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Frequency of Deletion of AZF Region of the Y Chromosome in Chhattishgarh, India

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Abstract: *Background:* In the work up of male infertility, Y chromosome microdeletion screening is crucial. PCR is a very sensitive technique to screen Y chromosome microdeletion. In the current study, Y chromosome microdeletion was detected by PCR based technique. To the best of our knowledge, no such study has been reported from Chhattishgarh state of India so far. *Material and methods:* A total of seventy-three subjects were enrolled for the study during the period of one year. Out of which forty-seven subjects were cases (infertile men with oligozoospermia and azoospermia) and twenty-five were controls (with normozoospermia and having child). Semen analysis was done in each case to evaluate spermatogenesis status. Sperm DNA fragmentation by sperm chromatin dispersion of cases with oligozoopermia was also performed to detect DNA fragmentation Index. *Results:* Y chromosome microdeletion was observed in one out of forty-seven infertile males who were oligozoospermic or azoospermic. The type of deletion was AZFbc. Thus 2.12% men among oligozoospermic or azoospermic men have Y chromosome microdeletion in Chhattisgarh. *Conclusion:* In Indian population, AZFbc deletion has been found to be the second commonest type of deletion. In our study, we have also found this as the only deletion. This test also provides etiological interpretation of male infertility to the patient. We believe that awareness about transmission of deleted gene to the offspring could prevent infertility up to certain extent in the affected couples.

Keywords: Azoospermic Factor (AZF), Oligozoospermic, Yq Microdeletion

1. Introduction

Infertility affects around 10% of the general population. Its prevalence is higher in affluent sections of the society. In around half of the couples reporting with infertility, the cause lies with the male partner. Proper and thorough counselling of couples with male factor infertility is a must before treatment can be considered. In many cases semenogram reports does not give conclusive results which can direct a patient towards Assisted Reproductive Techniques (ART). Other tests which are advisable are Karyotyping, Androgen receptor gene mutation assay test, Cystic fibrosis test, Y chromosome microdeletion analysis, Sperm chromatin dispersion assay, Flourescence in situ hybridization (FISH), metabolic assays and proteomic methods. Many factors affect spermatogenesis: cryptorchidism, endocrine disorders and obstruction in the pathway of seminal fluid. There are also immunological factors, infections and genetic causes which can affect spermatogenesis. Autosomal and sex chromosomal defects are responsible for 10-15% cases of male infertility. [1, 2] Genetic factors result in malfunctioning of testis, decreased spermatogenesis or shutdown of spermatogenesis.

A genetic factor on long arm of male Y chromosome has been established for germ cell development and multiplication. These genes are present on the long arm of chromosome from proximal to distal (figure 1). AZFa gene expresses on spermatogonial germ cell level. So, the effect of this deletion leads to Azoospermia with Sertoli cell only syndrome. Next distal genes are AZFb and AZFc. Partial overlapping is present over these genes. Deletion of AZFb presents as azoospermia [3, 4] but oligozoospermia is also seen. In AZFc region main gene is DAZ gene. Deletion of AZFc region has variable presentation both clinically and histologically. Here, presentation is either oligozoospermia or severe oligozoosermia. In rare cases, AZFc deletion being transmitted to male offspring have been reported [5]. Complete deletion of AZFbc region (both AZFb and AZFc) has variable presentation from oligozoospermia to azoospermia [3, 6, 7].

According to European Academy of Andrology (EAA), primarily two markers in each region detects 90% deletion [8]. They are as follow:

For AZFa	sY84, sY86
For AZFb	sY127, sY134
For AZFc	sY254, sY255

In India, overall incidence of Y chromosome microdeletion in azoospermic and oligozoospermic cases is reported to be 8.4%. To the best of our knowledge, no study has been reported from Chhattisgarh so far. The aim of our study was to detect Y chromosome microdeletion of AZF region in oligo and azoospermic males in this state of India. The relatively high proportion of microdeletion in our Indian population suggests for the need of strict patient selection criteria. In this study, we have attempted to follow the same. Endocrinal profile of these patients was also observed for correlation.

