Bacterial artificial chromosomes (BACs)-on-Beads™ as a diagnostic platform for the rapid aneuploidy screening of products of conception

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Abstract. The aim of the present study was to evaluate the use of KaryoLite™ bacterial artificial chromosomes (BACs)-on-Beads™ (BoBs) technology for the rapid screening of products of conception (POC). Validation and prospective studies were carried out on 85 and 95 patient samples, respectively. Validation studies had previously been analyzed using routine culture and G-banded karyotyping. BoBs resulted in an abnormality detection frequency of 27%, with a failure rate of <3%. The time required for processing was significantly lower compared with that of tissue culture. In conclusion, BoBs technology decreased the failure rate, while increasing the analytical sensitivity compared with G-banded karyotype analysis alone. Additionally, significant cost savings may be achieved with regard to the time of processing and analysis of specimens.

Introduction

Molecular diagnostic laboratories are routinely tasked with the cytogenetic analysis of tissues following miscarriage or intrauterine death. Compared with healthy prenatal specimens, such as amniotic fluid and chorionic villus, tissue culture failure for products of conception (POC) is a major problem. The non-viability of tissue following fetal demise, fungal contamination and bacteriological infection is a common cause of culture failure, resulting in an inability to obtain a conventional karyotype result in up to 40% of specimens (1).

Cytogenetic techniques that are not dependent on actively growing tissue are becoming more common, including quantitative fluorescence (QF)-PCR (2,3), multiplex ligation-dependent probe amplification (MLPA) (4,5), molecular karyotyping (6) and bacterial artificial chromosomes (BACs)-on-Beads™ (BoBs) (7-11). Of these techniques, BoBs offers the ability to assess small batch sizes at a relatively low cost with comprehensive coverage of all the chromosome arms, whilst complementing the highly interrogative array-based methods that are used in molecular karyotyping.

Briefly, BoBs technology is equivalent to a low-density whole-genome array in which beads replace a standard solid phase two-dimensional surface. Three BACs encompassing a small region of the human genome are attached to each of the beads, which are labeled with defined amounts of two dyes to provide a unique spectral signature. This allows an array comprising 96 uniquely labeled beads carrying defined human DNA fragments of a known genome location to be constructed. The DNA of a patient, which is labeled with biotin, is hybridized with the beads and any bound patient DNA is quantitated by hybridization with a fluorescent reporter.

The labeled beads are individually assessed using a Luminex platform (Luminex Corporation, Austin, TX, USA) to identify their spectral signature, together with their fluorescence intensity. The mean fluorescence intensity of the sample is compared with that of two controls (one male and one female; Promega, Madison, WI, USA). The analysis is performed using BoBsoft™ software (PerkinElmer Life Sciences Wallac, Turku, Finland), which converts the data to a numerical ratio. Normal diploid loci exhibit a ratio of 1.0, single copy gains exhibit a ratio of 1.3-1.4 and single copy deletions exhibit a ratio of 0.6-0.8. The advantages and disadvantages of this technique are summarized in Table I (11).

Due to the high failure rate of culturing cells from POC samples, we aimed to evaluate the efficiency of BoBs technology as a routine diagnostic assay in a clinical setting.

Materials and methods

Validation study. Ninety-three specimens from 85 patients were processed in the validation study. These specimens comprised 55 chorionic villi, 20 umbilical cord, five formalin-fixed paraffin-embedded tissue (FFPET), four fetal membrane, skin and cultured cell samples and one whole-blood sample. The tissue samples were microscopically dissected and 5-30 mg of chorionic villus was disassociated in a mixture of 1.25%

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trypsin in PBS/Versene, 0.2 mg/ml collagenase (C1889; Sigma, St. Louis, MO, USA) and 20% fetal calf serum in Ham's F10 medium. Similar quantities of other tissues were disassociated in collagenase only. An aliquot from each specimen was placed in a micro-centrifuge tube and stored at -20˚C for up to 3 weeks. This approach was used to ensure the efficient batching of samples while maintaining the 28-day turnaround time mandated by the National Pathology Accreditation Council (NPAAC). The remaining specimens were used to produce slides for subsequent confirmation by fluorescence *in situ* hybridization (FISH). Slides were frozen at -20˚C until they were required for use. Explant and/or cell suspension cultures were established on all the specimens for routine G-banded karyotyping.

