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ORIGINAL ARTICLE

An alternative to array-based diagnostics: a prospectively recruited cohort, comparing arrayCGH to next-generation sequencing to evaluate foetal structural abnormalities

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ABSTRACT

Molecular diagnostic investigations, following the identification of foetal abnormalities, are routinely performed using array comparative genomic hybridisation (aCGH). Despite the utility of this technique, contemporary approaches for the detection of copy number variation are typically based on next-generation sequencing (NGS). We sought to compare an in-house NGS-based workflow (CNVseq) with aCGH, for invasively obtained foetal samples from pregnancies complicated by foetal structural abnormality. DNA from 40 foetuses was screened using both 8×60 K aCGH oligoarrays and low-coverage whole genome sequencing. Sequencer-compatible libraries were combined in a ten–sample multiplex and sequenced using an Illumina HiSeq2500. The mean resolution of CNVseq was 29 kb, compared to 60 kb for aCGH analyses. Four clinically significant, concordant, copy number imbalances were detected using both techniques, however, genomic breakpoints were more precisely defined by CNVseq. This data indicates CNVseq is a robust and sensitive alternative to aCGH, for the prenatal investigation of foetuses with structural abnormalities.

IMPACT STATEMENT

- **What is already known about this subject?** Copy number variant analysis using next-generation sequencing has been successfully applied to investigations of tumour specimens and patients with developmental delays. The application of our approach, to a prospective prenatal diagnosis cohort, has not hitherto been assessed.
- **What do the results of this study add?** Next-generation sequencing has a comparable turnaround time and assay sensitivity to copy number variant analysis performed using array CGH. We demonstrate that having established a next-generation sequencing facility, high-throughput CNVseq sample processing and analysis can be undertaken within the framework of a regional diagnostic service.
- **What are the implications of these findings for clinical practice and/or further research?** Array CGH is a legacy technology which is likely to be superseded by low-coverage whole genome sequencing, for the detection of copy number variants, in the prenatal diagnosis of structural abnormalities

KEYWORDS

Next-generation sequencing; copy number variation; foetal structural anomaly

Introduction

For several decades, invasive prenatal genetic testing has been offered to women at an increased risk of having a child with a chromosome abnormality. While G-banded karyotypes, which typically offer a resolution of between 5–10 Mb, were the original gold-standard assay, this technique has now largely been superseded by array comparative genomic hybridisation (aCGH). By using aCGH genomic copy number, variants can be detected at an increased resolution to karyotyping, allowing the identification of smaller deletions and duplications than was previously possible (Bi et al. 2008; Hillman et al. 2011; Park et al. 2011; Breman et al. 2012; Wapner et al. 2012). Consequently, higher diagnostic detection rates have been reported for aCGH analysed cohorts of foetuses with structural abnormalities (American College of Obstetricians and Gynecologists Committee on Genetics 2013; Hillman et al. 2013; Saldarriaga et al. 2015).

Recent advances in genomic analysis have been dominated by next-generation sequencing technologies, with successive models of Illumina instrumentation leading the field in terms of data volume and sequence quality. This has resulted in both diagnostic and research laboratories developing a range of novel sequence-based assays and informatics solutions. One such workflow, CNVseq, utilises low-coverage whole-genome sequencing for the detection of copy number variants (CNVs). Sequence reads are aligned to

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Supplemental Data for this article can be accessed here.

a reference genome and read counts are compared between a test sample and 'normal' reference control (Chiang et al. 2009; Xie and Tammi 2009; Yoon et al. 2009; Zhao et al. 2013). Our laboratory has implemented this technique as a UK Genetic Testing Network approved assay that is currently being used for the molecular diagnosis of postnatally ascertained cases (Hayes et al. 2013).

As part of our ongoing research and development initiative, we undertook a proof-of-concept study demonstrating how CNVseq can be usefully applied to foetal material from which poor quality DNA yields precluded an aCGH result (Cohen et al. 2015). The resolution of the abnormalities detectable from these data was limited by the per-run sequencing output, generated at that time, from an Illumina GAIIx. As NGS instrumentation has improved, the per-run data yield has increased, a result of both extended read lengths and an increased number of clusters sequenced per run. In addition to increasing the resolution of detectable abnormalities, falling per-base sequencing costs are making sequence-based assays ever-more affordable.

