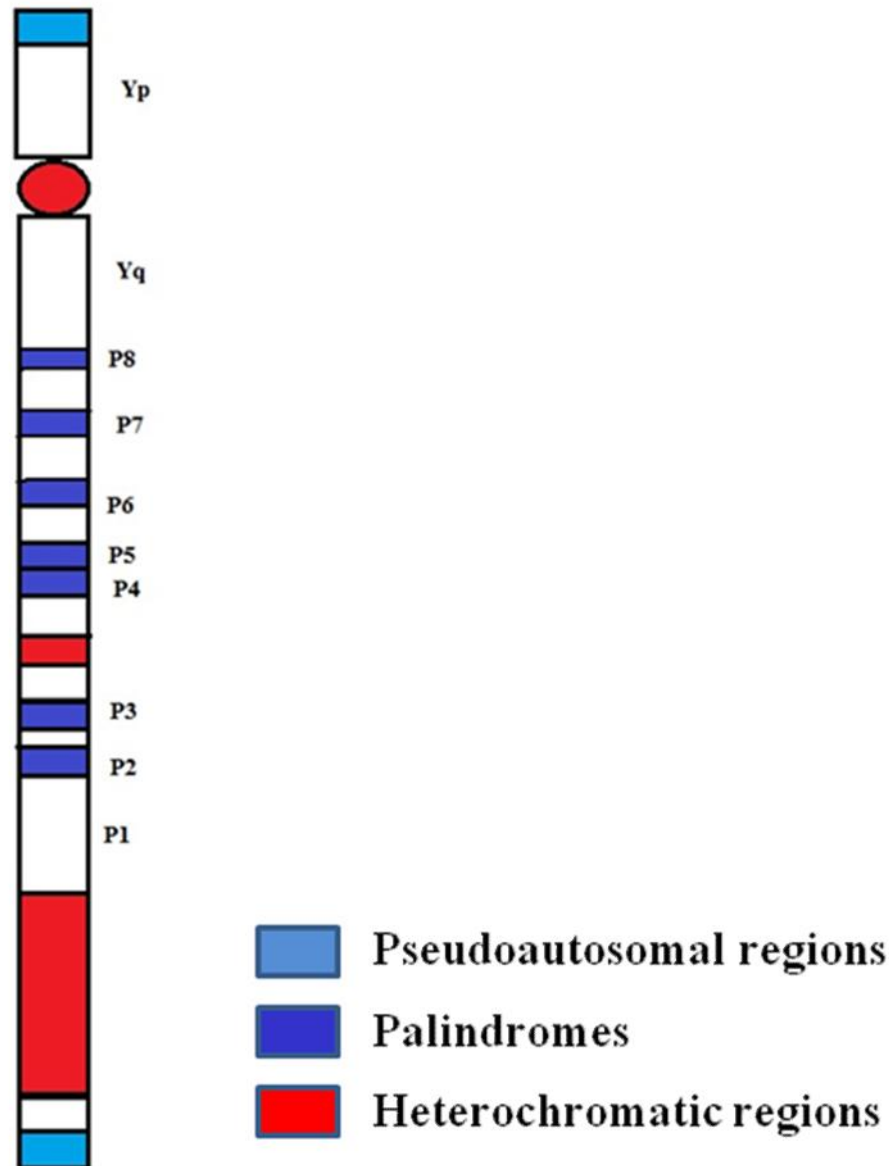

Figure 4.1: Schematic representation of Y chromosome depicting PAR's (Pseudoautosomal regions), Palindromes and heterochromatic region.

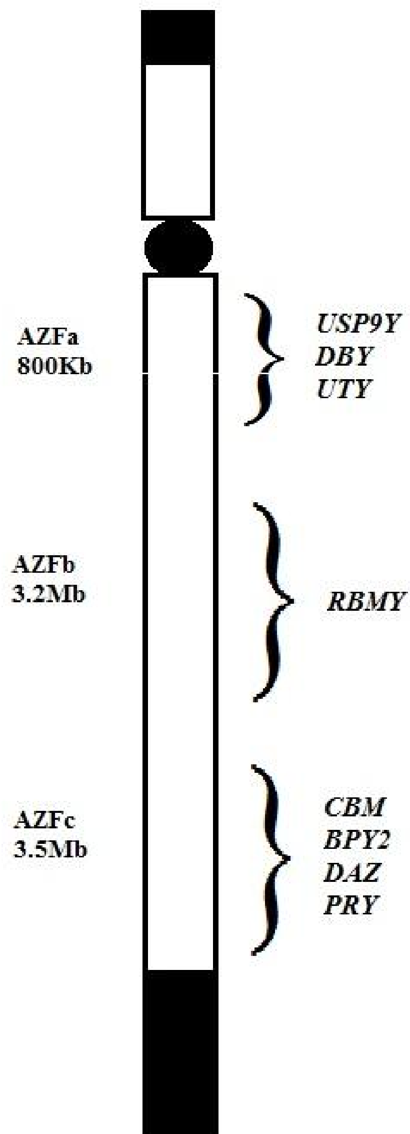

Figure 4.2: Schematic display of major genes on the Yq which are associated with male infertility