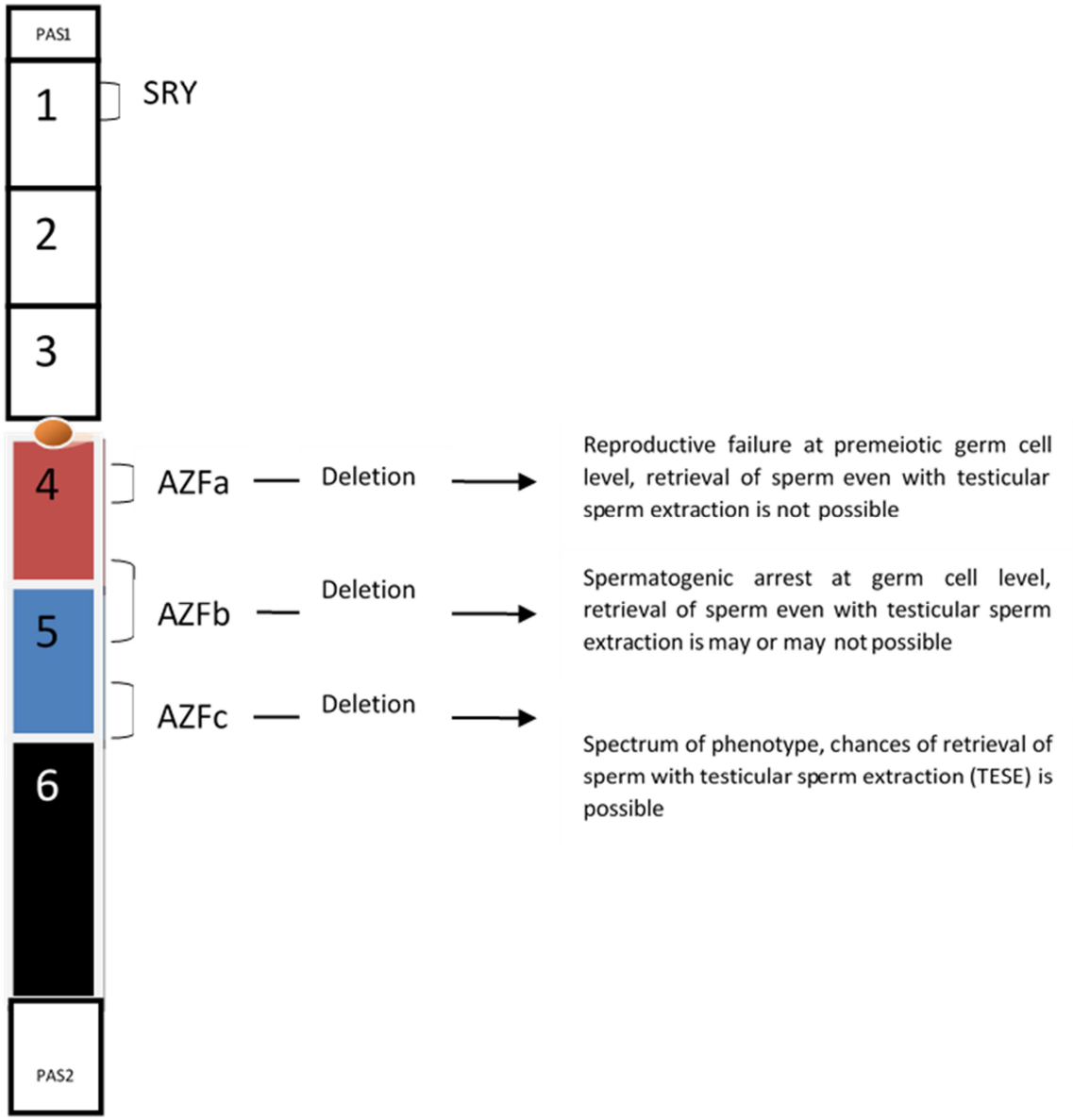

Figure 1. *Schematic representation of Y chromosome and location and effects of deletion AZF regions.*

2. Material and Methods

2.1. Patient Selection

Subjects of the study were enrolled during the period August 2018- February 2020 after ethical clearance. The study was carried out in forty-seven cases presenting to the OPD of Department of Obstetrics and Gynecology, Department of Urology and Department of Endocrinology at All India Institute of Medical Sciences, Raipur, India. We included 25 fertile men as controls. Cases were male infertile couples not having any issue. They were classified on the basis of sperm count; Normozoospermia (>15 x 10^6/ml) oligozoospermia (<15 x 10^6/ml), severe oligozoospermia (<5 x 10^6/ml) and Azoospermia (Nil). Oligozoospermic and azoospermic men were enrolled as cases and normozoospermic men with one or more issues were enrolled as controls. Normozoospermic men of infertile couple, men of infertile couple having history of testicular cancer, under treatment for any cancer, testicular maldecent, testicular infection including testicular torsion were excluded from the study. Age of cases and controls were in range 22-45 years to prevent bias.

