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Development Of Multiplex Pcr To Assess Male Infertility

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Introduction

The second most populous country on earth, India accounts for more than 17 per cent of world population. Rather population growth is a major concern, yet infertility is a devastating health problem. According to WHO, infertility is the inability of the female partner to attain pregnancy after one year or more of the unprotected sex. Infertility affects about 15% of the couple of which half of the reproductive problems are associated with the male counterpart (Agarwal *et al*, 2015). Causes of infertility in men include a variety of things but can be grouped into a number of genetic and non genetic categories. Non-genetic conditions include hypogonadotrophic hypogonadism, testicular maldescence, structural abnormalities of the male genital tract, genital infections, previous scrotal or inguinal surgery, varicoceles, chronic illness and medication (Crosignani et al., 1993). Genetic causes include chromosomal abnormalities [Klinefelter syndrome (XXY) and specific translocations, point mutations in the androgen receptor and the cystic fibrosis transmembrane conductance regulator (CFTR) gene], **Y chromosome microdeletions**, mitochondrial DNA (mtDNA) mutations, monogenic disorders, multifactorial disorders and endocrine disorders of genetic origin. Genetic causes of infertility are an important etiological factor leading to irreversible partial or complete spermatogenetic arrest among which Y chromosome microdeletions receives prime focus. The microdeletions that occur in the AZF [AZoospermic Factor] region of the long arm of Y chromosome affect genes that are involved in spermatogenesis. There are three recurrently deleted nonoverlapping subregions in the proximal, middle and distal Yq11 regions in the deleted interval designated as AZFa, AZFb and AZFc (Kent First et al., 1999). It has been identified in 12.9% of south Indian infertile men (Poongothai et al., 2008). Still now no compact method is available for general public for Y chromosome microdeletion analysis (YCM) in India. European Academy of Andrology (EAA) recommends that 90 % of YCM can be detected by the use of 2 STS markers for each AZF loci. In the present study, method to assess male infertility in Indian population using multiplex PCR employing 3 STS markers for each AZF loci was devised.

Methodology

This study was conducted on subjects attending infertility clinics in Coimbatore District of Tamil Nadu, South India. Blood samples were collected from men with proven fertility (n = 10) who served as control. A female sample was included as negative control. Samples were collected with informed consent following clearance from Institutional human ethics committee, PSG institute of medical sciences & research, Coimbatore (Proposal number: 10/357). Karyotyping and semen analysis was done for all the male samples.

DNA extraction

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DNA was extracted from 2 ml of peripheral blood using standard procedure (Thangaraj et al., 2003).

Multiplex PCR analysis for Y chromosome microdeletions

Pentaplex and quadraplex PCR containing Sex determining Region of Y Chromosome (SRY) as internal control was designed. Each multiplex contains atleast one primer for each *AZF* loci. Primer sequences were collected from NCBI. The primer combinations were as follows:

Quadraplex: sY86 (*AZFa*), sY99 (*AZFb*), sY127 (*AZFb*), sY254 (*AZFc*) + SRY

Pentaplex: sY84 (*AZFa*), sY746 (*AZFa*), sY134 (*AZFb*), sY255, sY156 (*AZFc*) + SRY

Details of the STS markers are given in Table 1. Multiple primer analyser (primer analysis software) was used to find out the cross complementarity among the primers.

S. No.	Locus	STS	Primer sequence	Product size
1	NRY	SRY	F: GAATATTCCCGCTCTCCGGA R: GCTGGTGCTCCATTCTTGAG	472 bp
2	AZFa	sY84	F: AGAAGGGTCTGAAAGCAGGT R: GCCTACTACCTGGAGGCTTC	326 bp
3	AZFa	sY86	F: GTGACACACAGACTATGCTTC R: ACACACAGAGGGACAACCCT	320 bp
4	AZFa	sY746	F: TTGACTGCTTATTCTACACAAGGC R: CAGGGGAAATTGGGTTTT	216 bp
5	AZFb	sY99	F: GACTCAGGGATCCAGGTTG R: GCACTGCAACTTTTATGCCT	350 bp
6	AZFb	sY127	F: GGCTCACAAACGAAAAGAAA R: CTGCAGGCAGTAATAAGGGA	274 bp

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7	AZFb	sY134	F: GTCTGCCTCACCATAAAACG R: ACCACTGCCAAAACTTTCAA	301 bp
8	AZFc	sY156	F: AGGAACTGGCAGGATTAGCC R: ATGTCAGGGTTTCCTTTGCC	950 bp
9	AZFc	sY254	F: GGGTGTTACCAGAAGGCAAA R: GAACCGTATCTACCAAAGCAGC	380 bp
10	AZFc	sY255	F: GTTACAGGATTCGGCGTGAT R: CTCGTCATGTGCAGCCAC	123 bp

Table 1: STS markers for Y chromosome microdeletion

Qudraplex and pentaplexPCR was carried out in 40 µl of the final PCR mix containing 150 ng of genomic DNA, 20 pm of each primers, 800 µM of dNTPs, 5 U of Taq DNA polymerase subjecting to PCR amplification of initial denaturation for 3 min at 94 ºC and 35 cycles of 94 ºC for 1 min, with the annealing temperature of 58 ºC for 30 sec and extension at 72 ºC for 70 sec. The final extension time was 5 min at 72 ºC. The PCR products were analyzed on a 2.5 % agarose gel containing ethidium bromide (0.5 µg/mL).