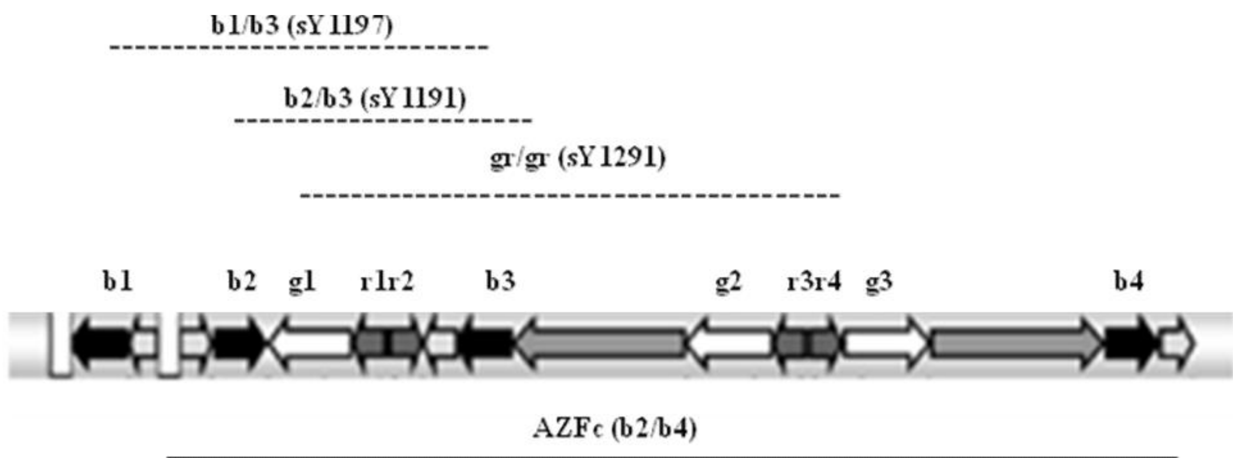

Figure 4.3: Map of the AZFc region on long arm of the Y chromosome

Table 4.3: Frequency of different types of deletions in RPL groups and control

STS	**RPL** **N=100**	**Control** **N=50**
AZFa (sY84 and sY86)	-	-
AZFb (sY127 and sY134)	-	-
AZFc (sY254 and sY255)	-	-
sY1291 alone	2	-
sY1191 alone (b2/b3)	3	-
sY1197 alone	-	-
sY1291 & sY1191	-	-
sY1291 & sY1197	1	-
sY1191 & sY1197	-	-
sY1291, sY1191 & sY1197	1	-
Total	**7 (7%)** **($p>0.05$)**	-

Table 4.4: Semen profile along with sperm function test values among the subjects with AZFc sub deletions.

Sample ID	**Age**	**No. of pregnancy losses**	**Subregions**	**Deleted markers**	**Volume (ml)**	**Sperm concentration (10^6/ml)**	**Total motility**	**NCD**	**HOS**	**AIT**
9	40	2	b2/b3	sY1191	3.3	34	65	70	50*	55
13	31	2	gr/gr, b1/b3	sY1291, sY1197	1*	20	65	60*	80	40*
17	32	2	gr/gr, b2/b3, b1/b3	sY1291,sY1191, sY1197	3	13*	50	70	70	20*
54	24	2	b2/b3	sY1191	0.7*	14*	32*	80	80	45*
56	32	2	b2/b3	sY1191	2	31	55	70	60	50
65	31	2	gr/gr	sY1291	3	19	45	30*	50*	60
78	39	3	gr/gr	sY1291	1.5	36	50	50*	40*	60

