Comparison of QF-PCR and FISH for Aneuploidy Detection in Prenatal Diagnosis

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ABSTRACT

Introduction: Among all chromosomes (46) in the human genome, particular significance has been given to chromosomes 13, 18, 21, X and Y. This is primarily because of aneuploidy in these chromosomes that result in viable pregnancies with congenital defects. As a result, standardised methods like Rapid Aneuploidy Test (RAT) for detection is the need of the hour in addition to Non-Invasive Prenatal Testing (NIPT) and Chromosomal Microarray (CMA).

Aim: To compare and analyse the diagnostic utility of Fluorescent In-Situ Hybridization (FISH) and Quantitative fluorescent Polymerase Chain Reaction (QF-PCR) in aneuploidy of detection.

Materials and Methods: In the present observational study, 120 pregnant women suspected of having fetal aneuploidies were subjected to amniocentesis and Chorionic Villus Samplling (CVS). Following DNA extraction, FISH and QF-PCR were carried out using pre-designed chromosomal markers and specific FISH probes for trisomy of 13, 18 and 21.

Results: Of 120, 5 prenatal samples showed Trisomy (T) 13, 18 and 21 chromosomes, amounting to a frequency of 4.2% (5/120). These results were concordant by both tests i.e FISH and QF-PCR trisomy 18 and 21 detected. Four amniotic fluid samples, two each respectively (4/108; 3.7%), and one Chorionic Villus Sampling (CVS) (1/12; 8.3%) were tested positive for trisomy of chromosome 13.

Conclusion: From the present study, it can be concluded that QF-PCR is a better technique for detection of aneuploidies. However, both these techniques, together called RAT of Invasive Prenatal Screening (IPS) should be performed for errorless results before termination of pregnancy (TOP).

Keywords: Amniotic fluid, Chorionic villus sample, Rapid aneuploidy test, Trisomy of 13, 18 and 21

INTRODUCTION

Since the late 1960s, conventional methods such as karyotyping are used for Prenatal Diagnosis (PD) of chromosomal anomalies. With advancement in technology, various prenatal invasive procedures such as CVS and amniocentesis were for detection of chromosomal anomalies such as aneuploidies, unbalanced and balanced structural anomalies. However, CVS testing is done in 10th week and amniocentesis are done in the 14th week of pregnancy possess high potential risk of miscarriages and development of fetal abnormalities. Based on existing data, these techniques have been known to result in 0.5% and 1% of cases of fetal loss respectively. Due to associated risk, these procedures have limited usage and screening tests currently used have limited confirmatory testing value. Therefore, as a result, it has become increasingly important to ascertain good confirmatory tests that are rapid, reliable, cost-effective and objective in their interpretation [1].

Samples obtained through invasive techniques can be analysed by various techniques to detect the presence of chromosomal abnormalities in fetus. Karyotyping is the gold standard analysis for detection of several conditions including mosaicism, polyploidy and chromosomal rearrangements such as deletions, translocation and inversions but its labour intensive nature, low resolution and a longer turnaround time limits its uses. These limitations have led to the development and utilisation of other techniques.

In 1990, FISH was developed to overcome conventional cytogenetics analysis, where the interphase cells remained undivided and therefore hindered rapid analysis of trisomy cases. FISH is based on specific fluorescent dye coupled to a probe complementary to a particular chromosome region; it targets only limited regions of the genome and are dependent on the viability of cells, specificity of probes and operator capability. Over the past years, quantitative QF-PCR is increasingly used for rapid diagnosis of aneuploidy in prenatal specimens [2-5]. Amplification of QF-PCR depends on Short Tandem Repeat (STR) markers generating a fluorescent product and is directly proportional to the amount of target sequence present in the initial template. This method is more applicable and cost-efficient than FISH [6-9].

In the present study, FISH and QF-PCR, collectively known as RAT, were compared in their ability to detect chromosomal aneuploidy of chromosome 13, 18 and 21 including sex chromosomes in prenatal samples of pregnant women with suspected fetal aneuploidies, since NIPT, CMA, whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) are costlier and their prescription depends mainly on clinicians choice.

MATERIALS AND METHODS

This observational study was designed to compare two techniques i.e FISH and QF-PCR to investigate trisomic cases for rapid analysis.