We aimed to report our experience using a CNVseq informatics workflow, in combination with an updated sequencing platform, to assess genomic copy number variants in a cohort of patients prospectively recruited with foetal structural abnormalities.

Materials and methods

We present a cohort of 53 prospectively recruited cases from our centre, a tertiary referral foetal medicine unit, which covers a population of approximately 5 million people across the Yorkshire and Humber region of Northern England. Our study inclusion criteria included either; (i) one or more structural anomalies identified on an ultrasound scan, (ii) an isolated nuchal translucency of $\geq$3.5 mm, (iii) two or more ultrasound variants. The patients underwent an invasive testing comprising either amniocentesis or chorionic villus sampling (CVS). Each participant was counselled in person and provided with a detailed patient information leaflet. Phenotypic descriptions were collected on anonymised data collection forms with a unique patient identifier. Women under the age of 16 were not eligible for recruitment. Ethical approval for this study was granted by the Bradford Leeds Research Ethics Committee (reference: 15/YH/0508).

DNA was extracted from prenatal samples using QIAamp DNA micro (QIAGEN Ltd., Manchester, UK) and iGENatal (IGEN Biotech, Madrid, Spain) extraction kits. Tissue cultures were established for samples with low concentration DNA extractions, as determined using a Quibit® fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Diagnostic quantitative fluorescence (QF)-PCR was performed to exclude aneuploidy of chromosomes 13, 18 and 21, triploidy and monosomy X. Array-CGH was subsequently processed on a BlueGnome ISCA 8 × 60 k oligoarray, following the manufacturer's protocols (Illumina Inc, San Diego, CA, USA). The data was analysed using BlueFuse Multi Software Version 4.1. Identified variants were reported according to the criteria defined by the Association for Clinical Genetic Science, the Royal College of Obstetricians and Gynaecologists, the American College of Medical Genetics standards and the guidelines and local policy (Association for Clinical Cytogenetics. 2009; Kearney et al. 2011; The Royal College of Pathologists. 2015). The expected values for the quality control metrics calculated by the BlueFuse software are detailed in Supplementary Table I. All of the samples not meeting these criteria were classified as being 'suboptimal' (Table 1) (Illumina 2014).

In addition to aCGH, each sample was processed using a previously validated CNVseq workflow (Watson et al. 2014). Briefly, 200 ng (batches 1, 2 and 4) or 500 ng (batch 3) of genomic DNA, quantified using a Qubit® dsDNA Broad Range Assay (Thermo Fisher Scientific, Waltham, MA, USA), was sheared using a Covaris S2 (Covaris Inc., Woburn, MA, USA). The fragment size was assessed using an Agilent Bioanalyzer high sensitivity chip (Agilent Technologies Ltd., Stockport, UK). Illumina compatible whole genome sequencing libraries were prepared using NEBNext® Ultra™ reagents (New England Biolabs, Ipswich, MA, USA). AMPure bead size selection producing a library insert size of approximately 200 bp was performed. The end-repair and adaptor ligation were undertaken as outlined in the manufacturer's protocol. The quality and concentration of each final library were determined using an Agilent Bioanalyzer and a Quant-iT™ Picogreen® assay (Thermo Fisher Scientific, Waltham, MA, USA). Equimolar concentrations of 10 libraries (11 for batch 4), were pooled for sequencing. This was performed using single-end 51 bp reads across two lanes of a HiSeq2500 Rapid flowcell (Illumina Inc., San Diego, CA, USA). Raw sequence data was converted to FASTQ.gz format using

Table 1. Categories for the overall classification of each aCGH assay.