*indicates lower reference value than the WHO guidelines

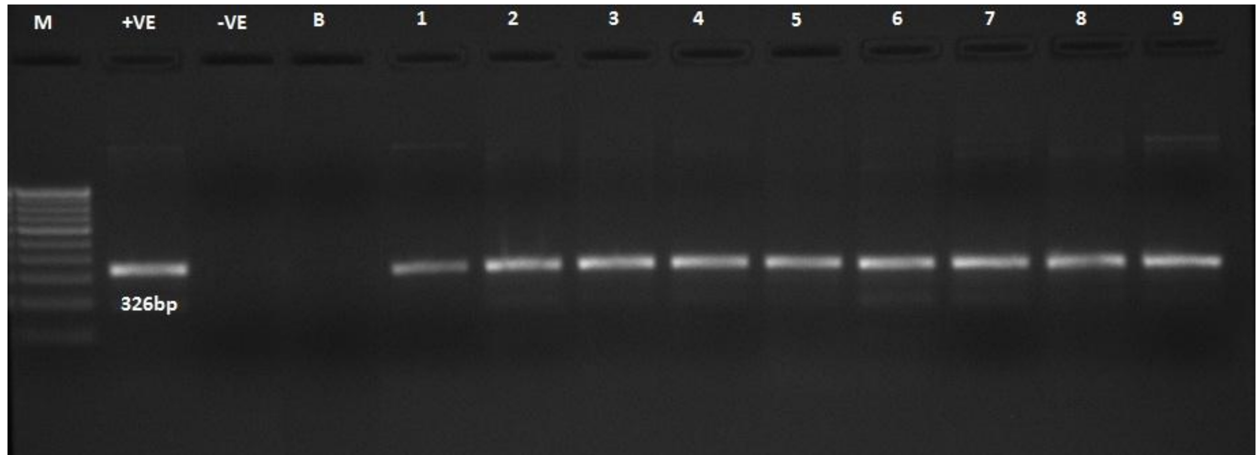

Figure 4.4: PCR of sequence tagged sites (STS) marker sY84 to screen deletion in AZFa region. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 1-9 showing PCR amplified product for the STS marker sY84 shows absence of deletions.

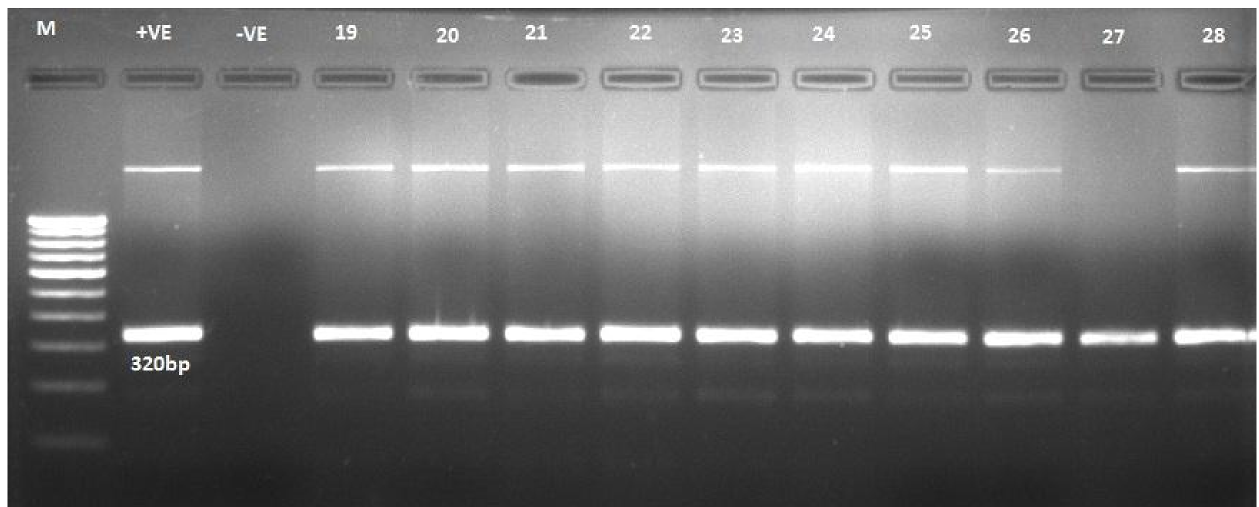

Figure 4.5: PCR of sequence tagged sites (STS) marker sY86 to screen deletion in AZFa region. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, 19-28 showing PCR amplified product for the STS marker sY86 shows absence of deletions.