Patient selection: Pregnant women as suggested by the clinician ranging in age from 20 to 46 years, suspected of having fetal aneuploidies were subjected to amniocentesis/CVS post approval and under the ambit of registered physicians.

These samples were analysed at Supratech Micropath Laboratory and Research Institute, Ahmedabad by QF-PCR and FISH. The CVS (12) and AF (108) samples after consultation were collected from different states of India and were tested during January 2016 -December 2017. For QF-PCR, predesigned chromosomal markers [Table/Fig-1,2] and specific FISH probes for trisomy 13, 18, 21 and sex chromosomes (Metasystem, Germany) were used.

The study was approved by Internal Ethical Committee of Gujarat University (IEC 001 GU) Ahmedabad.

DNA extraction: The DNA was extracted from CVS and Amniotic Fluid (AF) samples using a Qiagen DNA extraction kit. The kit was used according to the manufacturer's instructions. The extracted genomic DNA was used as a template and was kept at 4°C until further use.

Marker	Label	Chromosome location	Known alleles (base pairs)
AMXY	6-Fam	Xp22.1-22.31 - Yp11.2	X 104 Y 109
SRY	6-Fam	Yp11.2	Y 463
X22	6-Fam	Xq28 Yq (PAR2)	189-194-199-204-209-214-219-224-226-229-234-239-242-247-253
DXYS218	PET	Xp22.32 Yp11.3 (PAR1)	266-270-274-278-282-286-290-294
HPRT	6-Fam	Xq26.1	264-268-272-276-278-280-284-288-292-296-300-313
DXS6803	VIC	Xq12-Xq21.33	106-110-114-118-120-124-128
DXS6809	VIC	Xq	238-242-246-250-252-254-258-260-262-266-268-270-274
DXS8377	NED	Xq28	213-216-219-222-225-228-238-241-244-248-252
SBMA	VIC	Xq11.2-Xq12	166-169-172-175-178-181-184-187-190-193-196-199-202-205-208-211
D21S1414	6-Fam	21q21	328-330-334-338-342-346-350-352-354-356-358-360-362-443
D21S1411	VIC	21q22.3	246-262-266-274-278-282-286-290-294-298-302-306-316-319
D21S1446	PET	21q22.3-ter	200-204-208-212-214-218-220-224-228
D21S1437	VIC	21q21.1	120-124-128-132-136-140-144
D21S1008	6-Fam	21q22.1	196-200-204-208-212-216-220
D21S1412	6-Fam	21q22.2	384-388-392-396-400-406-410-414-418
D21S1435	PET	21q21	142-160-164-168-172-176-180-184-188
D18S391	VIC	18pter-18p11.22	144-148-152-156-160-164-168
D18S390	VIC	18q22.2	398-402-406-410-414-418-422-426-430
D18S535	NED	18q12.2	126-130-134-138-142-146-148-152-156
D18S386	NED	18q22.1	319-330-334-338-342-344-350-354-358-362-366-370-372-376-380-387
D18S858	PET	18q21.1	186-190-192-196-200-204
D18S499	6-Fam	18q21.32-q21.33	386-392-396-400-404-408
D18S1002	6-Fam	18q11.2	122-130-134-138-142
D13S631	VIC	13q31-32	192-196-200-204-208-212-215-218
D13S634	VIC	13q14.3	460-464-466-470-474-478-482-484-486-490-496-500
D13S258	NED	13q21	230-232-234-236-238-240-242-244-248-265-267-269-271-273-277-279-281
D13S305	PET	13q12.1-13q14.1	426-430-434-438-442-446-450-454-458
D13S628	6-Fam	13q31-q32	436-440-444-448-452-456-460-464
D13S742	VIC	13q12.12	254-258-262-266-268-270-274

[Table/Fig-1]: Markers included in Aneufast-QF PCR for detection of chromosome 13, 18, 21, X and Y copy number.

