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Amplification of X- and Y-Chromosome-Specific Regions from Single Human Blastomeres by Polymerase Chain Reaction

Amani N. Azarah and Fadel A. Sharif, Medical Laboratory Sciences-Islamic University of Gaza.
Correspondence to: Prof. Fadel A. Sharif, Department of Medical Laboratory Sciences, Islamic University of Gaza. P.O.Box 108, Gaza-Palestine, E-mail: fsharif@iugaza.edu.ps.
Tel: +970(8)2860700, Fax: +970(8)2860800.

Short title : PCR on DNA from single blastomeres.
Key Words: Polymerase Chain Reaction (PCR), Whole Genome Amplification (WGA), blastomere, Sex determination.

Abstract

Objective: This study was conducted in order to investigate the feasibility of applying whole genomic DNA amplification (WGA) and polymerase chain reaction (PCR) techniques for sex determination of single human blastomere for the first time in Gaza Strip.

Samples and Methods: In this study, WGA technique was employed on single blastomeres that were biopsied from 30 surplus human embryos donated for this research. The obtained genomic DNAs were then subjected for PCR amplification of the Y-linked SRY (Sex-Determining Region on Y chromosome) and the X-linked glucose-6-phosphate dehydrogenase (G6PD) genes.

Results: Whole genomic DNA amplification efficiency from single blastomeres was 100 % and the level of amplification ranged from about 40 to 100 fold. All amplified DNA samples were included in the subsequent sex determination PCR analysis. Of the 30 embryos, 7 (23%) were found to be male and the remaining 23 embryos (77%) were of female gender.

Conclusion: Based on these results, WGA is effective for obtaining sizable amounts of DNA from single blastomeres. Moreover, the obtained DNA proved amenable to PCR and this will pave the way for pre-implantation genetic diagnosis of various inherited disorders and for non-medical sex selection in Gaza strip.

من خلية جنين ناتج من عملية التلقيح Y و X البحث عن منطقتين محددتين على كروموسومي المجهري باستخدام تقنية PCR

ملخص: يهدف البحث الى دراسة امكانية تطبيق تقنية إكثار الجينوم من خلية جنين واحدة لاستخدامها في تحديد جنس الأجنة الناتجة من التلقيح المجهري وذلك لأول مرة في قطاع غزة. فقد تم استخدام تقنية الـ PCR على المادة الوراثية التي تم اكثارها لتحديد جنس 30 جنين وذلك من خلال البحث عن الجين (SRY) المحدد للجنس الذكري والموجود على كروموزوم Y وجين الجلوكوز فوسفات ديهيدروجينيز (G6PD) الموجود على كروموزوم X.

أثبتت نتائج الدراسة فاعلية إكثار الجينوم حتى على مستوى الخلية الواحدة مما سيفتح البـــاب فـــي قطاع غزة أمام تشخيص العديد من الأمراض الوراثية وتحديد جنس الاجنة قبل إرجاعها إلى رحم الأم.

Introduction

Pre-implantation genetic diagnosis (PGD) is currently one of the practical options available for couples at-risk to avoid the birth of children with genetic and chromosomal disorders. PGD involves removing a single cell (blastomere) from a 3-days old in vitro fertilized (IVF) embryo (containing from 6 to 8 cells) by a process called blastomere biopsy followed by analyzing that cell for gender, specific genetic disorders, or chromosomal abnormalities [1,2].

Whole genomic DNA amplification (WGA) is a technique that amplifies the entire genome extracted from single cells up to microgram levels. Sufficient DNA can be obtained, allowing for diagnosis of any known single gene defect that would have been impossible otherwise, and multiple tasks which need abundant DNA can be performed from WGA products. Earlier approaches of WGA included the use of primers for repeated interspersed sequences. Several other methods have been later developed to amplify the whole genome by using random or partly degenerate primers [3]**.** Recently, a new isothermal WGA method was introduced by using bacteriophage phi29 DNA polymerase (that has strand displacement activity) and random hexamer primers [4,5].

Polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are the two most commonly used methods in PGD in many IVF/ICSI laboratories [2]. Additionally, FISH is less prone to contamination and can also provide the copy number for each chromosome tested. FISH, however, cannot serve as a screening test for all chromosomes in a single cell because most FISH techniques can only detect limited numbers of chromosomes and may fail to detect single gene disorders and microdeletions smaller than 90 kb [2,6].

The more novel and the most successful approaches for global genome screening of single cells are comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) analyses [7,8].