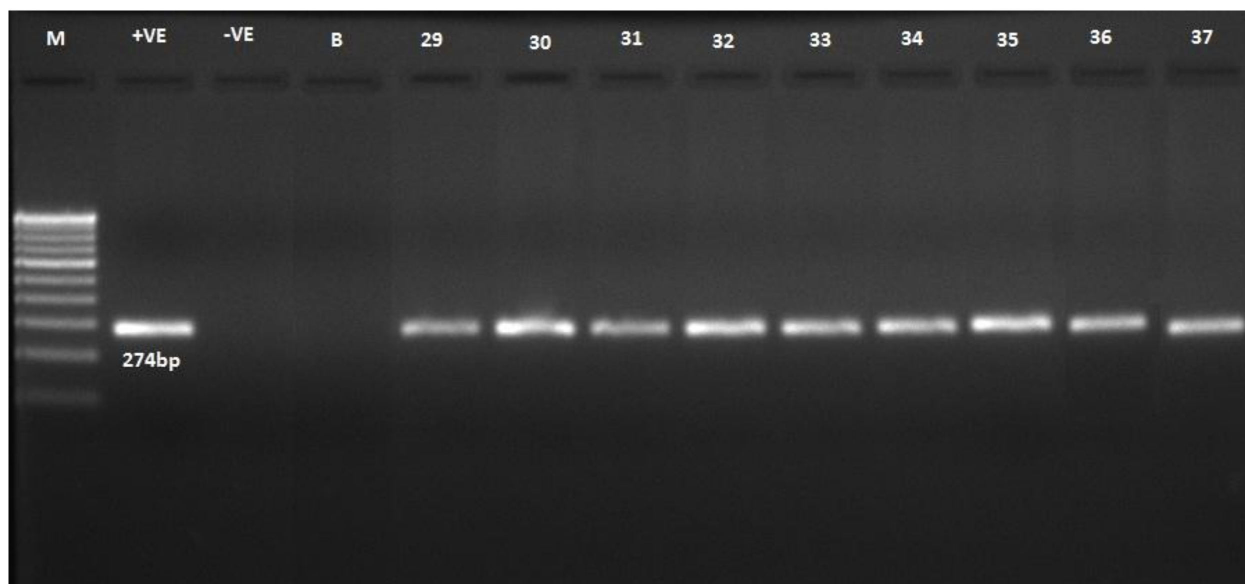

Figure 4.6: PCR of sequence tagged sites (STS) marker sY127 to screen deletion in AZFb region. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 29-37 showing PCR amplified product for the STS marker sY127 shows absence of deletions.

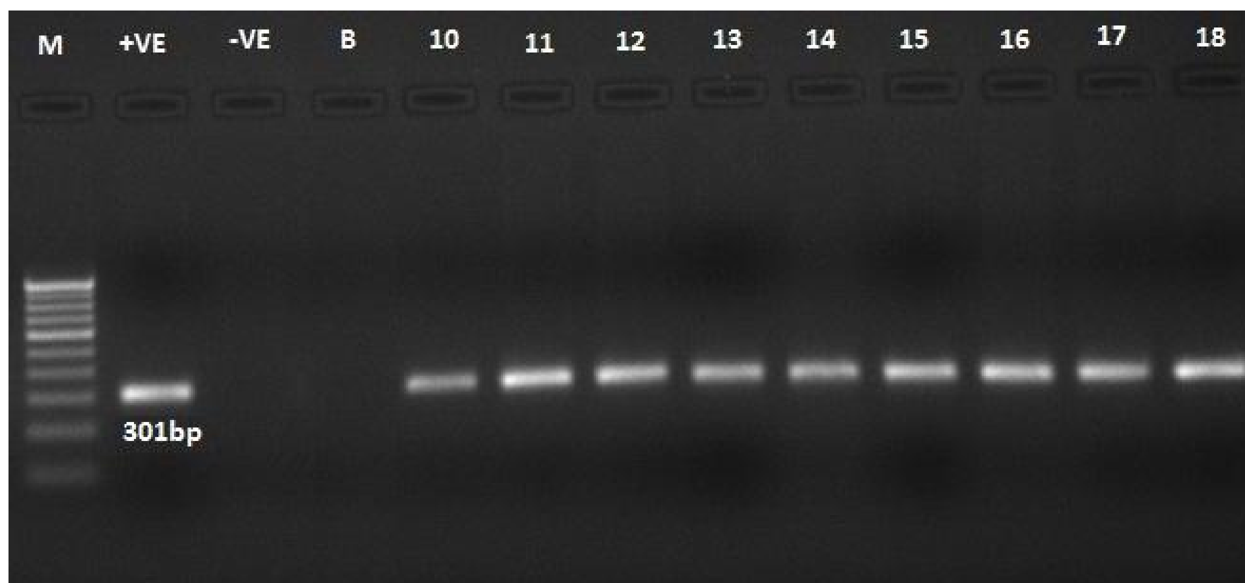

Figure 4.7: PCR of sequence tagged sites (STS) marker sY134 to screen deletion in AZFb region. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 10-18 showing PCR amplified product for the STS marker sY134 shows absence of deletions.