S1	S2	MXY	M21	M18	M13
AMXY	SRY	SRY	D21S1411	D18S386	D13S631
DXYS267	X22	AMXY	D21S1435	D18S391	D13S634
D21S1414	DXYS218	HPRT	D21S1437*	D18S858*	D13S742*
D21S1446	HPRT	TAF9L*	D21S1412*	D18S499*	D13S628*
D21S1442	D21S1411	DXYS156*	D21S1809*	D18S1002*	
D18S535	D21S1435	DXS6803*			
D18S391	D13S634	DXS6809*			
D18S976	D13S258	DXS8377*			
D13S797	D18S386	DXS981*			
D13S631	D18S390	DXS1187*			
D13S305					

[Table/Fig-2]: Markers amplified with the Aneufast™ QF-PCR Kit the markers included in each of the six Primer sets are shown in the table.
*Additional markers for selected chromosome are added with the correspondent chromosome specific assays including two markers already present in the S1 and S2 assays to conform the identity of the sample.

QF-PCR amplification and capillary electrophoresis: the amount of target sequence present in the initial template is directly proportional to QF-PCR amplification of STR markers that generates a fluorescent product.

The Aneufast™ QF-PCR Kit by Molgentix SL (Spain) with six multiplex marker sets of STRs was used for amplification of selected microsatellites and Amelogenin-SRY region of a chromosome. This combination of markers allowed the detection of aneuploidies involving chromosomes X, Y, 21, 18 and 13 with 100% sensitivity and specificity for non-mosaic trisomies. Two multiplex sets i.e S1and S2 of the kit were used to perform initial aneuploidy diagnosis and these assays were analysed through electrophoresis. In addition, four chromosome-specific marker sets (M21, M13, M18 and MXY) were used for the detection of trisomy 21, 13, 18 and sex chromosomes aneuploidies and as back-ups in case all the markers of S1 and S2 were non-informative (homozygous).

Five-dye DNA fragment analysis: The Aneufast™ QF-PCR kit (Molgentix, SL) is based on five-dye fluorescent system for automated DNA fragment analysis. Fluorochromes such as 6-FAM™, VIC™, NED™ and PET™ were used in conjunction with GS 500 LIZ™ size standard.

The data were analysed using GeneMapper IDX Version1.2 Applied Biosystems fragment analysis software following Aneufast QF-PCR kit (Spain).

Detection of trisomy 13, 18, 21 and sex chromosome: In a trisomic sample, three copies of a chromosome were detected by the presence of three peaks corresponding to chromosome specific STRs, having the same fluorescent intensity and a ratio between the areas of 1:1:1 (Trisomic Triallelic). In quantitative PCR the two chromosomes with the same repeat number, produced two unbalanced fluorescent peaks with an area ratio of 2:1 (Trisomic Diallelic). Triploid samples thus produced trisomic diallelic and triallelic patterns for informative STRs on all chromosomes.

FISH analysis: The trisomy of 13, 18, 21 and sex chromosomes were detected using probes i.e. X/Y D5608-100-OG; chromosome Y (Orange), chromosome X (Green); D5607-100-TC; for chromosome 13 (Blue), chromosome 18 (Green) and chromosome 21 (Red) as per FISH probe Metasystem protocols (Germany; catalogue 2017-2018).

RESULTS

Screening tests: Of 120 patients screened, in case of CVS, one fetus showed absence of nasal bone. Ultrasound was also done for nuchal translucency in some cases, suggested by the Clinician [Table/Fig-3].

FISH Analysis revealed, four abnormal signals, two samples depicted trisomy of chromosome 18 (blue dots) and two samples depicted trisomy of chromosome 21 (red dots), constituting 3.7% (4/108) aneuploidy in amniotic fluid samples. Among 12 CVS samples, only one trisomy of 13 (Patau) case (green dots) was detected (1/12; 8.3%) [Table/Fig-4a, 5a, 6a].

QF-PCR: Two samples showed trisomy of 21 and three homozygous STRs peaks of same intensity and same area ratio 1:1:1 called trisomy triallelic was observed. In trisomic aneuploidy of chromosome 18 and 13, two unbalanced which fluorescent peaks are ratio of 2:1 called trisomic diallelic was detected [Table/Fig-4b, 5b, 6b]. In the present study, no sex chromosomal trisomy was observed.

Geographic pattern: Out of 120 AF and CVS samples, higher frequency was obtained from Rajasthan followed by Assam, Gujarat and other states of India [Table/Fig-7].