DNA extraction from defrosted cell suspensions was performed using a column-based procedure (ZR Genomic DNA™ Tissue MiniPrep; Zymo Research, Orange, CA, USA) according to the manufacturer's instructions, and DNA was recovered in 25 μl elution buffer. The DNA from FFPET was extracted using a standard phenol-chloroform procedure after the specimen was dewaxed using xylene. The eluted DNA concentration and purity were assessed using a NanoDrop1000 spectrophotometer (Thermo Scientific, Thermo Electric North America LLC, Waltham, MA, USA).

KaryoLite™ BoBs was performed according to the manufacturer's instructions (PerkinElmer Life Sciences Wallac, Turku, Finland) and the beads were analyzed using a Luminex 100 platform equipped with a BMD FIDIA system and MLX booster™ software (Biomedical Diagnostics, Marne La Vallee, France). Two male and two female controls were included in each session for normalization. The results were analyzed and converted to numerical values using BoBsoft 1.0 software (PerkinElmer Life Sciences Wallac). Mosaicism and maternal cell contamination yielded ambiguous results, with ratios that differed from those expected. Suspected mosaicism was confirmed using FISH. Standard karyotyping was performed on all the viable tissues and the results were compared with the BoBs data. Abnormal results that were not detected by G-banding were confirmed by FISH, using locus-specific probes.

Prospective study. Specimens from an additional 95 patients were prepared as outlined above, with the exception that a small amount of cell suspension was cultured as a back-up only. This was not processed further unless required for the confirmation of dosage changes.

Statistical analysis. ezANOVA (McCausland Centre, Columbia, SC, USA) and SigmaXL (version 2.1; Sigma XL Inc., Toronto, Canada) programs were used for data analysis. A non-parametric Kruskal-Wallis test was also performed (raw data not presented).

Results

Validation study. The validation study yielded 63 normal results with approximately equal male/female ratios. Since 18 tissues failed to grow in culture, the results were verified by FISH alone. Thirty abnormal results were obtained, including two samples in which results were obtained from >1 tissue type. This gave an adjusted abnormality frequency of 32.9%, which is skewed as a number of tissues were selected on the basis of an abnormal G-banding result in order to validate the BoBs technique.

Table I. Advantages and disadvantages of the BACs-on-Beads™ technology.

Advantages	Disadvantages
May be used on non-viable, paraffin-embedded (PET) and fixed tissues.	Unable to identify maternal cell contamination.
Identifies small, unbalanced, sub-telomeric rearrangements.	Unable to identify balanced rearrangements, including translocations, inversions and Robertsonian translocations.
Detects mosaicism in >20-30% (no artefacts since cell culture is not required).	Unable to identify mosaicism in <20%.
Turnaround time (TAT), <24 h[a].	Samples require batching.
Able to analyze 100 regions in one test.	
Minute quantities of DNA are required (usually 125 ng).	
Able to run tens of samples simultaneously.	

[a]While TAT is <24 h, this is off-set by the need to batch samples. BACs, bacterial artificial chromosomes.

The majority of abnormalities were identified as common aneuploidies associated with fetal loss, namely trisomies 13, 16, 18, 21 and 22 (Table II). Approximately 30% of the abnormal results represented an abnormal ploidy level. BoBs was unable to unequivocally identify these and thus required FISH verification. Among the discrepant results, an unbalanced translocation, described by G-banding as 46, XY, der(6) t(6;10)(p25.1;q25.2), was identified to be male with additional 10qter, and a marker from a case with a 92,XXX-X,+mar karyotype was shown to be 9qter material. A double trisomy (male +14, +22) was identified by BoBs and verified using FISH in 100% of ≥50 cells that were examined, while G-banding on cultured cells identified only the trisomy 14 (47,XY,+14); the additional chromosome 22 was not observed in any of the metaphase cells examined. A gain of 20qter was identified by BoBs and verified by FISH; however, this gain was likely to be below the resolution of the light microscope as G-banding identified it as a normal female.

Prospective study. The prospective study involved an additional 95 patients and yielded 71 normal results with a slightly higher female/male ratio and an overall adjusted abnormality frequency of 27%. As experienced in the validation study, the commonly encountered trisomies associated with pregnancy loss dominated the range of abnormal results. A trisomy 18 case also had an apparently balanced translocation between

Table II. Details of abnormalities identified in the validation and prospective studies.