Category	Explanation	Number of specimens
No clinically significant imbalance	No imbalances detected or known benign polymorphisms. Limited to imbalances <150 kb within a gene region and <200 kb outside a gene region.	29
Array failed	No result obtained.	0
Suboptimal array	This was used when quality metrics were suboptimal but consensus opinion was that the array was reportable. Reported results were qualified with the statement: 'The quality of the data was slightly outside quality control guidelines. Although this is unlikely to have compromised the validity of the study we cannot exclude the possibility that very small imbalances may not have been detected.'	7
VOUS	Variant of uncertain clinical significance.	0

Table 2. Genomic positions of clinically significant variants identified in the aCGH and CNVseq processed samples. Genomics coordinates are reported for human genome build hg19.

Sample number	Clinical features	Karyotype	aCGH min positions			aCGH max positions			CNVseq window boundaries		
			Start	Stop	Size (Mb)	Start	Stop	Size (Mb)	Start	Stop	Size (Mb)
CS19	IUGR, posterior fossa abnormality, echogenic bowel	10p deletion	136,391	20,825,562	20.7	1	21,431,063	21.4	73,503	20,888,107	20.8
CS25	Echogenic kidneys, polyhydramnios	17q12 deletion	34,856,085	36,248,889	1.4	34,611,377	36,510,769	1.9	34,851,278	36,249,761	1.4
CS27	Bowel dilatation, polyhydramnios	22q11 deletion	18,894,865	21,540,318	2.6	18,847,965	21,561,492	2.7	18,886,235	21,461,135	2.6

min: minimum; max: maximum; Mb: Megabase; IUGR: Intrauterine growth restriction.

CASAVA Version 1.8.3. Sequence reads from each sample were aligned to an indexed human reference genome (hg19) using bwa aln Version 0.6.2 (http://bio-bwa.sourceforge.net) (Li and Durbin 2009). The duplicate reads were marked and removed from coordinate sorted BAM files using Picard Version 1.85 (http://broadinstitute.github.io/picard/). The genomic coordinates of uniquely mapped test and reference reads (those with a MAQ value $\geq$37) were extracted using samtools Version 0.1.18 (https://sourceforge.net/projects/samtools) (Li et al. 2009). Read counts were adjusted to account for variations in local GC% and the resulting output was loaded into the R module DNA copy Version 1.32.0, which segments the data into regions of an equal copy number (Venkatraman and Olshen 2007). Quality control criteria for CNVseq analyses were based on empirically determined metrics established while validating our post-natal CNVseq workflow.

Genomic databases including OMIM (https://omim.org), the Database of Genomic Variants (http://dgv.tcag.ca) (MacDonald et al. 2014), and Decipher (https://decipher.sanger.ac.uk) (Firth et al. 2009) were used to determine the clinical significance of variants identified by both the aCGH and CNVseq workflows.

Results

Fifty-three women consented to participate in the study between January and August 2016. An abnormal QF-PCR result was obtained for thirteen samples and these were excluded from further analysis (Supplementary Table II).

The remaining 40 samples were analysed using both the aCGH and CNVseq workflows (sample CS08 was repeated as initial output data was uninterpretable). Twenty-three samples were obtained by amniocentesis and 17 by CVS. Thirty-two were used directly while 8 required culturing to increase the total cell count prior to DNA extraction (6 samples were obtained by amniocentesis and 2 were obtained by CVS). Of the 8 specimens that required culturing, 3 produced suboptimal microarray results, yet robust CNVseq results were generated from all of the samples.

The DNA concentration of extracted samples ranged from 13–656 ng/μl. For 11 samples these were categorised as being low ($<$100 ng/μl). These samples provided an insufficient mass of DNA to meet the suggested input requirement for CNVseq library preparation. Despite this, all of the low concentration DNA samples provided robust CNVseq results and only 1 had a suboptimal array result.

Seven of the 40 microarray results were determined to be of 'suboptimal' quality for reporting purposes following the application of manufacturer recommended quality control parameters and review of these data by experienced cytogeneticists. A mean of 4 (range 1–8) variant calls per case was generated by the BlueFuse Multi algorithm. These automatic calls included variants smaller than our reporting size threshold.