DISCUSSION

In this cohort, RAT jointly FISH and QF-PCR were used to detect common aneuploidies as an adjunct to karyotyping. The results

| Sr.nos/Sample types	Abnormality	Gestational age (Weeks)	Maternal age (Years)	Screening markers	Nasal bone	NT (mm)	FISH result normal	FISH result abnormal	QF-PCR result normal	QF-PCR result abnormal
1. Amniotic fluid (108)	Normal (104) 46 XY/XX	12-20	27	Double/triple/ Quadruple	-		104	-	104	-
	Abnormal trisomy 18 (2) 47,+18	17. 1 16. 5	42/28	Double (+ve) Quadruple (+ve) NIPT (+ve)	Present/-	1.9/-	-	04	-	04
	Abnormal trisomy 21 (2) (47,+21)	19. 2/ 15.2	35/27	Double (+ve) Triple (+ve) NIPT (+ve)	Present/-	3.2/	-	-	-	-
2. CVS (12)	Normal (11) (46 XY/XX)	8 -12	27	Double/triple/ Quadruple	-	-	11	-	11	-
	Abnormal trisomy 13 (1) 47 + 13	12.5	27	Double (+ve)	Absent	2.5	-	01	-	01

[Table/Fig-3]: Trisomy screening result of amniotic fluid and chorionic villus sampling.
CVS=Chorionic villus sampling; AF=Amniotic fluid; NIPT=Non invasive prenatal testing; +ve=Positive; NT=Nuchal translucency; QF-PCR=Quantitative Fluorescence-Polymerase Chain Reaction; FISH=Fluorescent in situ hybridization Total cases 120; Trisomy total=5/120 (4.2%); AF 4/108 (3.7%) and CVS=1/12 (8.3%). Figures in Parentheses indicate sample type analysed; RAT=Rapid Aneuploidy Testing

Techniques	Trisomy-18
FISH	
QF-PCR	
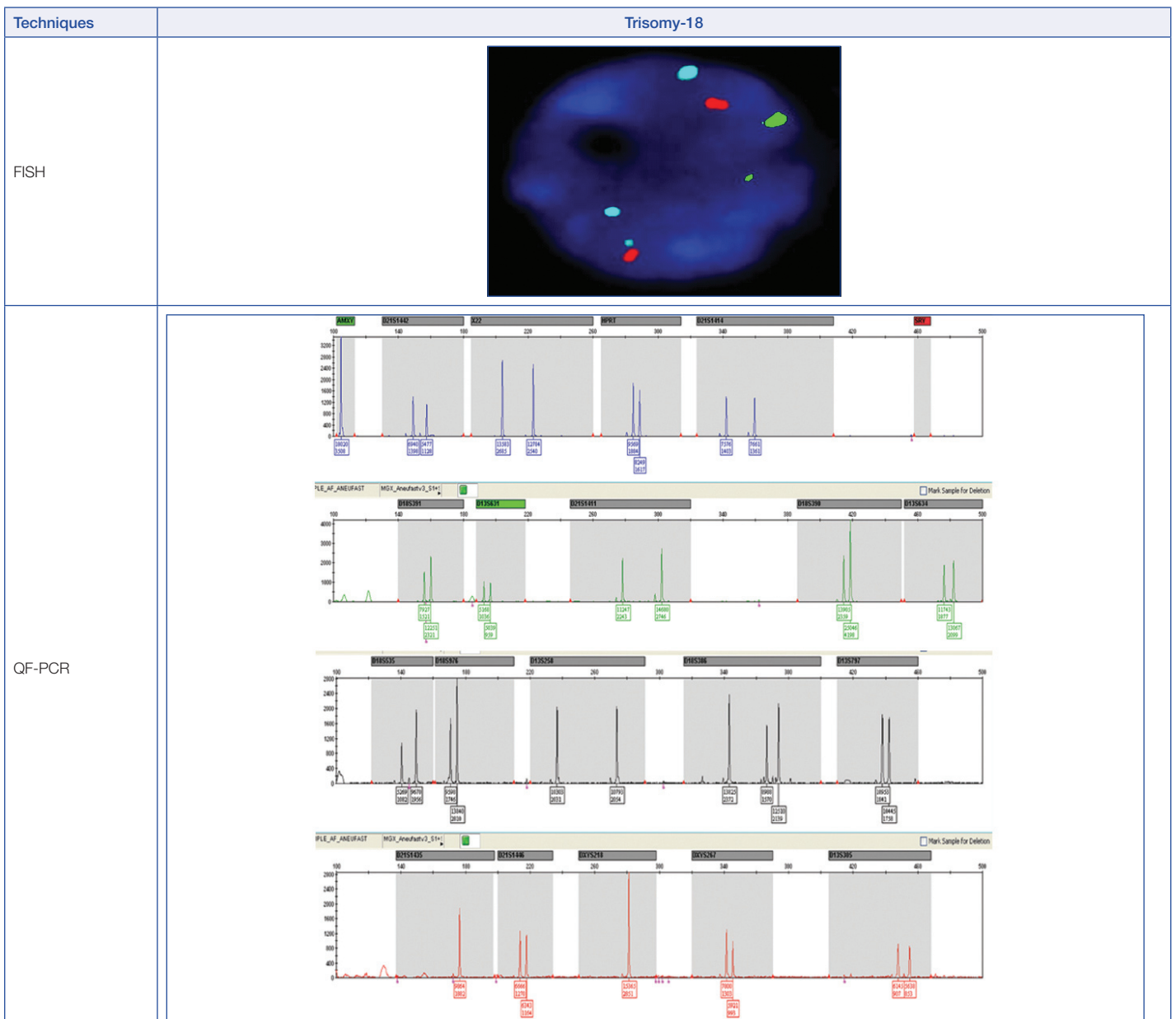
[Table/Fig-4]: Results of FISH (a) and QF-PCR (b) for detection of trisomy of 18.