Results and Discussion

Method for assessing Y chromosome microdeletion in infertile men was formulated with pentaplex and quadraplex primer sets both containing Sex determining Region of Y (SRY) as internal control. As no compact method is available for general public for "Y chromosome microdeletion analysis (YCM)" in India, Infertility specialist who is examining the patient, if considered necessary will collect blood sample from infertile men and transport it to research institutes like Centre for Cellular and Molecular Biology- Hyderabad, All India Institute of medical Sciences- New Delhi, Ganga Ram Hospital- New Delhi, Christian Medical College- Vellore for YCM analysis. The laboratories will isolate DNA from blood sample and check for YCM with Polymerase Chain Reaction or will employ Promega kit [Imported from USA]. Out of 300 STS available for YCM screening, until now three were no clear idea about the panel of STS to be used for YCM detection. Since male infertility is alarming development of robostic technique for YCM is need of the hour. Based on the recommendations of European Academy of Andrology and a

vivid analysis of the published Indian literature from 2001 onwards pentaplex and quadraplex PCR was designed.

Amplification of all primers in pentaplex and qudraplex was obtained (Fig:1) An STS was considered absent only after at least three amplification failures in the presence of internal control (SRY).Astonishingly, two non specific bands were seen in pentaplex PCR.

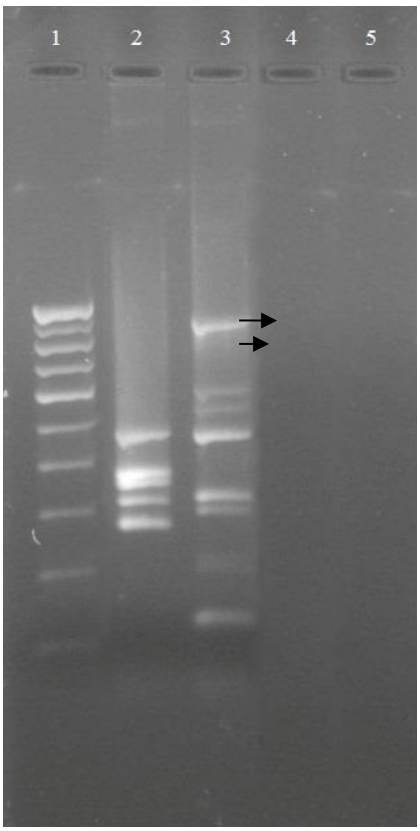

Fig 1: 2.5% agarose gel containing resolved multiplex PCR products for fertile male.

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Lane:1-100 bp ladder
Lane 2 & 3 corresponds to quadraplex and pentaplex PCR products respectively. Lane 2 has 5 bands corresponding to, from the top, SRY (472 bp), sY254 (380 bp), sY86 (320 bp), sY127 (274 bp),sY99 (357bp).
Lane 3 has 6 bands corresponding to, from the top, sY156 (950 bp), SRY (472 bp), sY84 (320 bp), sY134 (301 bp), sY746 (216 bp), sY255 (123 bp). The bands with arrow marks in lane 3 are undesired products.
Lane 4: negative control for quadraplex
Lane 5: negative control for pentaplex

To rule out non specific bands in pentaplex PCR, a gradient PCR with varying annealing temperature from 54 ºC to 59 ºC was performed with same conditions. No undesired products were seen after standardisation. But primer dimer was found in all lanes . It should be furthur standardised by reducing primer volume.

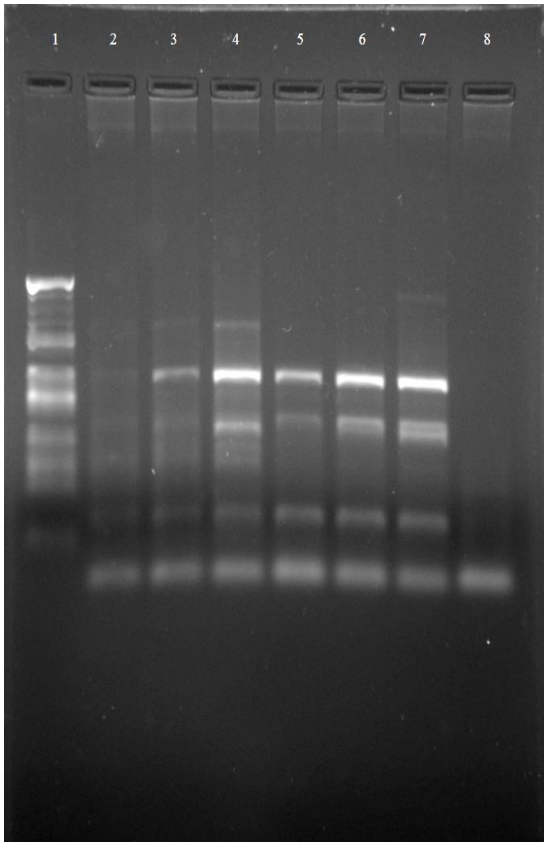

Fig 2: 2.5% agarose gel of gradient PCR for pentaplex
Lane 1: 100 bp ladder.
Lane 2 to 7 : Gradient PCR for pentaplex, with annealing temperature from 54°C to 59°C, with lane 7 corresponding to PCR products amplified, after standardisation of the conditions, with annealing temperature being 59°C. Lane 7 has 6 bands corresponding to, from the top, sY156 (950 bp), SRY (472 bp), sY84 (320 bp), sY134 (301 bp), sY746 (216 bp), sY255 (123 bp).
Lane 8: negative control (female DNA).

Conclusion

This easy and quick method for Y chromosome microdeletion method will be useful for Indian infertile couples if it is further validated with more number of fertile and infertile couples.

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