Short Communication

Non-invasive Prenatal Test - A Pilot Pan-India Experience of an Indian Laboratory

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ABSTRACT

Background: Serum-based tests are considered to be a very affordable screening option for pregnant women. The non-invasive prenatal test (NIPT), among prenatal screening for chromosomal aneuploidies has a sensitivity of >99%. The focus of this report is to present an overview of NIPT outcome from samples tested in our national laboratory over a period of six months. **Materials and Methods:** NIPT was done by whole genome shot gun sequencing and a total of 513 pregnant women were tested in our laboratory. The samples were analyzed for reporting on risk of trisomy 21, 18 and 13 as well as sex chromosome aneuploidies. **Results:** A total of 513 blood samples were received for NIPT testing and 9.3% were cancelled at the pre-analytical stage. Frequency of routine and IVF pregnancies was detected to be 83% and 12% respectively. Frequency of high risk including trisomy 21, trisomy 18 and sex chromosome aneuploidies was detected to be at 3.0% respectively. A total of 7% of cases which were reported high risk by serum screening were detected to be low risk by NIPT. **Conclusion:** NIPT is an efficient aneuploidy screening tool as it studies fetal DNA and our analysis indicates that the same can be beneficial in screening for fetal chromosomal risk with high sensitivity to identify cases which need confirmatory tests.

Key words: Cell free DNA, Next-Generation Sequencing, Non-Invasive Prenatal Testing, Pregnancy, Screening.

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BACKGROUND

Non-Invasive Prenatal Test/Screening (NIPT/NIPS) was first commercially introduced in the year 2011 in the United States and Hong Kong. It continues to gain popularity as an efficient prenatal screening tool to assess for risk of aneuploidies involving chromosomes 21, 18, 13 as well as the sex chromosomes in unborn fetus. Today, NIPT is available in over 60 countries and with many different inclusions to the basic test wherein some panels screen aneuploidies of only chromosomes 21, 18 and 13, while some include microdeletions and sex chromosome anueploidies. Examples of a few popular NIPTs include Panorama (Natera Inc., USA), Harmony (Ariosa Diagnostics Inc., USA), Verifi® (Illumina Inc., USA), NIFTY® (BGI Diagnostics Co., Ltd, China). Some tests include reporting on the gender of the fetus especially in countries where the legal boundaries are set compatible with the cultural norms.[1,2] NIPT involves utilizing cell-free fetal DNA (cffDNA) extracted from the venous blood sample of the pregnant woman and screening it for presence of chromosomal aneuploidies. It is marketed to be the best adjunct to serum-based prenatal screening tests like the double, triple and quadruple marker as NIPT bears the potential to identify aneuploidies of sex-chromosomes, microdeletions as well as aneuploidies of the common chromosomes 21, 18 and 13 with sensitivity of >99%. In addition to advantages of sensitivity the test can be recommended from as early as 10 weeks of pregnancy and does not need any ultrasonography (USG) indications to draw conclusion. Risk calculations in NIPT are based on genetic analysis irrespective of age of the pregnant lady, ethnicity and smoking status. In NIPT, depending

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on the bioinformatics module, ability to report on the fetal fraction (FF) becomes possible which is used as a clinical indicator for many adverse developmental conditions of the fetus in high risk cases. However, reporting by NIPT is largely dependent on requirement of a minimal FF of at least up to 3.5% in most cases.

NIPT has emerged as an efficient alternative before recommendation for the confirmatory invasive tests like amniocentesis and Chorionic Villus Sampling (CVS) after discovering that the cffDNA in maternal blood becomes an accurate alternative genetic material to study fetal development. The placenta has been detected to be the main source of cffDNA and for testing, the sample collection tube plays a key role in ensuring contamination with maternal DNA fragments is minimal. The Streck Cell-Free DNA BCT™ has been shown to be better at ensuring sample stability for up to 14 days under ambient conditions, while blood sample in EDTA tubes need to be processed for plasma separation within 6 hr of collection. Studies have shown Streck BCTs to be better at stabilizing concentration of short fragment cffDNA when stored for 72 hr at ambient temperature.[3,4] In terms of technology many studies have also compared the efficiency of NIPT with karyotyping and chromosomal microarray (CMA). These studies indicated the sensitivity of NIPT and karyotyping to be 70% and 30% in detecting pathogenic deletions and 5-20 Mb duplication. These indicate efficiency of NIPT to be comparable with karyotyping for detecting chromosomal imbalance in the second and third trimesters.[5]