The per-batch sequencing metrics for all of the CNVseq data are summarised in Supplementary Table III. The intra-batch per-sample read distributions were most dispersed for pool 1 (range 6.6–16.0%) and tightest for pool 3 (range 8.5–11.2%). The CNVseq assay resolution is dependent on the number of uniquely mapped reads per-sample; the minimum number was 17.4 million reads (maximum 46.5 million; mean 28.2 million) providing a minimum average resolution of 17 kb (maximum 46 kb; mean 29 kb) (Supplementary Table IV). The mean number of calls generated per-sample was 38. Sample CS01 was an apparent outlier, having 191 calls, of which only 46 were $>$30 kb. The median number of calls per case for the CNVseq cohort was 33. This increased the number of calls, identified in comparison to the aCGH dataset, is not surprising given the non-targeted nature of these data.

Excluding benign CNVs, and those CNVs that did not intersect disease-causing genes, clinically significant sub-chromosome-level imbalances were identified in 3 cases (Table 2). The detection of identified CNVs was concordant between both aCGH and CNVseq datasets. A low-level trisomy 2 mosaicism was evident in foetus CS12, which presented with coarctation of the aorta. As the sample was obtained by CVS, and in light of the unlikely foetal phenotype, it was reported as a likely confined placental mosaicism (Figure 1).

Case CS19 presented with intrauterine growth restriction, posterior fossa abnormality and echogenic bowel. Both the aCGH and CNVseq workflows identified a heterozygous terminal-arm deletion of 21 Mb between 10p12.31 and 10p15.3 (Figure 2). This region encompasses 88 genes of which 16 are listed as pathogenic in the OMIM database. This was the only terminal-arm deletion identified in the cohort.

The smallest clinically significant variant was identified in case CS25 which presented with echogenic kidneys and polyhydramnios. Although the aCGH quality control metrics classified the data from this array as being of suboptimal quality, the 1.4–1.9 Mb interstitial deletion, located at 17q12, was clearly distinguishable. Furthermore, the presence of this variant was corroborated by the CNVseq data (Figure 3). This phenotype has been previously described secondary to mutations or deletions of *TCF2* (OMIM: 137920) (Gilboa et al. 2016).

