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Screening of Y-Chromosomal Microdeletions Using 8 STS Primers in Idiopathic Infertile Men from Coimbatore District, Tamilnadu

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Abstract—India is one of the most populous country in the world with 2.5 as an average fertility rate. Nevertheless, the trend of fertility rate has been tumbling down in Indian states. Earlier, women were considered to be lone reason for infertility. It took several decades to understand that infertility in males which may either be due to genetic or non-genetic factors. Y-Chromosomal microdeletion is one of the major genetic factor which cause infertility in males. Analysis of Sequence Tagged Sites (STS) markers in Azoospermia Factor regions (AZFa, AZFb, AZFc) at Yq part helps us to know about the deletion frequency in infertile males. Infertile males who adopt Assisted Reproductive Technique (ART) are likely to transmit the infertility to their sons. Molecular diagnosis of Y chromosomal microdeletion is one of the paramount and prognostic techniques to acquire information about the infertility passing from one generation to another. Only little research has been carried out on Y chromosomal microdeletions in Tamilnadu. The aim of our research is to scrutinize the frequency of Y chromosomal microdeletions in infertile males of Coimbatore district, Tamil Nadu. We utilized eight STS primers (sY84, sY86 for AZFa region; sY 99, Sy100, sY 134 for AZFb region; sY 156, sY 254, sY 255 for AZFc region) to detect the microdeletions. Blood samples of thirty infertile men were scrutinized for Y chromosomal microdeletion. Our results showed that the frequency of deletion in AZFa region accounted for 53%; AZFb – 50%; AZFc region- 60% respectively. AZFc region was highly deleted when compared to AZFa and AZFb.

Keywords— Y-Chromosomal Microdeletions, Azoospermic Factor Regions, Sequence Tagged Sites Primer.

I. INTRODUCTION

World Health Organization (WHO) reports that infertility affects 8-12% of the couples in the world [1]. The infertility rates differ among the Indian states. In olden days, women are the only reason to be blamed for infertility. A research in India indicated that male reproductive disorders contribute 50% to infertility [2,3,4]. The predominant factors to know about the infertility in male are sperm count, motility, structure and shape. Later, Scientists has intrigued about the genetic factors behind male infertility. Y chromosomal microdeletion is one of the breakthrough technologies to acquire knowledge about genetic factors behind infertility [5].

The Y Chromosome is divided into short arm (Yp) and long arm (Yq). The X chromosome will recombine with the ends of Y chromosome which is known as pseudo autosomal regions (PARs). The Y chromosome region which does not involve in recombination is termed as nonrecombining region(NRY). The euchromatin part of NRY consists of (Azoospermia factor region) *AZFa*, *AZFb* and *AZFc* gene clusters play a predominant role in spermatogenesis [6].

The deletion of *AZF* locus which can be detected by using STS PCR rather than cytogenetic test. The first identified candidate gene *RBMY* (RNA binding Motif on the Y chromosome) from *AZFb* expressed in the testis and its actual function in the male germ cell development is ambiguous [7]. After *RBMY*, *DAZ* (deleted in azoospermia) candidate gene from *AZFc* has been noticed and it is expressed in testis. Though there are other candidate gene such as *CDY1* (Chromo domain Y) in *AZFc*, the high ubiquity of *DAZ* deletion in infertile men make them as an important candidate gene [7,8]. The candidate gene of *AZFa* is USP_9y (Ubiquitin-specific protease 9, Y chromosome) and it has been expressed ubiquitously. *DBY* (Dead box on the Y chromosome) is another candidate gene from *AZFa* [9, 10,11].

The basic set of STS Primers has been used for detecting the deletions in *AZF* regions [12,13]. In this study, we tried to analyze the microdeletions in *AZF* regions from thirty infertile male samples in Coimbatore, India using eight STS primers.

II. MATERIALS AND METHODS

A. Collection of Samples

Ethical clearance for human research (Proposal number: 10/357) was issued by Institutional Human Ethics Committee of PSG Institute of Medical Sciences and Research in Coimbatore, India. Blood samples were collected from thirty infertile males with unspecified factors which caused infertility or also termed as idiopathic cases. Five samples

which were obtained from fertile male and female were used as positive and negative control respectively.