In this study we employed a PCR assay for the simultaneous detection of X and Y chromosomes on human blastomeres. The Y-linked SRY (Sex-Determining Region on Y chromosome) and the X-linked glucose-6-phosphate dehydrogenase (G6PD) genes were amplified by PCR using specific primers [9,10]. Fragments specific to each chromosome are

generated: both X and Y fragments in male embryos and, due to the absence of Y chromosome, only an X-specific fragment is produced in females.

The study was conducted in order to investigate the feasibility of WGA and PCR for analysis of DNA extracted from single blastomeres for the first time in Gaza strip.

We believe that WGA and subsequent detection of the SRY and G6PD genes is suitable for gender determination in preimplantation diagnosis for couples at risk for X-linked genetic diseases and for non-medical sex selection purposes.

Materials and Methods

Study Sample

The study sample consisted of 30 human embryos (45 blastomeres) obtained from 7 ICSI cycles. Embryos used in this study were surplus embryos following embryo transfer.

Ethical Considerations

Informed consent was obtained from all embryo donors and the objective of the study was thoroughly explained to them.

Blastomere Processing

One or two cells were removed through a hole made in the zona pellucida, from each of 30 human embryos at the 4-8 cell cleavage stage three days after ICSI. By using an inverted microscope and a micromanipulator, the zona pellucida was opened by using a blastomere pipette (35-49 μm dimeter) containing acid Tyrode's solution (pH 2.4). During processing, the embryo was immobilized by a holding pipette and one or two blastomeres with visible nuclei were removed by gentle suction. Under a stereomicroscope each single or double blastomeres from each embryo were then separated by mouth pipetting using a fine glass pipette (pulled on flame to approximately the size of a blastomeres), washed in phosphate buffered saline (PBS) and transferred into 0.2 ml eppendorf tube containing 2.5 μl PBS [11].

Blastomere genomic DNA isolation and amplification

Blastomeres were lysed and genomic DNA was amplified by using REPLI-g Mini Kits (Qiagen, USA) adhering to the manufacturer protocol. Genomic DNA extracted from peripheral blood served as positive control. The quality of the amplified DNA was verified by running aliquots on ethidium bromide-stained 1% agarose gels. Additionally, the quantity of the DNA was measured by a nanophotometer (Imblen GmbH, Germany) at 260 nm.

Molecular analysis

SRY and G6PD gene specific PCR

PCR was carried out in a uniplex fashion for each primer set (See Table 1). The primer sequences were those described by Daloii et al. [9] and Katz et al. [10]. PCR was carried out in 0.2 ml PCR tubes in a 20 µl reaction volume containing: 2 µl template genomic DNA (100-200 ng), 10 µl PCR Master mix (Promega, USA) , 2 µl (2.7 µmol) of each primer, and nuclease free sterile distilled water to 20 µl. Amplification was started with an initial denaturation step at 94˚C for 15 minutes, followed by 35 sequential cycles each including 1 minute denaturation at 94˚C, 1 minute primer annealing (57˚C for SRY and 61˚C for G6PD) 1 minute extension at 72˚C. The protocol was followed by a final extension step at 72˚C for 10 minutes and then cooling to 4˚C. PCR products were resolved by electrophoresis on ethidium bromide containing 1.5% agarose gels. Positive control (G6PD amplified from genomic DNA isolated from peripheral blood) and negative control (reactions containing water instead of DNA) were run concurrently with each PCR reaction.

Results

DNA isolated separately from the 45 blastomers (that were obtained from the 30 embryos) was successfully amplified and therefore was included for subsequent sex determination by PCR. The gel electrophoresis showed that the amplified DNA was of high molecular weight as depicted in Figure 1. The DNA concentration, as measured by spectrophotometr, ranged from 200 to 600 ng/ µl (Table-2). Assuming that a single blastomere contains an average of ~ 6 ng/µl [12], the amplification level thus ranged from ~ 30 to 100 fold.

Of the 30 embryos, 7 (23 %) were found to be male where PCR amplicons resulted from the amplification of both SRY and G6PD genes with corresponding sizes of 472 and 583bp (Figure 2). The remaining 23 embryos (77 %) were of female gender as only the G6PD amplicon (583 bp) appeared on the gel (Figure 3).