Demographic profile of cases and control were also noted. Semen samples were analyzed twice before microdeletion test. We considered only oligozoospermic and azoospermic men as cases for the microdeletion test.

2.2. Screening for Y Chromosome Microdeletion

DNA was isolated from blood samples by using the QIAamp Blood Kit (Quiagen). Extracted genomic DNA was amplified using polymerase chain reaction (PCR) to look for microdeletion in Y chromosomes. A normal male control and a normal female control were included with each PCR run. To avoid contamination, PCR composition were prepared in a laminar air flow hood. PCRs were run in the Veriti 96 well thermal cycler, Applied Biosciences by Thermofisher Scientific. Centrifuge used during the process was model no 5424R Eppendrof.

A series of Y chromosomes specific sequence tagged site (STS) and primer sequence had been characterized previously [9]. Among these 6 sets of primers i.e. sY84, sY86, sY127, sY134, sY254 and sY255 were used in this study. The primers correlate respectively with AZFa, b and c region. As an internal control, one more primer was used i.e. SRY (which correlates with sex determining region). Each case was checked by multiplex PCR through co-amplification with the SRY marker. The primer sequence, their product size and locus are depicted in Table 1.

Multiplex PCR method was used for screening. Two sets of master mix (A & B) were prepared. Mix A contained sY84, sY127, sY254, SRY and mix B contained sY86, sY134, sY255, SRY. Each PCR-tube had volume of 13 µl which contained 3.5 µl sample DNA, 10x buffer (Tris with 15 mM $MgCl_2$), dNTP mix (2.5mM each), primer (10 pm each) and Taq polymerase (1 U). Final volume was adjusted by Milli Q water upto 13 µl. Further reaction was carried out in a thermocycler with the following schedule- initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 59.5°C for 1 min and extension at 72°C for 1 min followed by final extension at 72°C for 7 min.

PCR products were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide (EtBr) (10 ug/ml). Gel image was captured using gel documentation unit. Every sample was amplified for three times. After three PCR attempt the sample was confirmed to be normal if it included all the four bands and if one or two bands were missing then the deletion was confirmed. Failure of multiplex PCR was checked by subsequent PCR analysis.

Table 1. *Sequence-tagged site / primer details.*

Y DNA marker (STS)	Sequence	PCR product length (bp)	Gene or locus
SRY-F: SRY-R:	5'-GAATATTCCCGCTCTCCGGA-3' 5'-GCTGGTGCTCCATTCTTGAG-3'	472 bp	SRY
sY86-F: sY86-R:	5'-GTGACACACAGACTATGCTTC-3' 5'-ACACACAGAGGGACAACCCT-3'	320 bp	AZFa
sY127-F: sY127-R:	5'-GGCTCACAAACGAAAAGAAA-3' 5'-CTGCAGGCAGTAATAAGGGA-3'	274 bp	AZFb
sY254-F: sY254-R:	5'-GGGTGTTACCAGAAGGCAAA-3' 5'-GAACCGTATCTACCAAAGCAGC-3'	400 bp	AZFc
sY84-F: sY84-R:	5'-AGAAGGGTCTGAAAGCAGGT-3' 5'-GCCTACTACCTGGAGGCTTC-3'	326 bp	AZFa
sY134-F: sY134-R:	5'-GTCTGCCTCACCATAAAACG-3' 5'-ACCACTGCCAAAACTTTCAA-3'	301 bp	AZFb
sY255-F: sY255-R:	5'-GTTACAGGATTCGGCGTGAT-3' 5'-CTCGTCATGTGCAGCCAC-3'	126 bp	AZFc

3. Result

Twenty-five controls and forty-seven cases were screened for the presence of Yq microdeletions. Among forty-seven cases, oligozoospermic, severe oligozoospermic and azoospermic were 19, 14 and 14 cases respectively (Table 2). Average age and duration of infertility in these patients were 32.48 ± 6.48 years and 5.95 ± 4.77 respectively. Obstructive etiology was ruled out in all the azoospermic patients either by standard clinical evaluation or scrotal USG or acid phosphatase test. Among cases, one deletion was observed in azoospermic infertile male. The deletion was for AZFbc region and markers were sY127, sY134, sY254, sY255

(Figure 2, Figure 3). Among controls, no deletion was observed. We also evaluated oligozoospermic and severe oligozoospermic for sperm DNA fragmentation Index which was in the range of 5-58%.