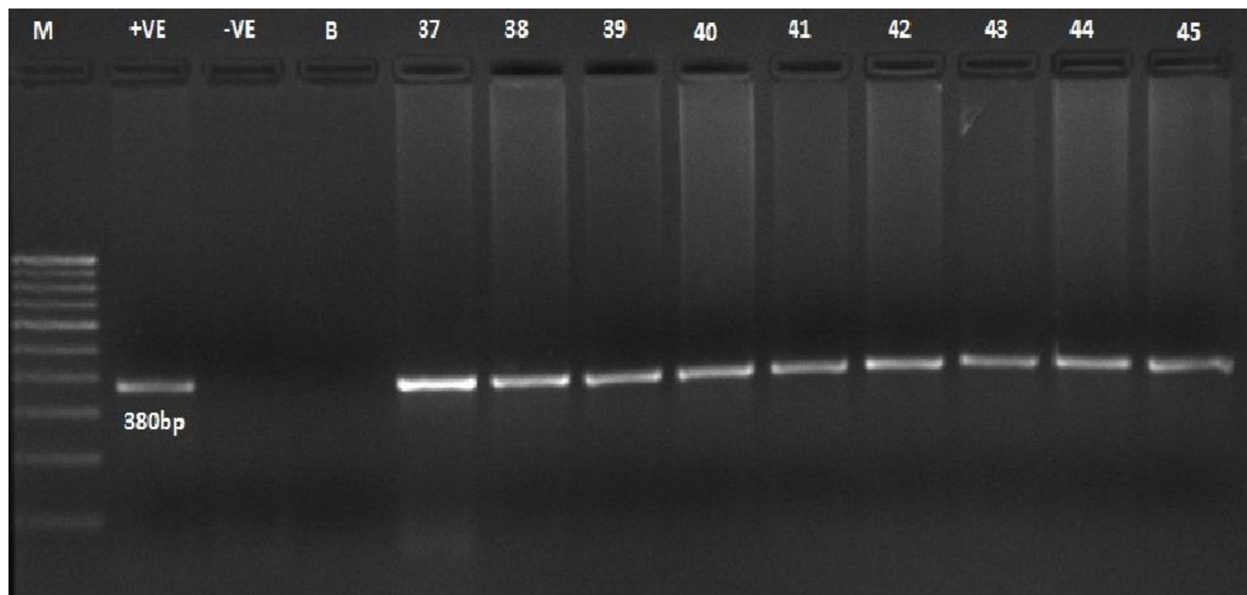

Figure 4.8: PCR of sequence tagged sites (STS) marker sY254 to screen deletion in AZFc region. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 37-45 showing PCR amplified product for the STS marker sY254 shows absence of deletions.

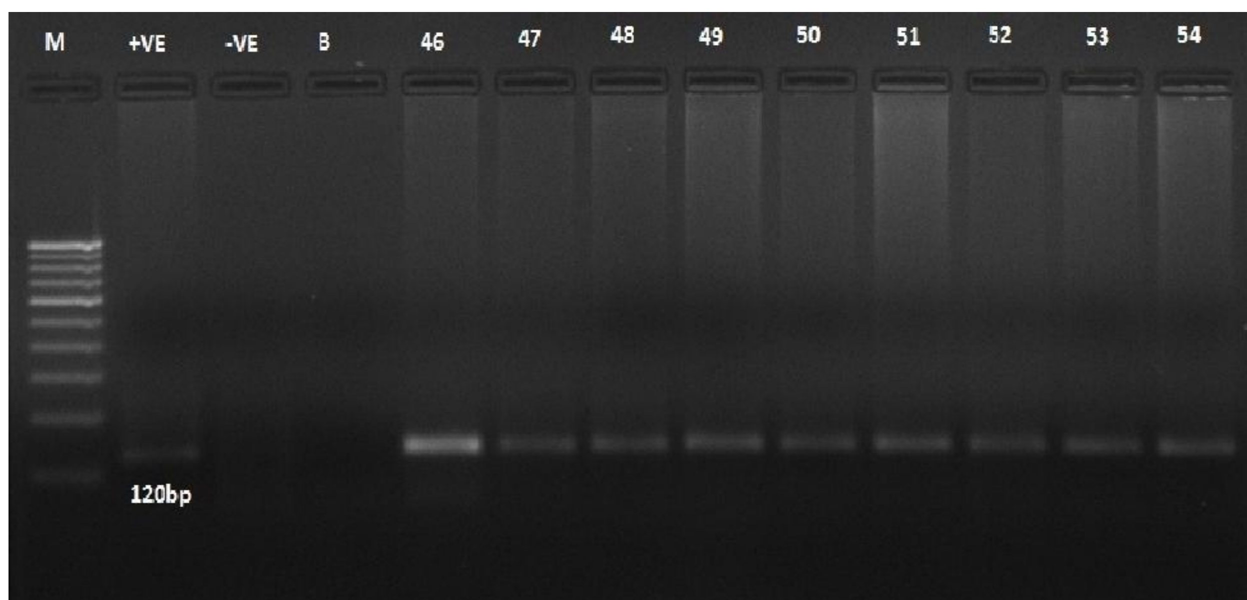

Figure 4.9: PCR of sequence tagged sites (STS) marker sY255 to screen deletion in AZFc region. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 46-54 showing PCR amplified product for the STS marker sY255 shows absence of deletions.