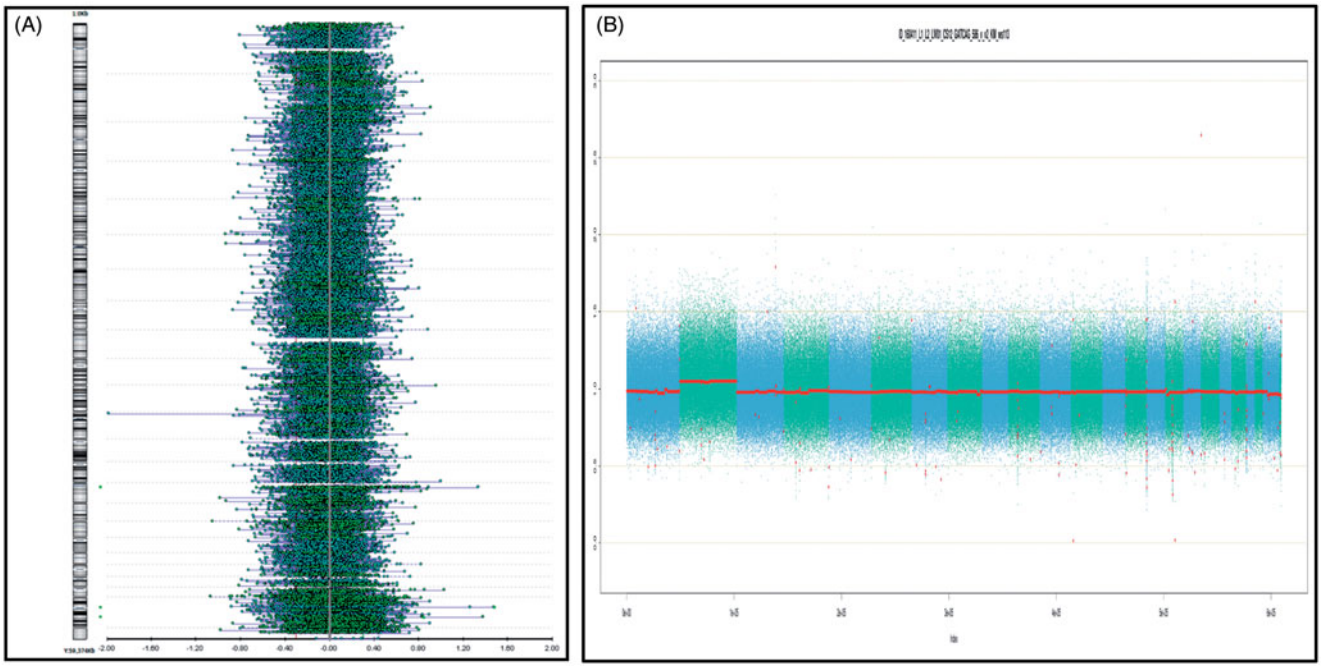

Figure 1. Case CS12 showing (A) A BlueFuse Multiview data points across all chromosomes and (B) a comparable karyogram view from the CNVseq workflow. The low-level trisomy 2 is detectable by the raised baseline segmentation for this chromosome (horizontal red lines).

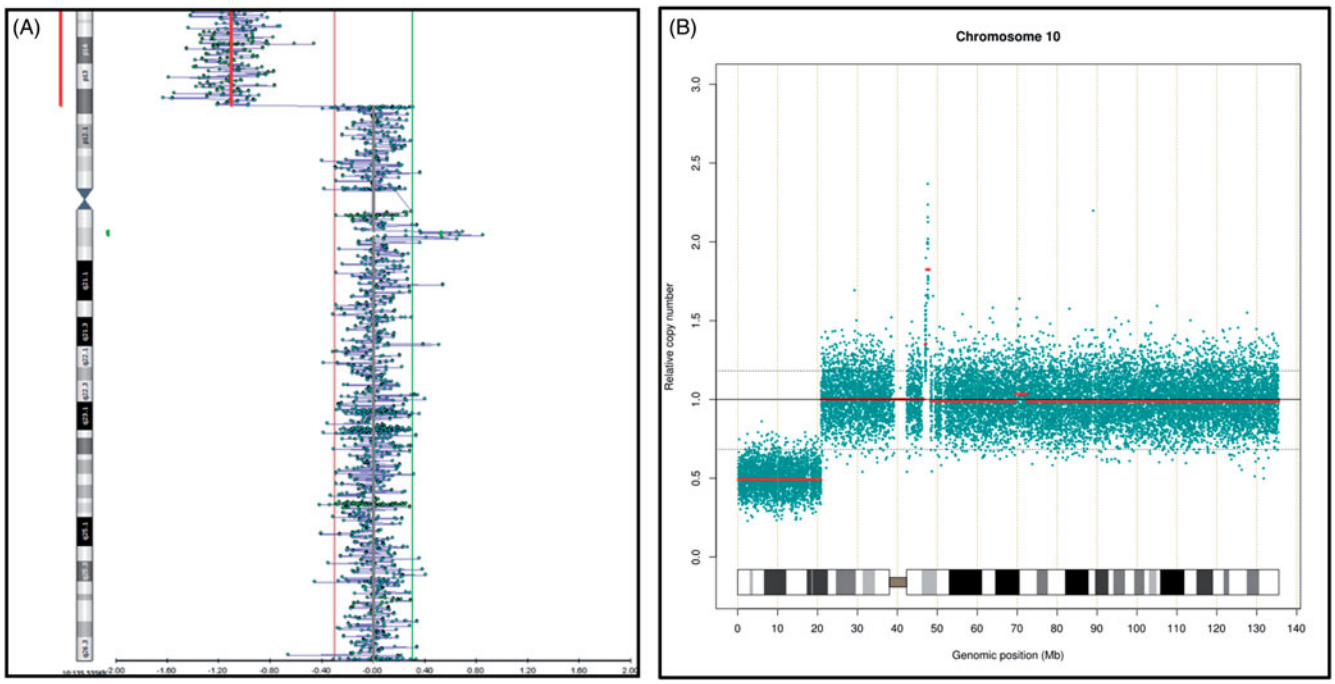

Figure 2. Case CS19 showing (A) a heterozygous terminal deletion of the chromosome 10 p-arm from the BlueFuse Multi software. (B) The corresponding deletion as detected by the CNVseq workflow.