suggest that FISH could possibly be replaced by QF-PCR for prenatal aneuploidy detection where skilled manpower or cytogenetics infrastructure are crucial. The Association of Clinical Cytogeneticists (ACC) reported that 1% of all prenatal samples are likely to have a chromosomal abnormality which remains undetected sometimes. [10]. While, NIPT, A non-invasive prenatal test using cell free fetal DNA (cffDNA) has very high sensitivity and specificity for T21, it is slightly sensitivity for T13 and T18 detection. Therefore,

NIPT should not be used for total diagnosis [11,12]. Insufficient fetal fraction and high associated cost makes it unsuitable for retesting [13]. And 25% of invasive diagnostic procedures could be avoided [14]. In the present study cohort, 120 referral samples including AF and CVS were used for detecting aneuploidies during pregnancy. Given that these samples were collected from a wide geographic distribution across India, a variety of initial screening tests i.e. double, triple, quadruple and ultrasound were done for

Techniques	Trisomy-21
FISH	
QF-PCR	
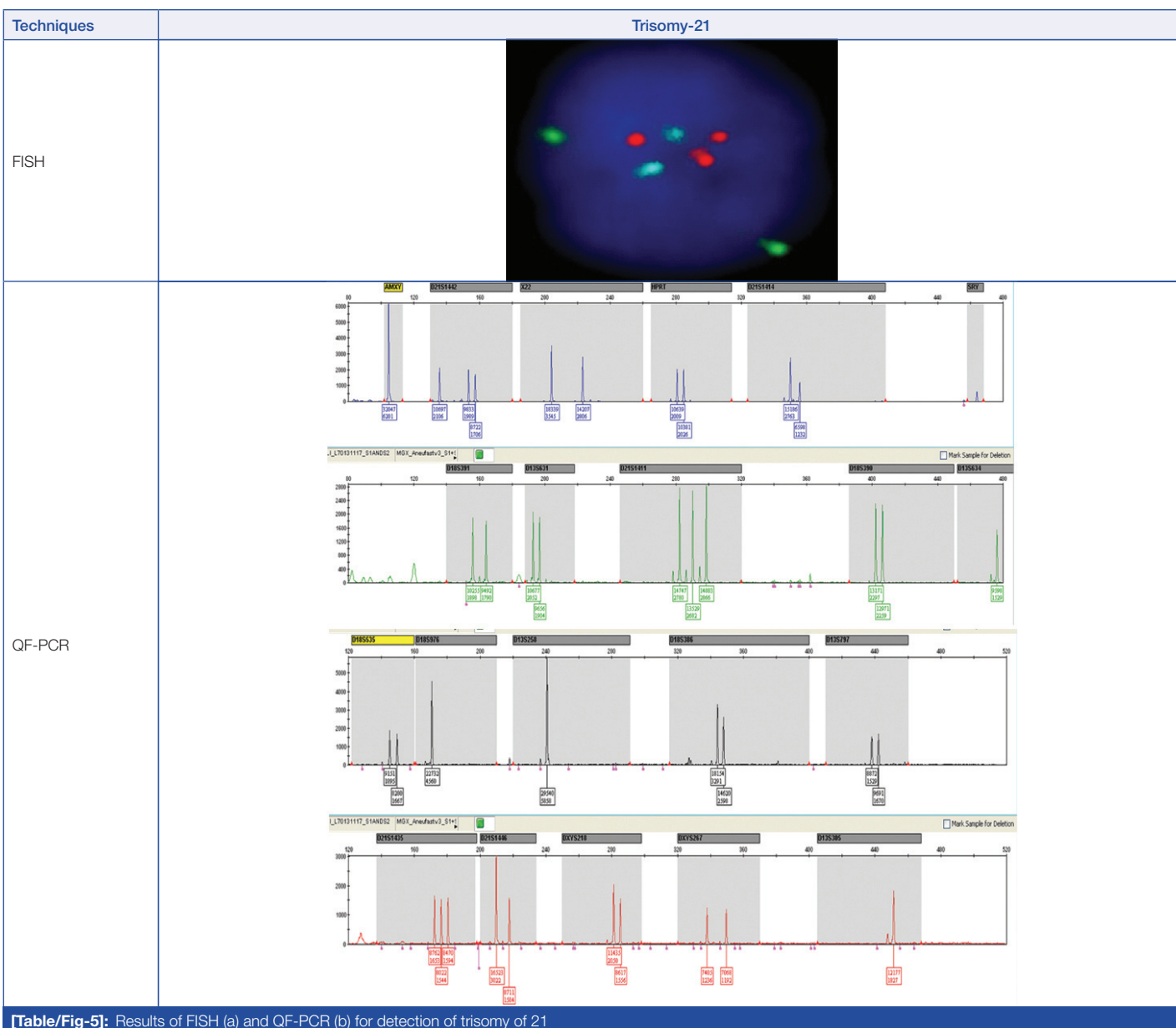
[Table/Fig-5]: Results of FISH (a) and QF-PCR (b) for detection of trisomy of 21