	Method of analysis and/or confirmation		
Abnormality	G-banding	KaryoLite™ BoBs™	FISH
Validation study			
2x male trisomy 13	47,XY,+13	M+13	No
Male trisomy 16	46,XY,+16	M+16	No
2x female trisomy 16	47,XX+16	F+16	No
2x male trisomy 18	47,XY,+18	M+18	No
Female trisomy 18	47,XX,+18	F+18	No
2x female trisomy 21	47,XX,+21	F+21	No
4x male trisomy 22	47,XY,+22	M+22	No
Female trisomy 22	47,XX,+22	F+22	No
Monosomy 21	45,XY,-21	M-21	Yes
Monosomy X	FFPET (no karyotype)[a]	F-X	Yes
Triploidy	Culture failed	Ambiguous M? XXY	Yes - triploidy XXY
3x triploidy	1x culture failed, 2x FFPET	F	Yes - triploidy XXX
Triploidy	FFPET[a]	M	Yes - triploidy XXY
Triploidy	FFPET[a]	M	Yes - triploidy XYY
t(6;10) unbalanced	46,XY,der(6)t(6;10)(p25.1;q25.2)	-6ptel; +10qtel	Yes
Male trisomy 14 and 22	47,XY,+14	M+14,+22	Yes (on direct specimen)
Tetraploidy	92,XXX,-X, +mar	F+9qtel	No
Trisomy 5	47,XX,+5	F+5	No
Add 19q	Culture failed	F+19qtel	No
Dup 20q	Culture failed	M+20qtel	Yes
Prospective study			
Male trisomy 13	47,XY,+13	M+13	Yes
Female trisomy 13	Culture failed	F+13	Yes
Male trisomy 16	47,XY,+16	M+16	No
Female trisomy 16	47,XX,+16	F+16	No
Male trisomy 18	47,XY,+18	M+18	Yes
Female trisomy 18	N/A	F+18	Yes
Female t(5;10) + T18	47,XX,t(5;10) +18	F+18	Yes
2x male trisomy 21	47,XY,+21	M+21	Yes
Female trisomy 21	47,XX,+21	F+21	Yes
Female trisomy X, trisomy 21	48,XXX,+21	F+21	Yes
2x male trisomy 22	47,XY,+22	M+22	No
Female trisomy 22	47,XX,+22	F+22	No
Monosomy X	45,X,-X	F-X	Yes
Triploidy	69,XXX	F	No
2x triploidy	1x culture failure, 1x FFPET[a]	M	Yes - triploidy XXY
Triploidy	FFPET[a]	M	Yes - triploidy XYY
Tetraploidy	96, XXXX	F	Yes - tetraploidy XXXX
Female trisomy 15	47,XX+15	F+15	No
Female trisomy 17	47,XX,+17	F+17	No
Female dup 4p	46,XX	F+4ptel	Yes
Male dup 14q	46,XY, add 4p	M+14q	Yes

[a]Formalin-fixed paraffin-embedded tissue (FFPET) only; fresh tissue was unavailable for karyotyping. BoBs™, bacterial artificial chromosomes (BACs)-on-Beads™; FISH, fluorescent *in situ* hybridization.

chromosomes 5 and 10; however, this was not identified by BoBs. Three aneuploidies (XO, trisomy 13 and monosomy 21) revealed slightly ambiguous results due to high correlation coefficient values (CV; 8.5-10.9), which may have occurred as a result of the quality of DNA extracted from the tissues. We also identified two samples that showed an XYY signal pattern

Table III. Retrospective analysis of G-banding data between 2006 and 2010 (inclusive).

Variable	G-banded analysis (actual)	BoBs™ /FISH (predicted)
Processed samples, n	1573	1573
Successfully analyzed samples, n (%)	1277 (80)	1525 (97)
Abnormal samples, n (%)	353 (27.6)[a]	424 (27)
Samples failed to be analyzed, n (%)	296 (20)	47 (3)
Potentially non-concordant results		
Case 1	46,XX, t(7;10)	XX
Case 2	46,XY/45,X,t(X;15)	XY/XX mos
Case 3	46,XY, del(4)(q21q21)	XY
Case 4	47,XX,del(5)(p13),+mar	XX, del(5pter), + marker identified
Case 5	48,XX, +16 + mar	XX, trisomy 16, + marker identified

[a]22.4% of total processed samples. BoBs™ , bacterial artificial chromosomes (BACs)-on-Beads™ ; FISH, fluorescence *in situ* hybridization.

of <2% on direct FISH due to extensive maternal contamination, which were later confirmed as entirely XYY by FISH probes applied to the paraffin-embedded tissues. A G-banded karyotype showing an add (4p) was characterized as a gain of 14p. The remaining abnormalities are shown in Table II.