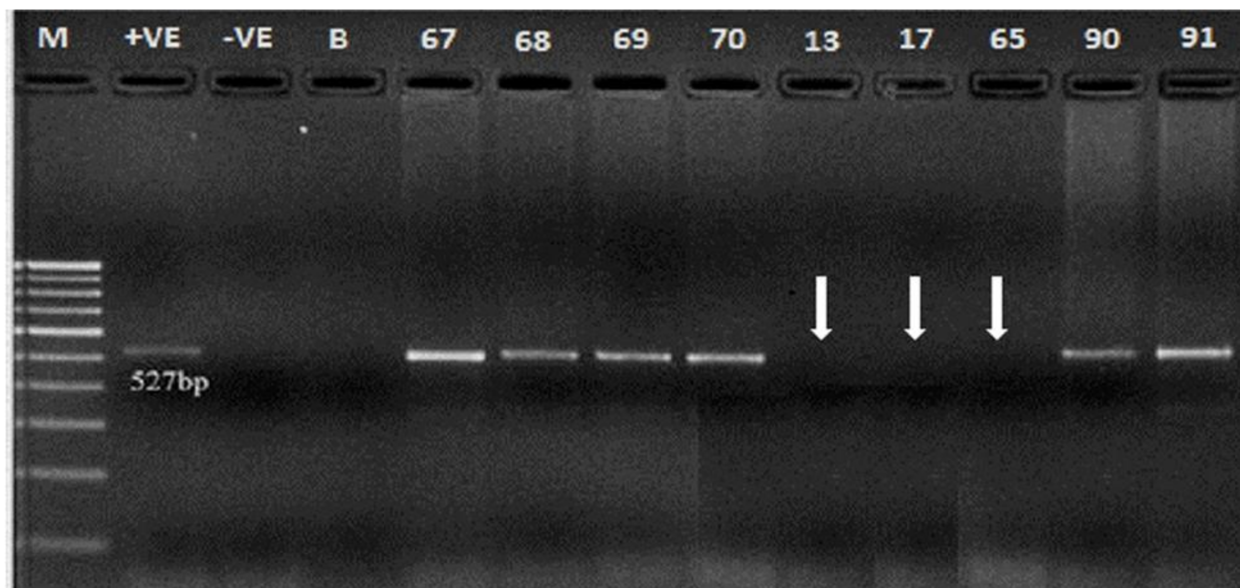

Figure 4.10: PCR of sequence tagged sites (STS) marker sY1291 to screen partial deletion in AZFc region for gr/gr. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 67-91 showing PCR amplified product for the STS marker sY1291 shows absence of deletions. 13, 17 and 65 shows the presence of deletion without showing any amplification for the specific STS marker.

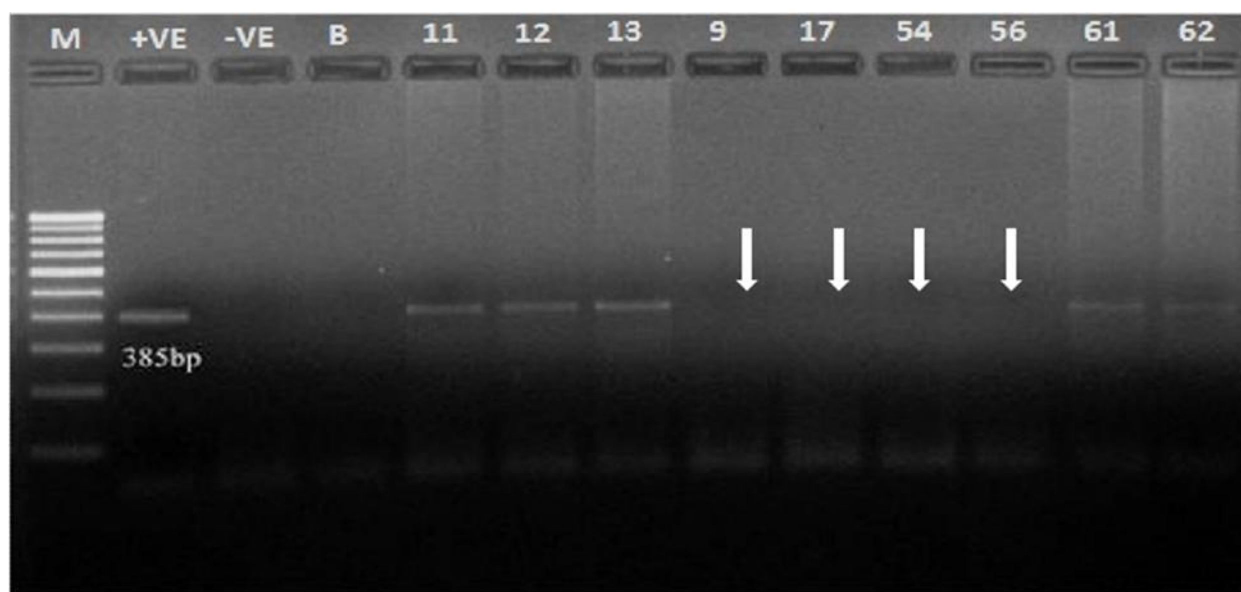

Figure 4.11: PCR of sequence tagged sites (STS) marker sY1191 to screen partial deletion in AZFc region for b2/b3. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 11-62 showing PCR amplified product for the STS marker sY1191 shows absence of deletions. 9, 17, 54 and 56 shows the presence of deletion without showing any amplification for the specific STS marker.