Table 2. *Percentage of different types of cases.*

SN	Types of cases	N-47
1	Oligozoospermia	19 (40%)
2	Sever Oligozoospermia	14 (30%)
3	Azoospermia	14 (30%)

FSH, LH and testosterone concentration of infertile subjects (cases) were 20.45 ± 16.75 mIU/ml (range 2.97-52.54 mIU/ml), 10.70± 5.02 mIU/ml 3.5-24.94 mIU/ml), and 358.78± 257.89 ng/dl (1.83 - 1012ng/dl) respectively. Among infertile subjects, the case with deletion did not have significant difference in FSH, LH level (31.7 mIU/ml, 19.5 mIU/ml) but Testosterone level was significantly low (1.84 ng/dl).

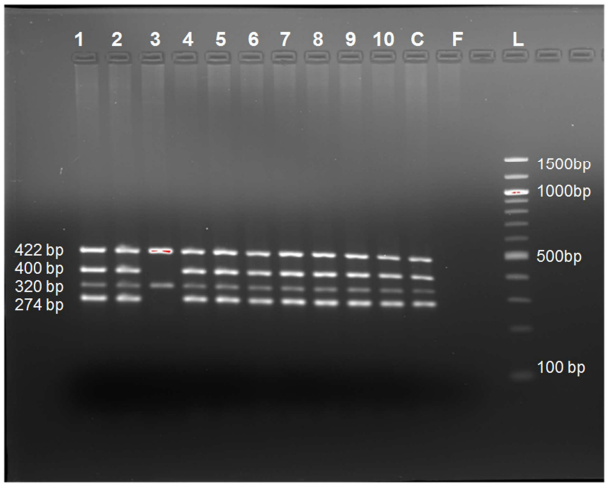

Figure 2. *Example of Multiplex PCR A mix: lane L length marker 100 bp DNA ladder, Lane 1-10 were cases lane F was negative controls, Lane C was positive control. Lane 3 showed bands SRY, sY86. (sY254, sY127 bands were missing).*

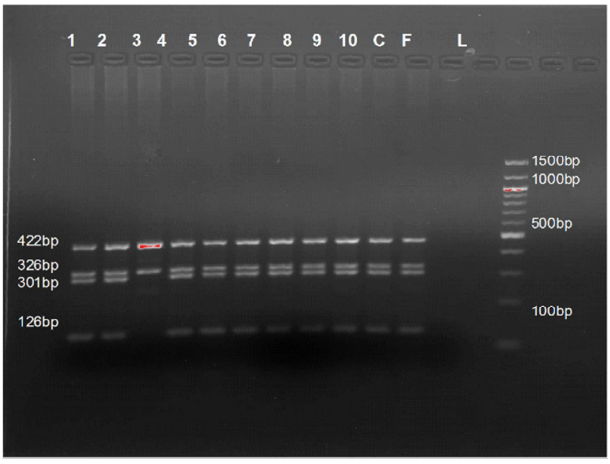

Figure 3. *Example of Multiplex PCR B mix: Lane L length marker 100 bp DNA ladders, Lane 1-10 were cases lane F was negative controls, Lane C was positive control. Lane 3 showed bands SRY, sY84. (sY134 & sY255 bands were missing).*

4. Discussion

After semenogram, Yq microdeletion is an important test to detect the etiology of infertility in men with azoospermia or oligozoospermia. One study showed no remarkable difference in Y chromosome microdeletion in groups with different semen parameters except oligoasthenoteratozoospermic group [2]. Detection of microdeletion can prevent a patient from undergoing unproven treatment with hormonal therapy. Furthermore, it also eliminates the risk of transmission of disease in offspring.

In India, Babu et al were the first to detect Yq microdeletion with PCR based technique [10]. With advancement in molecular genetic techniques, multiplex PCR has emerged as a cost effective, inexpensive and rapid technique to rule out male infertility due to Y chromosome microdeletion. This reveals many facts related to opting for ART. Deletions of AZFa region excludes the option of testicular sperm extraction (TESE). However, deletion of AZFc region offers the possibility of TESE. Some studies have reported better results with the sperm taken after TESE in ICSI and IVF [11].