A further copy number imbalance was detected in case CS27, a foetus that presented with bowel dilatation and polyhydramnios. The 22q11 deletion was ≈2.6 Mb in size (Figure 4). This microdeletion syndrome, also referred to as Velocardiofacial or Di George Syndrome, encompasses more than 50 genes and is a known pathogenic variant likely to be responsible for the ultrasound features seen in this case.

Discussion

We aimed to report our experience using a CNVseq informatics workflow in the assessment of genomic copy number variants in a cohort of patients prospectively recruited with foetal structural abnormalities and have successfully done so.

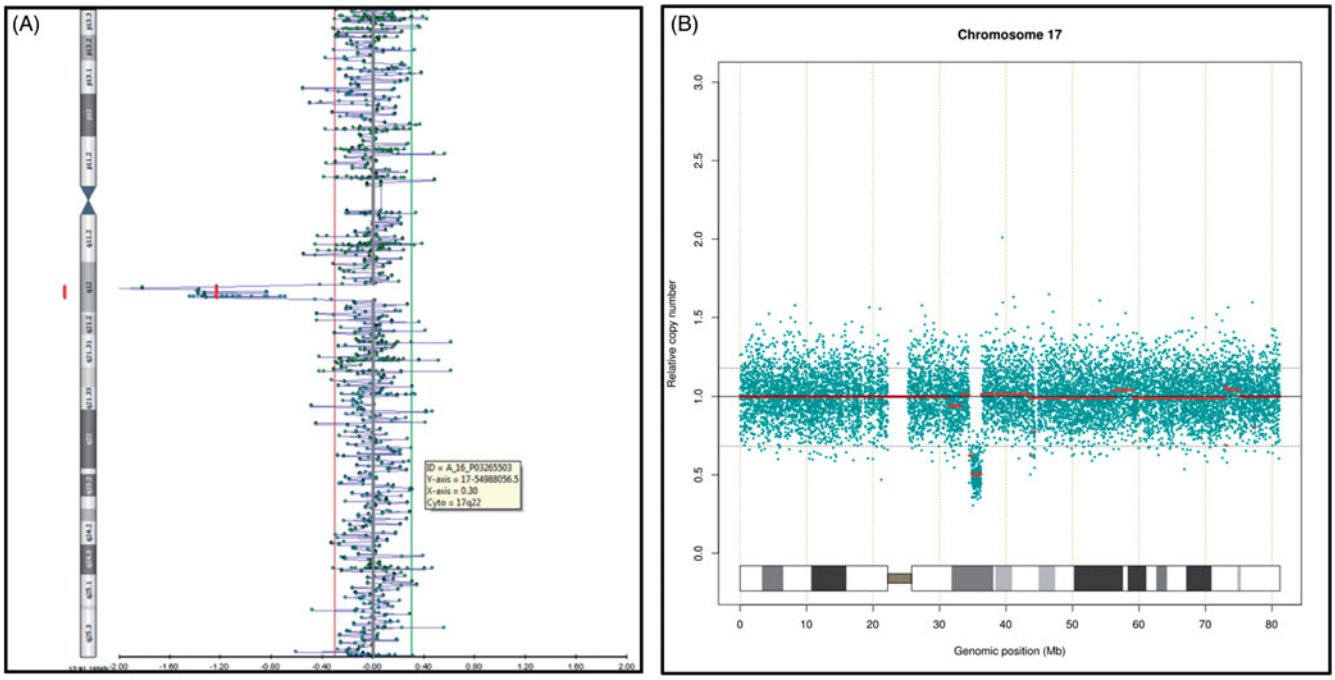

Figure 3. Case CS25 showing (A) a heterozygous interstitial deletion on chromosome 17 from the BlueFuse Multi software. (B) The corresponding deletion as detected by the CNVseq workflow.