B. DNA Isolation from Blood Samples

Salting out method was used to isolate the DNA from blood samples [14]. The integrity of isolated DNA from samples were checked using agarose gel electrophoresis. The purity of DNA was determined by the A_{260}/A_{280} ratios [15]. The quantification of DNA was done by using Nano-spectrophotometer.

C. Selection of STS Markers

The eight STS primers for this study were selected according to the guidelines of European Academy of Andrology and European Molecular Genetics Quality Network which helped to test the Y-Chromosomal Microdeletions [12,13]. The details of STS Markers are presented in the Table 1.

D. PCR Analysis

The conditions of PCR for eight STS markers were standardized by testing the estimated annealing temperature, annealing time, extension time, primer concentration and DNA concentration. The standardized PCR conditions for eight STS markers were listed in the Table II. 1µl (200 ng) of DNA template from the individual samples were used for each reaction. Total volume of PCR reaction mix was 20 µl and it consisted of 10X PCR buffer,10mM dNTPs, forward and reverse primers (50 ng/ µl), Taq polymerase (3U/ µl), Double distilled water and DNA samples. PCR products were electrophoresed at 75V in 2% agarose gel. The PCR products were visualized under UV trans illuminator and then documented by alpha imager gel documentation system.

S.No	STS Markers of *AZF* Regions with sequences				
	STS Marker	***AZF* Region**	**Forward Primer**	**Reverse Primer**	**Size of PCR Products (base pairs)**
1.	**sY84**	***AZFa***	AGA AGG GTC TGA AAG CAG GT	GCC TAC TAC CTG GAG GCT TC	326
2.	**sY86**	***AZFa***	GTG ACA CAC AGA CTA TGC TTC	ACA CAC AGA GGG ACA ACC CT	320
3.	**sY99**	***AZFb***	GAC TCA GGG ATC CAG GTT G	GCA CTG CAA CTT TTA TGC CT	357
4.	**sY100**	***AZFb***	TAA AGG AAC TTC TGT GTG TAA ACA	TAA GCC AGA TAG GGG CTT CT	111
5.	**sY134**	***AZFb***	GTC TGC CTC ACC ATA AAA CG	ACC ACT GCC AAA ACT TTC AA	301
6.	**sY156**	***AZFc***	AGG AAC TGG CAG GAT TAG CC	ATG TCA GGG TTT CCT TTG CC	950
7.	**sY254**	***AZFc***	GGG TGT TAC CAG AAG GCA AA	GAA CCG TAT CTA CCA AAG CAG C	380
8.	**sY255**	***AZFc***	GTT ACA GGA TTC GGC GTG AT	CTC GTC ATG TGC AGC CAC	126

Table: 1 STS markers of *AZF* regions with sequences and their Product Size

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	PCR reaction conditions for STS primers							
PCR Steps	**sY84 (*AZFa*)**	**sY86 *(AZFa)***	**sY99 *(AZFb)***	**sY100 *(AZFb)***	**sY134 *(AZFb*)**	**sY156 *(AZFc)***	**sY254 *(AZFc)***	**sY255 *(AZFc)***
Initial Denaturation	94˚ C, 3 min	94˚ C, 3 min	94˚ C, 3 min	94˚ C, 3 min	94˚ C, 3 min	94˚ C, 3 min	94˚ C, 3 min	94˚ C, 3 min
Denaturation	94˚ C, 1 min	94˚ C, 1 min	94˚ C, 1 min	94˚ C, 1 min	94˚ C, 1 min	94˚ C, 1 min	94˚ C, 1 min	94˚ C, 1 min
Annealing	56˚ C, 1 min	60˚C, 45 sec	58˚ C, 1 min	60˚ C, 1 min	56˚ C, 1 min	56˚C, 1 min	58˚ C, 1.5 min	60˚ C, 1 min
Extension	72˚ C, 1 min, 35 Cycles	72˚C,45s, 35 cycles	72˚ C, 1 min, 35 cycles	72˚ C, 1 min, 35 cycles	72˚ C, 1 min, 35 Cycles	72˚C,1 min, 35 cycles	72˚ C, 2 min, 35 cycles	72˚ C, 1 min, 35 cycles
Final Extension	72˚ C, 5 min, 35 cycles	72˚ C, 5 min, 35 cycles	72˚ C, 5 min, 35 cycles	72˚ C, 5 min, 35 cycles	72˚ C, 5 min, 35 cycles	72˚ C, 5 min, 35 cycles	72˚ C, 5 min, 30 cycles	72˚ C, 5 min, 35 cycles
Hold	4˚ C	4˚ C	4˚ C	4˚ C	4˚ C	4˚ C	4˚ C	4˚ C