Structural variations in chromosomes like deletions and duplication have been associated with mental and developmental disorders in the fetus and generally continue to persist throughout the term of pregnancy and beyond while embryos with deleterious aneuploidies generally undergo spontaneous abortion. Further submicroscopic chromosomal anomalies have been shown to exceed the frequency of aneuploidies among fetus who exhibit USG abnormality in third trimester. High resolution CMA has emerged as a method of choice by replacing karyotyping as the gold standard due to low failure rate and minimal turnaround time. However, considering aspects of cost and affordability NIPT emerges to be an equivalent option. A whole-genome shotgun assay by NGS becomes faster and economically feasible and also bears accuracy comparable with CMA for copy number variants.[6-9]

The NIPT assay done in our lab includes the Prenatal Screen SAGE™ test (Yourgene Bioscience CO., LTD.) and reporting for risk of trisomy 21, 18 and 13 and four sex chromosome aneuploidies; namely XO (Turner's syndrome), XXX (Triple X syndrome), XXY (Klinefelter syndrome) and XYY (Jacob's syndrome) was done. Gender determination was not included as a part of NIPT in our laboratory in compliance with the legal framework assisted by the PCPNDT Act of 1994 (Pre-conception and Prenatal Diagnostic Techniques Act, 1994). Our current short report is aimed at summarizing the pilot findings from NIPT in our laboratory.

METHODS

The NIPT data utilized for this study report has been generated by a reference laboratory and not hospital-based setting. Further, no patient identifiers which can compromise confidentiality have been discussed. Consent for test and use of data for research purpose has been obtained from every NIPT client in our laboratory in accordance with PCPNDT Act of 1994.

Data from a total of 465 women who signed up for NIPT at our laboratory has been considered for this communication. Average age of women recommended for NIPT in our case was detected to be 33 years with routine pregnancies being higher in recommendation at 83% in comparison to IVF pregnancies at 12%. Other types included the Intracytoplasmic Sperm Injection (ICSI) and Intrauterine Insemination (IUI) each at 0.9% respectively. The entire cohort characteristics have been summarized in Table 1.

Table 1: Cohort characteristics.

Sr. No.	Characteristic	Count/Frequency
1	Total cohort	465
2	Mean Age	33 years
3	Mean maternal weight	64 kg
4	Mean Fetal Fraction	11.3% +/-1.5
5	**First trimester**	5.80%
a	Routine pregnancy	82%
b	IVF Pregnancy	11.0%
c	Singleton	89%
d	Twins	None
e	Mean Age	34 years
f	Mean maternal weight	68 kg
6	**Second trimester**	93%
a	Routine pregnancy	84%
b	IVF Pregnancy	12%
c	Singleton	95%
d	Twins	3.90%
e	Mean Age	33 years
f	Mean maternal weight	64 kg

Prenatal Screen SAGE™ involves a whole genome shotgun assay and the workflow included library construction done with kit from Yourgene and sequencing from Thermo Fisher Scientific Inc. The Ion Torrent™ system (Life Technologies, United States) was utilized for sequencing and the bioinformatics included the Torrent Suite for raw data analysis, Premaitha Data Packer for demultiplexing and the SAGE™ Link for Trisomy Screening (Yourgene Bioscience CO. LTD).

The process flow included plasma separation followed by cfDNA extraction using MagCore®HF16 Automated Nucleic Acid Extractor (RBC Bioscience Corp.) The protocol for extraction of cfDNA included a final 60μL elute generated by a cycle of cell lysis, nucleic acid binding to magnetic beads, washing and elution in a total of 70 min protocol. Quantification of the extracted cfDNA was done using Qubit™ dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific Inc.) and a concentration of over 0.1 ng/μL was the minimum requirement. DNA libraries were constructed using Yourgene cfDNA Library Prep Dx available as a 576-reaction high throughput kit containing reagents for end repair, adaptor ligation, clean-up and amplification. The prepared libraries where then quantified by Plate Based Quant (PBQ) using Yourgene QS250 involving a fluorescence assay. A minimum library concentration of 1.5ng/μL was the requirement for further size selection. Size selection of pooled cfDNA library was done using 12 Channel 3% agarose 28.5μL gel cassette on the Yourgene QS250 instrument. The minimal reads required post sequencing was set at 1.8 million per sample. The final step involved use of a concentration reagent to wash the pooled cfDNA library.