Alkuraya *et al* (12) reported a high incidence of cryptic subtelomeric abnormalities in miscarriage products. Therefore, we anticipated a greater number of abnormal occurrences in telomeric regions compared with other chromosomal regions analyzed by the BoBs, even when FISH was unable to verify them. Notably, statistical analysis of all the abnormal calls by the BoBs software showed that there was no significant difference between subtelomeric calls and other chromosomal regions. Eight chromosomes were revealed to account for ~60% of the calls; however, this group also included the chromosomes most often encountered in pregnancy loss, namely chromosomes 16, 18, 20, 21 and 22 (raw data not shown).

Retrospective study. Following the validation and prospective studies, a retrospective analysis of our earlier G-banding data for the years 2006-2010 (inclusive) was performed (unpublished data). A total of 1,573 samples were processed by the laboratory. The overall culture failure rate was 20% and the abnormality frequency was 22.4%. FFPET samples that were received for ploidy studies were not included in this analysis.

We hypothesized that BoBs would correctly identify all the aneuploids and unbalanced karyotypes that were detectable by light microscopy. We predicted that five cases with abnormal karyotypes would yield non-concordant results using BoBs technology (Table III). Case 1 was a balanced translocation, case 2 was a mosaic with two cell lines, one of which was a translocation and case 3 was an interstitial deletion. These three cases represent <0.2% of the total cases processed. The remaining two cases involved abnormalities that BoBs was expected to detect. In both cases BoBs would also be expected to identify the origin of the marker chromosome by detecting increased dosage of centromeric material, provided the cell line containing the marker was above 30% in the uncultured sample. Bearing in mind, almost 300 cases did not yield any results due to tissue culture failure and assuming the BoBs failure and abnormality pick-up rates were correct at the time, we hypothesized that the use of BoBs may have identified an additional 60 abnormal results.

Discussion

BoBs technology reduced the failure rate in our laboratory from 20 to ~3% and increased the number of detectable abnormalities. The low failure rate was largely due to degraded DNA that was unsuitable for further analysis. There was a high concordance between BoBs and standard karyotyping and the only disadvantage to the technology was its inability to unequivocally detect differences in ploidy level. Samples with a 69,XXX karyotype were routinely identified as normal females, and 69,XXY could not be distinguished from normal male fetal specimens contaminated with maternal cells. To overcome this problem, all the referrals were screened by first applying routine aneuscreen FISH probes. Thus, not only was the identification of differences in ploidy level possible, the common aneuploids often associated with pregnancy loss were also identified. This strategy also allowed samples to be screened to a certain degree for maternal cell contamination prior to assessment on the Luminex platform. For example, samples demonstrating a low level male gender complement were not processed further by BoBs unless alternative tissue was able to be sourced. Consequently, this approach is not possible using FISH alone when the fetal tissue is female.

The BoBs assay was successful in characterizing unknown material as a result of unbalanced translocations and marker chromosomes. Such anomalies may be difficult to identify by conventional methods and often require interrogation using a number of FISH probes or array technology, which significantly increases costs. Therefore, loss or gain of material is able to be identified, but not the chromosome/s that may be involved in a balanced translocation.

The manufacturer's analysis guidelines state that acceptable CV values are <8, with values of <6 being optimal. This guideline is based on the use of DNA extracted from fresh prenatal samples; however, it is questionable whether it is

possible to routinely extract the same quality of DNA from POCs. In cases where there has been intrauterine death and a delay of unknown length occurs before the sample is sent to the laboratory for testing, there is the potential for DNA degradation. Samples with higher CV values are difficult to analyze due to the potential for false positives; therefore, caution is required.

In addition to the reduced failure rate and increased abnormality pick-up rate, there are also economic benefits to using the BoBs assay. Tissue culture is labor-intensive and routine karyotyping by skilled personnel is time-consuming and frequently made more so by the typically poor chromosome morphology of post-mortem tissue. In a busy laboratory with constantly increasing numbers of referrals and limited resources, this advantage is crucial.

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