Table2: PCR reaction conditions for Y chromosomal STS markers of *AZF* Regions

III. RESULTS AND DISCUSSION

Thirty infertile male samples were screened for Y chromosome microdeletions using eight STS primers (sY 84, sY 86 for *AZFa* region; sY 99, sY 100, sY 134 for *AZFb* region and sY 156, sY 254 and sY 255 for *AZFc* region). The PCR reactions were performed thrice to confirm the deletions in *AZF* regions. The number of samples with deletions and their percentage were listed in the Table III and IV respectively.

The deletion of *AZFa* region results in Sertoli cell only syndrome (SCOS) [16]. When we analyzed with sY84 marker, thirteen samples showed deletion in *AZFa* region among the thirty samples and the percentage of deletion in *AZFa* region using sY84 marker was 43%. The other marker sY86 for *AZFa* resulted deletions in eight samples and accounted 26.6% frequency of deletions in *AZFa* region. In this study, the percentage of deletions in *AZFa* region using sY84 and sY 86 were 53%.

Azoospermia and maturation arrest of germ cells prevalently occur, when there is a deletion in *AZFb* region [17]. Three STS markers such as sY99, sY100 and sY134 resulted deletions of *AZFb* region in eleven, ten and nine samples out of thirty samples respectively. The Percentage of deletions in *AZFb* region using sY99, sY100 and sY134 were 36.6%, 33% and 30% respectively. The *AZFb* deletions were analyzed by using these three markers which accounted for 50%.

The deletions in *AZFc* region reported that the conditions such as mild or severe oligozoospermia and azoospermia would become dominant [17]. While we analyzed samples for the deletions in *AZFc* region utilizing sY156, sY 254 and sY 255, the deletions were observed in four, eight and twelve samples out of thirty samples respectively. Hence the percentage of deletions in *AZFc* for sY156, sY 254 and sY 255 were 13%,66.6% and 40% respectively. The deletions of *AZFc* region was 60%, which seemed to be higher compared to the deletions in (53%) *AZFa* and (50%) *AZFb* region**.** Venn diagram depicted the distribution of *AZF* deletions in Fig.7.

Sample pictures of agarose gel analysis after PCR reactions exhibit microdeletions in the *AZF* region with respect to STS primers are displayed from Fig. 1. to Fig. 6.

S.NO	Sample number with *AZF* deletion	
	***AZF* Region**	***Sample Number with Deletions (S)***
1.	*AZFa*	S1,S3,S6, S7, S9, S10, S11, S12, S14, S15, S17, S18, S19, S20, S21, S28
2.	*AZFb*	S1,S2,S6,S7,S10,S11,S13,S14,S15,S16,S18,S21,S25,S27,S28
3.	*AZFc*	S1,S2,S4,S6,S7,S10,S11,S12,S14,S15,S17,S18,S19,S20,S21,S25,S29,S30

Table: 3 Microdeletions of *AZF* regions in infertile male samples

S.NO	Percentage of deletions in *AZF* region		
	***AZF* Region**	**Number of samples with deletion**	**Percentage of deletions**
1.	*AZFa*	16	(16/30) 53%
2.	*AZFb*	15	(15/30) 50%
3.	*AZFc*	18	(18/30) 60%
4.	*AZFa+b*	1	(1/30) 3.3%
5.	*AZFb+c*	2	(2/30) 6.6%
6.	*AZFa+b+c*	9	(9/30) 30%

Table: 4 Percentage of deletions in *AZF* regions for thirty infertile male samples

Surprisingly,six infertile male samples (S5,S8,S22,S23,S24,S26) did not show any deletion using eight STS primers and their percentage of undeletion accounted for 20%.Though the STS markers were selected based on the European Academy of Andrology and European Molecular Genetics Quality Network,six infertile male samples showed no deletions in the *AZF* regions.The undeleted infertile samples should contain a deletion possibly in some other loci which could not be able to diagnose by these eight STS primers. The observation of undeleted samples in infertile samples were also reported in Greek and Nilgiri districts, South India [18, 19].