The Ion Chef™ System was utilized for automated high-throughput template preparation and chip loading and the Ion PI™ Chip v3s was utilized for the assay (Life Technologies, United States). The Ion Proton™ Sequencer was utilized for high-throughput sequencing of the libraries loaded by Ion Chef™ System onto the Ion PI™ Chip v3s (Life Technologies).

The initial raw data analysis was handled by Torrent Suite Software analysis pipeline. The unmapped BAM files were then subjected to demultiplexing by the Premaitha Data Packer. The demultiplexed files were then subjected to trisomy Screening using SAGE™ LINK (Yourgene Bioscience CO. LTD.) which involves assessing for data noise, FF and gender. The z-score of each sample was used to assess quality of sequencing as well as library preparation. The reference ranges for drawing aneuploidy risk probability for chromosomes 21, 18 and 13 was $-6<Z$ score <2.8, while for the four sex chromosome aneuploidies it was $-3>Z$ score <3. Reduction in false positives and negatives was ensured for by the Yourgene algorithm which minimizes noise signals by accounting for a chromosome wide as well as genome wide view. The details of the algorithm are present in the patent application, US20160026759 (Yourgene Bioscience CO., LTD.). All personal client information and sequencing outcome was transferred from the Ion Torrent™ Server onto our local NAS device and archived.

RESULTS

A total of 513 samples were received in our laboratory for NIPT of which 465 samples passed the acceptance criteria pertaining to pre-analytical sample quality checks. A total of 9.3% were cancelled at the pre-analytical stage due to hemolysis and clot. In order to reduce cancellation rates, recommendations pertaining to proper sample mixing practice in the tube, storage and shipping of samples was done. The mean age of the cohort was 33 years and first trimester pregnancies accounted for 5.8% of the acceptable cohort while second trimester pregnancies accounted for 93%. The frequency of recommendation from routine pregnancy was detected to be 83%, while that of assisted reproductive technique like IVF was 12% and of IUI and ICSI was 0.9% each. Singleton and twin pregnancies in the cohort accounted for 94% and 3.6% respectively.

NIPT reporting by Z-score statistics involved chromosome counting method, wherein maternal cfDNA and the cffDNA is counted during trisomy screening to generate a risk score. Reporting for risk of aneuploidy in each of the chromosomes 21, 18, 13, X and Y included either low risk or high risk. The total frequency of low risk detected in our cohort was 93%. The spectrum of high risk cases reported in our laboratory includes trisomy 21, 18 as well as sex chromosome aneuploidies. No high risk for trisomy 13 has been reported as of writing this communication in our laboratory. The total frequency of high risk reported was 3%, of which trisomy 21 accounted for the highest at 43%, while trisomy 18 and sex chromosome aneuploidies were each reported at a frequency of 29%. For all the high risk cases amniocentesis confirmation was recommended from our end and follow up reports confirmed concordance for 21% while for the rest no reverts could be established. Risk outcome detected 0.4% of the high risk cases by NIPT to have exhibited positive serum screening. A total of 7% cases were detected to exhibit positive on serum screening and low risk on NIPT. A low risk frequency of 1.5% was detected among pregnancies which had history of previous newborn being affected.

Failure in quality control indices set in bioinformatics pipeline attributed to "no report" generation either due to high data noise, low reads and borderline risk scores. This was detected at a frequency of 4.1% in our acceptable cohort, of which high data noise cancellation were maximum at 53%, followed by borderline cases at 26% and low reads as well as failed library quantification at 5.2% each respectively.

FF is a crucial factor for generation of accurate NIPT reports and "no results" outcome can also occur due to low FF. In our case, no failure due to low FF was detected and the average FF in first trimester cases was detected to be 11.3% +/- 1.1, while that in second trimester case was detected to be 11.3% +/- 1.5 respectively. The analysis outcome has been summarized in Table 2.

Table 2: Summary of NIPT analysis outcome.

Sr. No.	Characteristic	Count/ Frequency
1	Total cohort	513
2	Analyzed cohort	465
3	Pre-analytical cancellation	9.3%
4	Low risk	93%
a	Mean fetal fraction	11.3% +/−1.5
b	Mean Z score - Chromosome 21	0.04 +/−1.0
c	Mean Z score - Chromosome 18	0.3 +/−1.0
d	Mean Z score - Chromosome 13	0.1 +/−0.9
e	Mean Z score - Sex chromosomes	0.6 +/−1.1
5	High risk	3%
a	Trisomy 21	43%
b	Trisomy 18	29%
c	Trisomy 13	None
d	Sex Chromosome Aneuploidy	29%
e	Mean fetal fraction	11.8% +/−1.9
f	Mean Z score - Chromosome 21	6.7 +/−2.6
g	Mean Z score - Chromosome 18	5.6 +/−1.6
h	Mean Z score - Sex Chromosomes	−2.0 +/−12.7
6	Serum screening positive and NIPT low risk	7%
7	Serum screening positive and NIPT high risk	0.40%
8	Previous child affected and NIPT low risk	1.50%
9	Singleton Pregnancy	94%
a	Low risk	94%
b	High risk	3.20%
10	Twin Pregnancy	3.60%
a	Low risk	82.30%
b	High risk	None