In our study, as we followed the selection criteria of EAA, one out of forty-seven infertile males (oligo and azoospermic men) showed deletion; ie 1/47 (2.12%). He was 34 years old azoospermic male who was detected to have deletion in AZFb, AZFc regions ie STS markers sY127, sY134, sY254, sY255. In some earlier Indian studies, the frequency of AZFbc deletion was found to be varying widely from study to study like 10.5% [12], 12.5% [13], 17.64% [14] 19.6% [2] and 51.7% [15]. They demonstrated higher frequency of double deletion (AZFbc) whereas other studies have shown lower frequency; 1.26% [16] and 1.76% [8]. In one cohort study from Indian population, the frequency of double deletion in azoospermic group was 37% while in oligozoospermic and severe oligozoospermic group, it was 12% and 3% respectively [2]. These studies showed that after AZFc, AZFbc is the second commonest deletion of AZF region.

Deletion of AZFbc has two presentations, one which extends from P5 to the distal arm of P1 (P5/distal-P1deletions) and other one from P4 to the distal arm of P1 (P4/distal-P1deletions). A deletion in P5/deletion represent deletion of 7.66Mb including 42 genes or transcripts and P4/distal-P1deletion represent deletion of 7.03 Mb along with 38 gene copies [17]. AZFbc deletion leads to impaired spermatogenesis and reduced probability of retrieval [4]. Based on the Poor outcome sperm retrieval procedure are not suggested for AZFbc deletion [18]. These patients should be offered the option of donor semen.

Only complete deletion of AZFa region is associated with sertoli cell only syndrome (SCOS). In cases of deletion of AZFa, sperm retrieval is still possible in partial deletion of AZFa [13, 19]. ART should be offered to those couples with AZFc deletion with own partner semen. Donor sperm insemination is a likely substitute to patients of AZFb and AZFa [20].

In this study, level of FSH, LH and Testosterone in case with deletion suggested primary gonadal failure. However, gonadal biopsy was not done in this patient to see the status of gonad. Mitra et al found no significant difference in cases with deletion in Indian scenario [8].

By analyzing Indian studies, we came to conclusion that EAA markers are not sufficient for Indian population. So, after using EAA markers, the patient should also be advised for Non EAA markers to detect the real cause of infertility. Among non-EAA markers sY746 and sY82 in AZFa region, sY121 and sY128, sY130 and sY143 in AZFb region and sY145 and sY160 in AZFc region are important. Additionally, if a couple opts for ART with deletion of these non EAA markers, what would be the long-term effects of these deletion? More research is needed in this area with the following questions: 1) Conception is possible or not? 2) If the couple wishes to use one's own sperm in ICSI, is it with or without any deleterious effect? 3) Is there any long-term adverse effect of deletion of these markers? 4) Is there any effect on fertility potential of the male offspring? 5) Is there any effect on metabolism of the offspring body?

5. Conclusion

In this study, we found that 2.12% cases had AZF deletions. Low prevalence of Y chromosome microdeletion found in the current study could be because of the characteristic of population or the use of only six primers. Despite this, the process is acceptable and may be adopted. Limitation of the study is that the samples were collected from only one institute. Although, the institute serves patients from all over the state, we accept that it may not be representative of the entire Chhattisgarh. However, in our knowledge, this is the first study reporting on this subject from Chhattisgarh region of India. All infertility cases must be analyzed for exact loci and extent of deletion. Yq microdeletion test is an important diagnostic step to counsel all couples with male factor infertility who are opting for ART.

Conflict of Interest

Authors have no conflict of interest.

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Contribution of Different Authors

Dr Manisha Barnwal Sinha contributed to design of project and writing of manuscript. Dr Suprava Patel was involved in supervision of semen analyses, patient description and follow up of patients. Dr Human Prasad Sinha was involved in drafting the article and revising it critically for important intellectual content. Dr Rima Dada performed discussion of results and critical review of the manuscript. Dr Manisha Barnwal Sinha was involved in supervision of laboratory experiments, discussion of results and critical review of the manuscript. Miss Apoorva Joshi was involved in laboratory experiments. Nilag Bagde was involved in enrolling the patients.

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