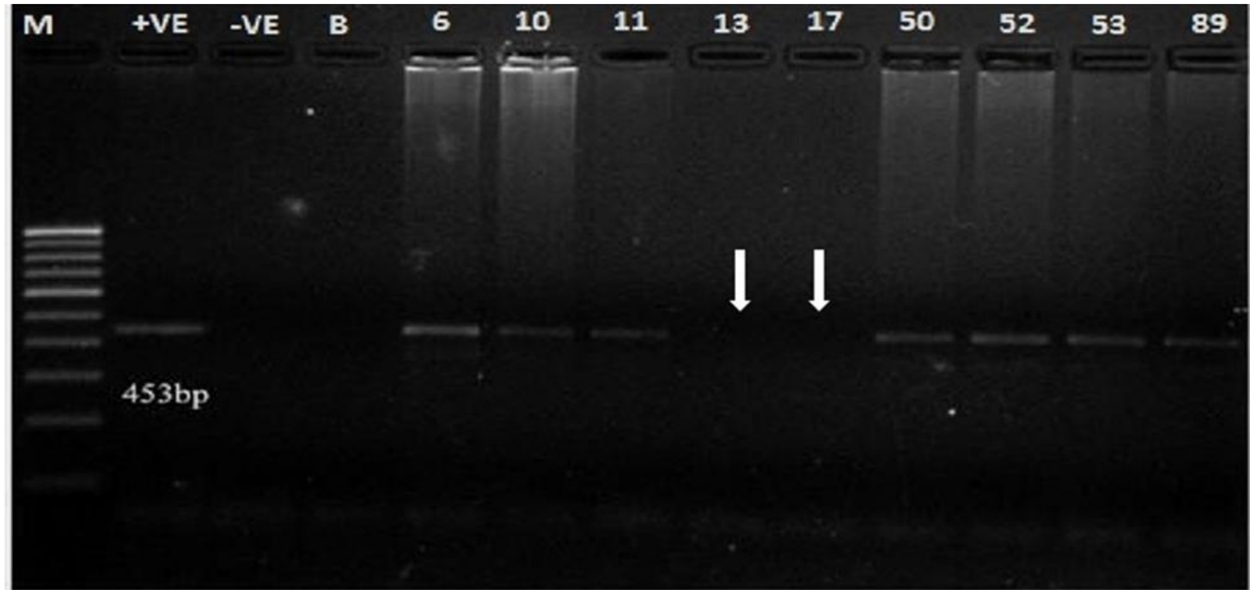

Figure 4.12: PCR of sequence tagged sites (STS) marker sY1197 to screen partial deletion in AZFc region for b1/b3. Lane M: 100bp DNA ladder, +ve: fertile men, -ve: female genomic DNA, B: Blank (double distilled water), 6-89 showing PCR amplified product for the STS marker sY1191 shows absence of deletions. 13 and 17 shows the presence of deletion without showing any amplification for the specific STS marker.

Discussion:

Etiologies behind RPL in 50% of the remains obscure and the investigations are focused on female partners alone. The male partner investigation is restricted to cytogenetic analysis alone. The most frequently observed structural abnormalities of Y chromosome are deletions in the male specific region of Y chromosome which ends in fertility complications. From past three decades researchers have demonstrated the role of Y chromosome on spermatogenesis. The frequency of AZFc deletions are more frequently observed type than AZFa and AZFb (Vogt *et al.,* 1997; Roberts, 1998). These have been extensively studied among infertile males. The first report to study the association between RPL and Y chromosome deletion was demonstrated by Dewan *et al.,* (2006). Later few studies are available demonstrating both positive and negative association. The present study demonstrated the positive association of Y chromosome microdeletions and RPL.

The deletions in the *DBY* of AZFa region among male partners of the RPL couples were reported only a single study (Said *et al.,* 2013). Deletion in the complete AZFa region is a result of homologous recombination between the identical sequence blocks (Blanco *et al.,* 2000; Kamp *et al.,* 2000; Sun *et al.,* 2000). The deletion of *DBY* is deleted in the infertile males which are having sever spermatogenic impairment which leads to azoospermia condition and sertoli cell only syndrome (Vogt *et al.,* 1996; Krausz *et al.,* 2000; Foresta *et al.,* 2000; Kamp *et al.,* 2001; Kleiman *et al.,* 2001; Hopps *et al.,* 2003). But the study conducted by Said and co-workers have not revealed the semen profile of the individual with the deletion of *DBY* (Said *et al.,* 2013). In male partners of the female with RPL will not show the semen profile of azoospermia and SCO I condition. In the present

study, no individual showed deletion in AZFa region for the selected markers sY84 and sY86 (Figure 4. 2 and 4.3).

The deletion in the AZFb is due to the homologous recombination between the palindromes P5 and P1 (Repping *et al.,* 2002). Gene that is present in this region *RBMY* are expressed specifically in testis and germ cells. The result of the deletion in this region is identical to the AZFa deletion like SCO or spermatogenic failure resulting in azoospermia (Simoni *et al.,* 2004). Deletion of this region with respect to male partner of RPL couples was reported in couple of studies (Karaer *et al.,* 2008 and Said *et al.,* 2013). Karaer and colleagues reported AZFb deletion in 7 (16.3%) out of 43 and the marker employed was DYS220 (Karaer *et al.,* 2008). In this study the marker chosen is not according to the guidelines and even this report do not give any information regarding the semen profile. Said and co-workers performed the real time PCR and they observed deletion in one subject off 40 individuals for RBMY gene (Said *et al.,* 2013). Even this study has not focused on the semen report and they have not quoted the STS marker which has been employed for the study. In the present study, out of 100 individuals none of them exhibited AZFb deletion for STS sY127 and sY134 (Figure 4.4 and 4.5) which accords with the previous studies where they employed the markers according to EAA/EMQN guidelines (Koc *et al.,* 2010 and Wettasinghe *et al.,* 2010).