DISCUSSION

Our report is focused at summarizing the pilot experience of a reference laboratory with NIPT. The Prenatal Screen Sage™ (Yourgene Bioscience CO. LTD.) was utilized for library preparation and multiplexing while the NGS technology of Ion Proton™ semiconductor platform (Life Technologies) was utilized for sequencing set at 450 flows.

NIPT recommendations are restricted in the first and second trimester as an adjunct to the serum aneuploidy screening as the former delivers risk analysis with sensitivity of over 99% while the latter is still a need in the Indian scenario to asses risk for Neural Tube Defects (NTDs). The power of a whole genome NIPT assay resides on the fact that apart from detecting aneuploidies, in low coverage the assay can also report on copy number variations (CNVs) sized at less than 5 Mb, but not chromosomal imbalances sized <2 Mb.[10,11] The need to have accurate screening tools to identify missed cases of trisomies at affordable rates continue to show an upward trend. Opinions have been registered wherein use of NIPT as a first-line screening tool has been shown to reduce need for invasive tests, reduces false positive results as well as cause fewer missed trisomies in as early as 10 weeks of pregnancy.[12]

Our test inclusion criteria was a minimum of 10 weeks gestation, though the frequency of recommendations in our case was seen to be lower in first trimester at 5.8% in comparison to second trimester at 93%. Further the need for minimum FF for successful reporting in our assay was set at 3.5% and in our pilot experience generation of "no result" due to low FF was zero. The frequency of "no results" in our cohort was 4.1% due to high data noise, borderline and low reads. Pre-analytical cancellations were at a rate of 9.3% which included rejections due to hemolysis and clot. Trisomy screening in our case involved counting of overall amount of DNA assigned to each sample as well as chromosome followed by comparing each of the chromosomes 21, 18 and 13 against the total amount. Proportion of each of the chromosomes is calculated and metrics of likelihood ratio and CV calculations are applied to assess for affected and unaffected. Our analysis detected high risk for trisomy 21 to be 43% of the total 3% high risk detected in our acceptable cohort. Amniocentesis outcome follow up for all the high risk reported by NIPT yielded a concordance of 21% while the rest did not share the information. The frequency of low risk was detected to be 93% in our analysis.

FF reporting in NIPT is crucial as levels of FF have been shown to be associated with many clinical con-

ditions, especially among high risk cases. Low FF has been associated with increased risk of chromosomal aneuploidy apart from which other causes can be high maternal weight and low gestation age. The average FF reported in our cohort was detected to be 11.3% +/− 1.5. The benefits of NIPT screening though remains indisputable with respect to its sensitivity and assay deliverable, theoretically its shortfalls pertaining to detection of balanced translocations, false negatives/positives due to placental mosaics exist. However, the possibility to draw a need for invasive amniocentesis becomes clear with a high sensitivity NIPT assay as our study did detect 7% of cases reported positive by serum aneuploidy screening to be low risk by NIPT. Filtering in true high-risk pregnancies on the basis of genetic factors independent of age or any other maternal factor becomes possible through NIPT.

CONCLUSION

Our study report though pilot and small in cohort inclusion, does add to the growing amount of literature on practical outcome of use of NIPT.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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ABBREVIATIONS

cffDNA: Cell-free fetal DNA; **cfDNA:** Cell free DNA; **CMA:** Chromosomal microarray; **CNV:** Copy Number Variation; **CVS:** Chorionic Villus Sampling; **EDTA:** Ethylenediaminetetraacetic acid; **FF:** Fetal Fraction; **ICSI:** Intracytoplasmic Sperm Injection; **IUI:** Intrauterine Insemination; **NGS:** Next-Generation Sequencing; **NIPT:** Non-Invasive Prenatal Test; **NTD:** Neural Tube Defect; **PBQ:** Plate-based Quant; **TAT:** Turnaround Time; **USG:** Ultrasonography.

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