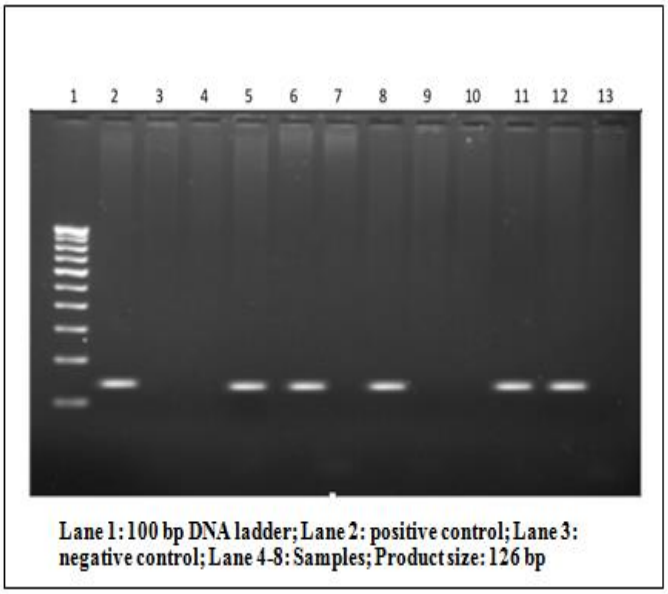

Fig.1: Analysis of microdeletions in *AZFc* region using sY255

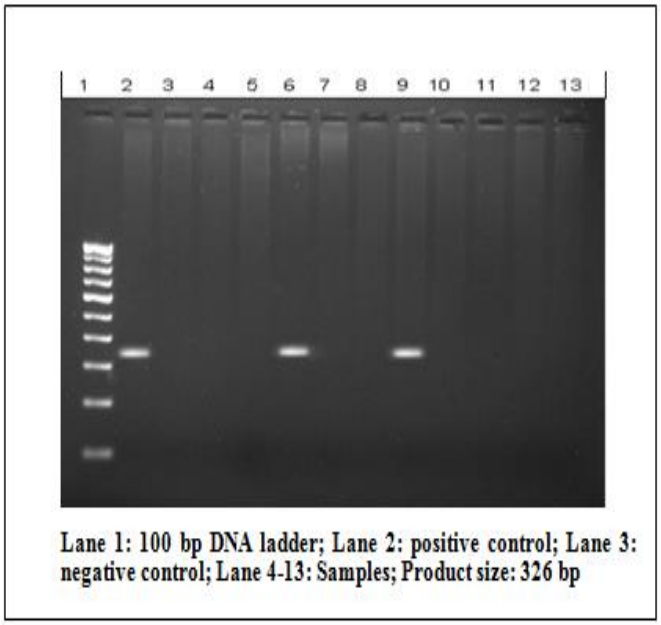

Fig.2:Analysis of microdeletions in *AZFa* region using sY84

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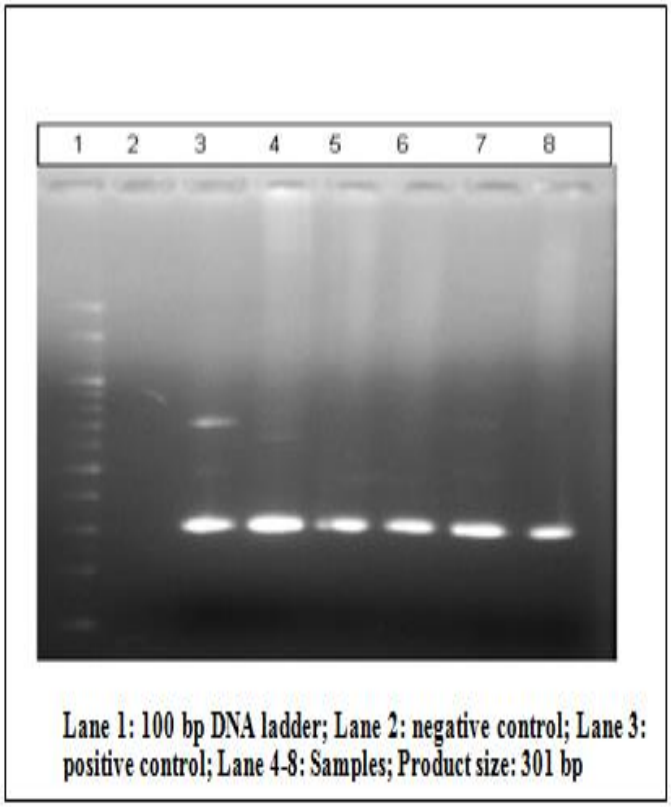