advance maternal age patients (35-42 years) based on physician's preference. To understand the geographic distribution of the Indian states in regard to these genetic disorders, the samples from various parts of India such as Rajasthan (25%), Assam (20%) followed by Gujarat (16%) and Maharashtra (15%) were analysed [Table/Fig-7].

Samples that were found to be positive in initial screening techniques were then processed by RAT i.e. FISH and QF-PCR, where the former is laborious, expensive and limited whereas the latter is fast, relatively cheaper and takes only approximately 24 hours for testing [6,14-18].

The clinical application of RAT was applied in the present study for prenatal diagnosis of aneuploidy samples comprising of AF and CVS that were analysed after initial screening tests. The results obtained by both techniques were comparable. Various authors such as Leung WC et al., Badenas C et al., and Gross S et al., reported that RAT is a reliable technique for detection of only trisomy of chromosome 13, 18, 21, and sex chromosome trisomy were detected when ultrasound detected anomalies were consistent suggesting that RAT does not detect all chromosomal abnormalities that would be picked up by traditional gold standard method karyotyping [4,14,15,18-20]. Similar findings were reported, and aneuploidy testing using invasive FISH and QF-PCR tests were recommended [16].

Among these techniques, FISH is direct examination of intact cells which is advantageous but is relatively expensive and