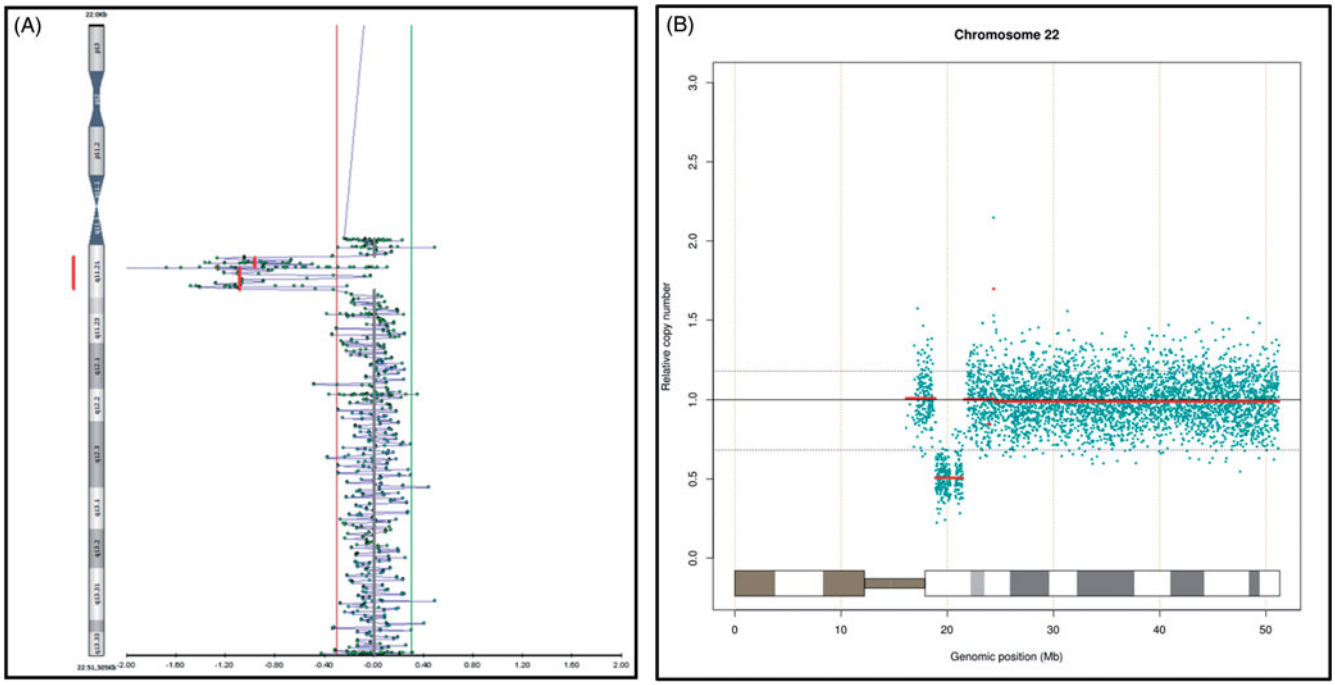

Figure 4. Case CS27 showing (A) a heterozygous interstitial deletion on the chromosome 22 q-arm using the BlueFuse Multi software. (B) The corresponding deletion as detected by the CNVseq workflow.

This study has demonstrated that NGS-based CNV-Seq technology can be used to investigate structurally abnormal foetuses with comparable genomic resolution, quality control metrics and turnaround times as array CGH in the prenatal setting. CNV-Seq successfully identified all of the genomic variants detected by array CGH, with a similar resolution was achieved despite the batch processing of ten samples in the CNV-Seq workflow. This potential for high-throughput analysis is an advantage of sequencing-based analysis over array CGH.