Fig.3: Analysis of microdeletions in *AZFb* region using sY134

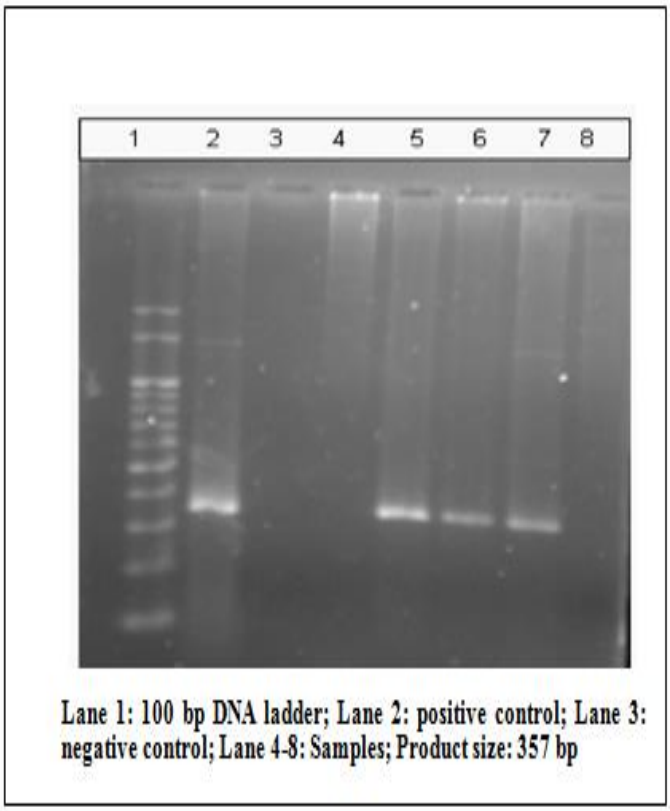

Fig.4: Analysis of microdeletions in *AZFb* region using sY99

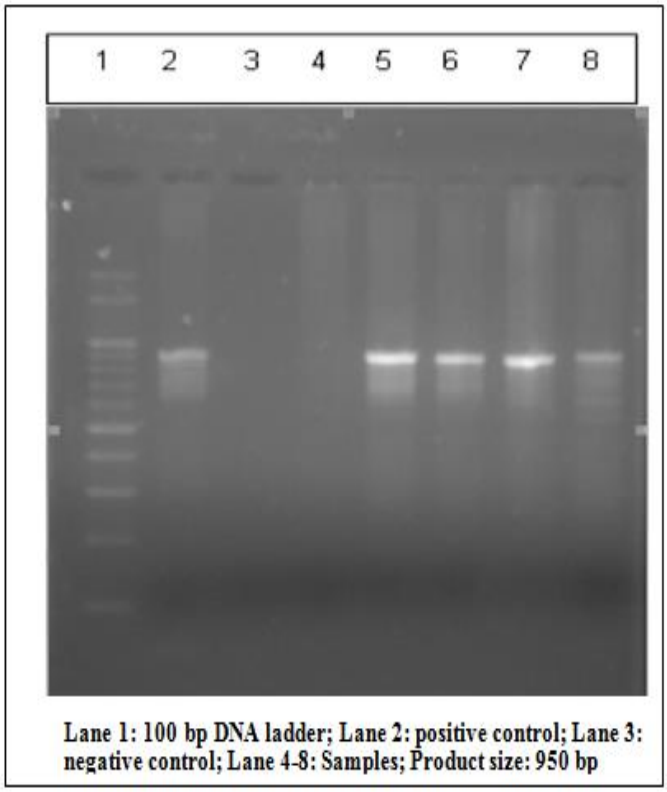

Fig.5: Analysis of microdeletions in *AZFc* region using sY156

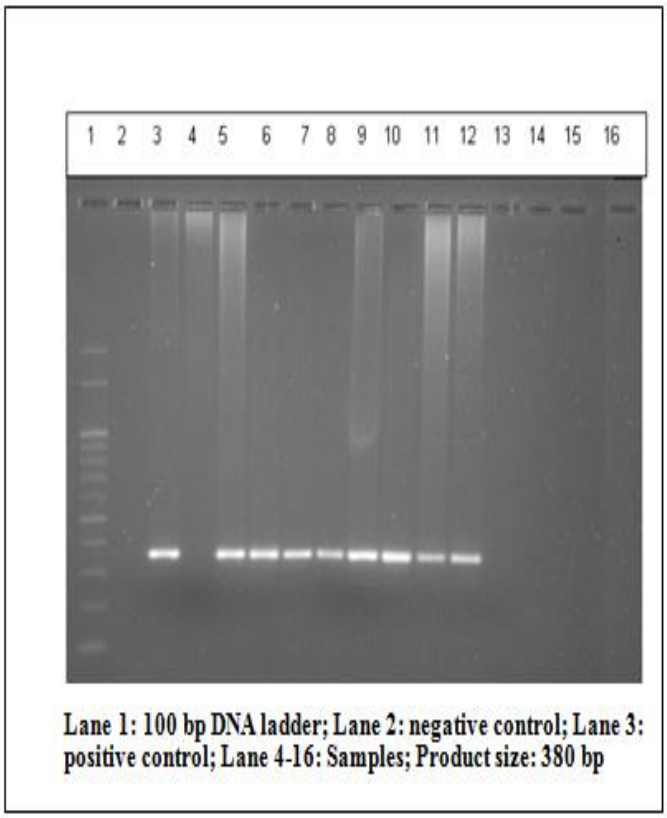

Fig.6: Analysis of microdeletions in *AZFc* region using sY254.

Fig.7: Venn diagram depicts the distribution of *AZF* deletion

AZFc region was highly deleted in infertile male patients compared to *AZFa* and *AZFb* region and this reports were congruous with previous research works [19,20,21,22].AZFc region contains palindromic sequences which are more susceptible to intrachromosomal recombination and therefore, this region has been deleted frequently in infertile male samples [23].A research conducted in North India reported that the deletion of AZFc region was 58.8% [21] whereas our results showed that 60% of deletion in AZFc region. Deletion in AZFa+b region was 3.3% which found to be lower than AZFb+c region (6.6%).Deletions in *AZFb*+c region lead to either asthenozoospermia (reduced sperm motility) or severe oligozoospermia (low sperm motility).Azoospermia (absence of sperm) or failure of testicular sperm retrieval are associated with the deletions in *AZFa+b* region [19,24].30% of deletion was observed in all the three regions.Eventhough infertile male who has a deletion in *AZFc* Region can adopt Intracytoplasmic sperm injection (ICSI) treatment to father a child, the deletion pattern which causes infertility is likely to transmit to their offsprings [25].It is because most of the *AZF* deletions are occurred in the germ cell [26]. Hence, the Y chromosomal microdeletion analysis helps the clinicians to predict the deletion pattern of AZF regions for the infertile male's future generation.Clinicians could able to inform infertile couples about the risks in adopting either ICSI or assisted reproductive techniques.

IV. CONCLUSION

This study showed that the *AZFc* region has been highly deleted when compared to the *AZFb* and *AZFa* regions.Increase in sample size would have helped us to explore more about the frequency of *AZF*deletions.Examining Y Chromosomal microdeletions assist the clinicians to decide the suitable assisted reproductive techniques for the infertile patients. Analysis of Y chromosomal microdeletions help the clinicians to counsel the infertile patients prior to assisted reproductive techniques.

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