laborious process. And QF-PCR should be prefered for detection of polyploidy STRs and prenatal rapid aneuploidy detection [5,17-22].
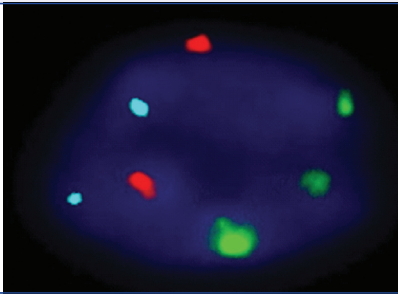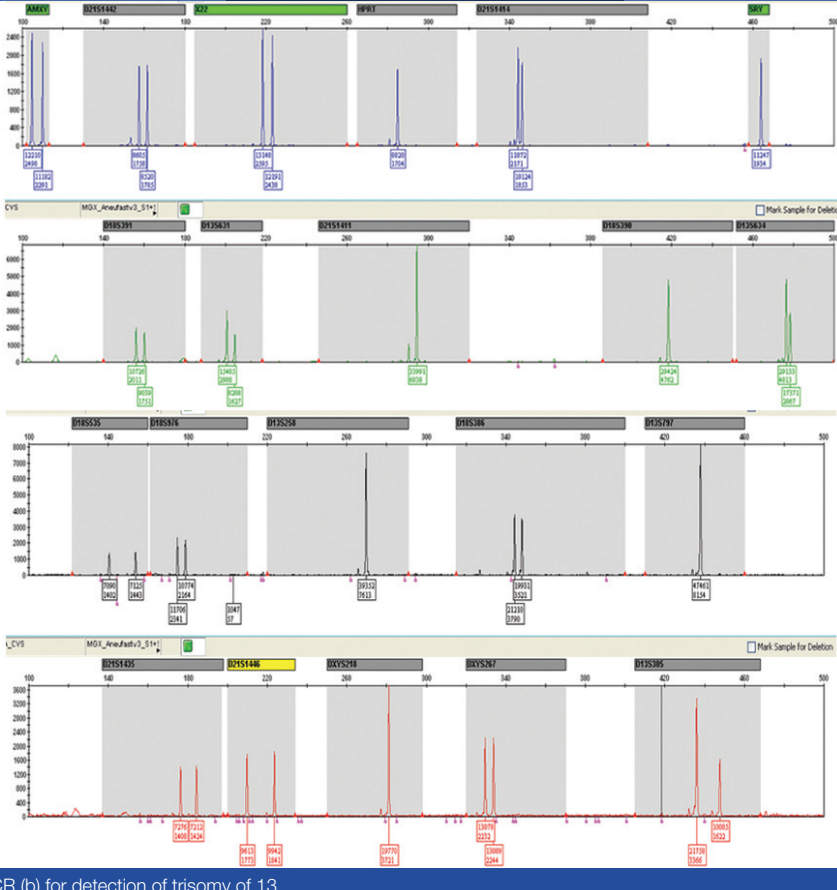
LIMITATION

The sample size was relatively small and conventional karyotypic analysis of the samples was not done. Further study with larger sample size are required to establish the utility of RAT for detection of aneuploidies. In addition, studies are required to determine the efficacy of RAT in detection of sex chromosome aneuploidy.

CONCLUSION

In this report, the data suggest that for rapid diagnosis of aneuploidy, QF-PCR is better in terms of cost as well as turnaround time compared to Fluorescent In-Situ Hybridization (FISH) and conventional karyotyping. For errorless results Rapid Aneuploidy Test (RAT) is suggested, though non-invasive (NIPT, NIPS) and invasive (CMA, WES, WGS) tests are available for diagnosis, that are expensive and therefore are not very commonly prescribed.

ACKNOWLEDGEMENTS

Techniques	Trisomy-13
FISH	
QF-PCR	

[Table/Fig-6]: Results of FISH (a) and QF-PCR (b) for detection of trisomy of 13.
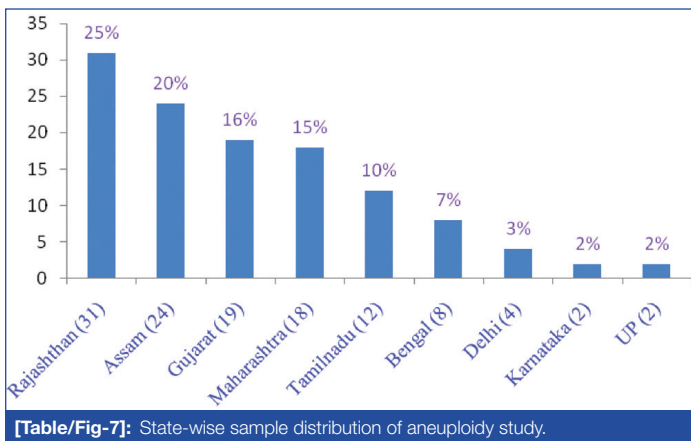
[Table/Fig-7]: State-wise sample distribution of aneuploidy study.

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