Frequently deletions are observed in AZFc region followed by AZFb and AZFa (Layman, 2002). Complete AZFc deletion is due to the homologous recombination in the palindromes P3 and P1 between the amplicons of b2 and b4 (Kuroda-Kawaguchi *et al.,*

2001). Deletions in the genes of AZFc are associated with wide range of phenotype from hypospermatogenesis to SCO II syndrome. Positive association of b2/b4 deletions in male partners of RPL couples were reported by few researchers (Dewan *et al.,* 2006; Karaer *et al.,* 2008; Soleimanian *et al.,* 2012; Said *et al.,* 2013). But the results of Dewan and Karaer group were criticized because of the flaws in their experimental design and STSs employed for the study. None of the test subject involved in this study demonstrated complete b2/b4 deletion (Figure 4.6 and 4.7).

Studies have reported even partial deletions in AZFc deletion might ends in spermatogenic alterations (Ferlin *et al.,* 2002; Fernandes *et al.,* 2002; de Vries *et al.,* 2002a &b; Ferlin *et al.,* 2004). Several studies on different populations are available with respect to AZFc partial deletions and infertile males (Lynch *et al.,* 2005; Wu *et al.,* 2007; Lu *et al.,* 2011; Shahid *et al.,* 2011). Only a single study is available evaluating partial deletions among males of RPL couples on Sinhalese population of Sri Lanka and they did not depicted on association (Wettasinghe *et al.,* 2010). In the present study the partial deletion was screened by employing the markers recommended by Repping *et al.,* (2002).

Subregions of AZFc region and the STS employed for the present study is as depicted in Figure 4.3 and Table 4.1. The penetrance of gr/gr deletion is far lower than complete b2/b4 deletion (AZFc) but it may lead to spermatogenic failure. The deletion of gr/gr is because of recombination between repeated sequences g and r resulting in the removal of two copies of *DAZ* and many other transcriptional units (Ferlin *et al.,* 2005; Lynch *et al.,* 2005; Giachini *et al.,* 2008; Visser *et al.,* 2009). Review of literature showed

that higher frequency of gr/gr deletion was reported among infertile male of Indian population when compared to US and European countries (Repping *et al.,* 2003; de Lianos *et al.,* 2005; Machev *et al.,* 2004; Hucklenbroich *et al.,* 2005). The gr/gr deletion was also identified among normozoospermic men ranging from 0 to 12% (Shahid *et al.,* 2011; Stouffs *et al.,* 2011). Two such deletions were observed with the condition of normozoospermia but both the individuals had subnormal scores for sperm function NCD and HOS (Table 4.4 and Figure 4.10).

b2/b3 deletion is caused as a result of gr/gr inversion or b2/b3 inversion removing 1.8Mb of the AZFc decreasing copy numbers without any effect on fertility (Fernandes *et al.,* 2004; Repping *et al.,* 2004). The association of b2/b3 with male fertility was reported alone in Chinese men both in infertile and control men but other population did not reported the same (Fernandes *et al.,* 2004; Repping *et al.,* 2004; Machev *et al.,* 2004; Lynch *et al.,* 2005; Hucklenbroich *et al.,* 2005; Wu *et al.,* 2007). Eloualid and coworkers reported b2/b3 deletion only in single individual with spermatogenic failure out of 339 men and none in the normozoospermic men (Eloualid *et al.,* 2012). In Indian population the of b2/b3 deletions was reported among SCOS and oligospermic men but none among control group (Shahid *et al.,* 2011). But in the present study three such deletions were demonstrated in male partners of RPL couples with two normozoospermic and one oligospermic men (Table 4.4 and 4.11).

b1/b3 deletion varies among population and is very rare type of deletion (Repping *et al.,* 2003; Giachini *et al.,* 2008; Shaqalaih *et al.,* 2009; Shahid *et al.,* 2011). The frequency is lower than gr/gr with unknown effect of spermatogenesis (Repping *et al.,*

2004). Deletion in b1/b3 is absent alone but is associated with gr/gr in one case and in another case with both gr/gr and b2/b3 in normozoospermic and oligospermic male partners of RPL couples (Table 4.4 and Figure 4.12). In the present investigation, partial deletions were observed in both oligospermic and normozoospermic men but the control males with proven fertility did not showed any of the deletion pattern.

The variations in the deletion frequencies and pattern could be due to varied genetic background, type and number of study subjects. Previous studies have specified that individuals with AZFc sub deletions can naturally father children. In case if not they can opt for assisted reproductive techniques but in this scenario the mutation will be transmitted in case of male offspring. In the present report the AZFc subdeletions are not associated with spermatogenic failure or SCO's. The deletion patterns are observed among normozoospermic and oligospermic men with the partner experiencing pregnancy loss. This demonstrates the strong positive association of AZFc subdeletions with recurrent pregnancy loss. Further similar studies are required in Indian population and worldwide with large cohorts to derive a conclusion.