In the cases where a clinically-significant imbalance was detected, genomic breakpoints were more easily defined using CNV-Seq. The digital nature of sequencing-based technology allows exact genomic positions to be elucidated, and genomic breakpoints and variant sizes can be inferred from these positions. Deleted probes on the array platform may

fall out with of the actual genomic breakpoints and impact on the $\log_2$ ratio for that region, reducing the ability to accurately pinpoint genomic size or positions. Such information may be of clinical value, especially where breakpoints are close to or intersect with clinically-important genes. It has been reported previously that low-level mosaicism is easier to identify using a CNVseq, rather than aCGH workflow (Grotta et al. 2015). The specimen with confined placental mosaicism in this cohort was more easily visualised in the CNVseq karyogram compared to the array result. However, it is not possible to be certain of this when only a single mosaic sample has been analysed.

One of the chief advantages is the ability to influence platform resolution by *in silico* manipulation of window size, which can be performed after processing. This is not possible using an aCGH platform where genomic resolution is fixed by the probe density defined by the reagents. CNV-Seq resolution is also adjustable by alterations in the number of samples processed within the batch; a smaller number of samples will share the same number of reads, increasing the read-count per patient and allowing smaller imbalances to be detected. Higher order multiplexing of tens of samples is possible but at the cost of reduced platform resolution. Such compromises may be important if the desired analysis was limited to large-scale genomic abnormalities such as trisomies or large deletions and duplications. Overall, our current CNVseq workflow configuration produces a per-patient resolution that significantly exceeds that obtained from the aCGH assays.

For the purposes of this study, the genomic resolution was chosen to mimic that of the array CGH platform across the genome represented by probes within. In this study, no additional clinically-significant variants were detected by the CNV-Seq platform, but a larger number of detected calls were made by the analysis pipeline when compared to array CGH. This is not surprising given the non-targeted nature of the platform. Importantly, the CNV-Seq platform did not generate any false positive calls which would have impacted on the clinical management of the foetal structural abnormality, and the majority of the calls were below the pre-defined thresholds for further investigation.

This increase in potential calls has an effect on the post-processing time required to analyse and report a CNVseq sample although the processing time for the technology was similar for both platforms. Strategies could be employed to further reduce analysis time, improving clinical utility of this platform. Filters can be introduced to remove frequently-occurring benign copy number variants, and reporting thresholds altered to limit the requirement for molecular confirmation studies. It is important to note that despite the increased genomic resolution of the CNVseq workflow, no additional variants of uncertain significance were detected. This finding will provide reassurance to clinicians who are naturally wary of the challenges posed by prenatal variants of uncertain significance.

The strengths of this study include the introduction of a novel technology into the prenatal setting, offering a credible alternative to array CGH analysis. Although beyond the scope of this work, analysis of post-mortem samples or DNA from pregnancy loss tissue such as stillbirths may also benefit from this technology, especially when DNA is fragmented or in small quantities. Currently, failed array experiments lead to a 'no result' clinical report. The addition of NGS-based technology allows repeat analysis and potentially, increased information for parents.

The study is limited by the number of participants recruited, which resulted in a small number of clinically-significant variants to base an analysis upon. The relative infrequency of genomic abnormalities in structurally abnormal foetuses is a barrier common to most studies of genomic analysis. The initial capital costs of establishing a CNVseq workflow are undoubtedly more significant than those associated with purchasing a microarray scanner. Despite this, many regional genetics laboratories in the UK are now equipped with sequencing core facilities that provide an opportunity to perform high throughput NGS.

Conclusion

We demonstrate CNVseq to be a reliable and robust alternative to aCGH when used for the prenatal diagnosis of structural abnormalities. Sequencing has a similar turnaround time and comparable detection rate of copy number variants when compared to aCGH. The most significant initial difficulty to widespread implementation is the high capital cost of establishing the required next-generation sequencing infrastructure. As large, population-scale programmes such as the 100 K Genome Project are conducted, it is likely that sequencing based-methods will become a first-line test for prenatal diagnostics in the near future.

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No potential conflict of interest was reported by the authors.

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