Discussion

The present study investigated the feasibility of isolating blastomeres, whole genomic amplification and locus specific PCR for the first time in Gaza strip. The results indicate that WGA using the REPLI-g Mini Kit (Qiagen, USA) is very successful in amplifying DNA extracted from single cells up to 100 fold. Moreover, the technique is quite efficient in that genomic DNA amplification was obtained from all tested samples. This kit relies on multiple displacement amplification (MDA)-based genome amplification. As compared with other WGA methods (e.g., primer extension

preamplification (PEP) and degenerate oligonucleotide primed (DOP), MDA has been shown by Burtt (2011) and Spits et al. (2006) to generate DNA with a higher molecular weight and shows better genome coverage [4,5]. The DNA obtained in this study, and as is evident in Figure 1, was also of high molecular weight. The whole procedure takes about 16 hours and generates appreciable amounts of DNA from single blastomeres which is suitable for multiple downstream applications, such as locus-specific PCR, sequencing, STR analysis or array comparative genomic hybridization (array-CGH) [2,7,8,13,14].

In this work we employed a PCR assay for the detection of loci on X and Y chromosomes in human blastomeres. Both X- and Y-specific fragments were amplified with primers which are common to both chromosomes and are derived from the SRY and the G6PD genes. Both an X and a Y fragments were amplified in male embryos and due to the absence of a Y chromosome only X fragment was amplified in females. The efficiency and accuracy of this assay are high; it generates no false positive amplification signals and allows sexing within 4 hours after WGA. We therefore believe that the whole procedure presented in this work is suitable for gender determination in PGD for couples at risk for sex-linked genetic diseases and for non–medical sex selection. Moreover, the obtained DNA should be amenable to other molecular analyses such as array CGH and SNP analysis, and may enable the detection of a wider spectrum of specific inherited disorders [2,8].

However, the technique described here has the disadvantage that fails in detection of fetuses with aberrant numbers of X or Y chromosomes. For instance, XXY and XO embryos will be diagnosed as being male and female, respectively.

In conclusion, results of the present study prove the feasibility of WGA-PCR assay for the detection of specific DNA fragments from single human blastomeres. This will pave its use in preimplantation gender determination genetic diagnosis.

Acknowledgements

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Table 1. PCR primers used to amplify parts of SRY and G6PD genes.

Primer	Primer sequence	Amplicon size (bp)	Melting temperature	Reference
G6PD-F	CCCCGAAGAGGAATTCAAGG GGGT	583	69 °C	8
G6PD-R	GAAGAGTAGCCCTCGAGGGT GACT			
SRY-F	GAATATTCCCGCTCTCCCGGA	472	53 °C	9
SRY-R	GCTGGTGCTCCATTCTTGAG			

Table 2: Concentration of DNA (ng/µl) post–amplification of blastomeres' genomic DNA using WGA technique.

No. of balastomere	Amplified DNA concentration (ng/ µl)
Single blastomere	460-470
Double blastomere	400-580
Three blastomeres	230-280

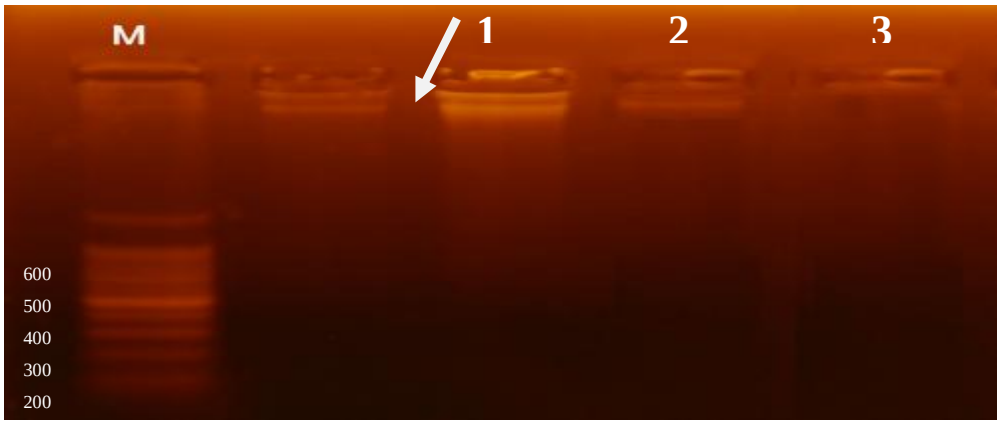

Figure 1. Ethidium bromide stained 1% agarose gel for whole human genomic DNA extracted and amplified from blastomeres. M: 100 bp DNA ladder, Lane1: single cell blastomere, Lane 2: positive control (peripheral blood genomic DNA), Lane 3: two blastomeres, Lane 4: negative control. Genomic DNA bands are evident near the gel wells (the arrow is pointing to one).

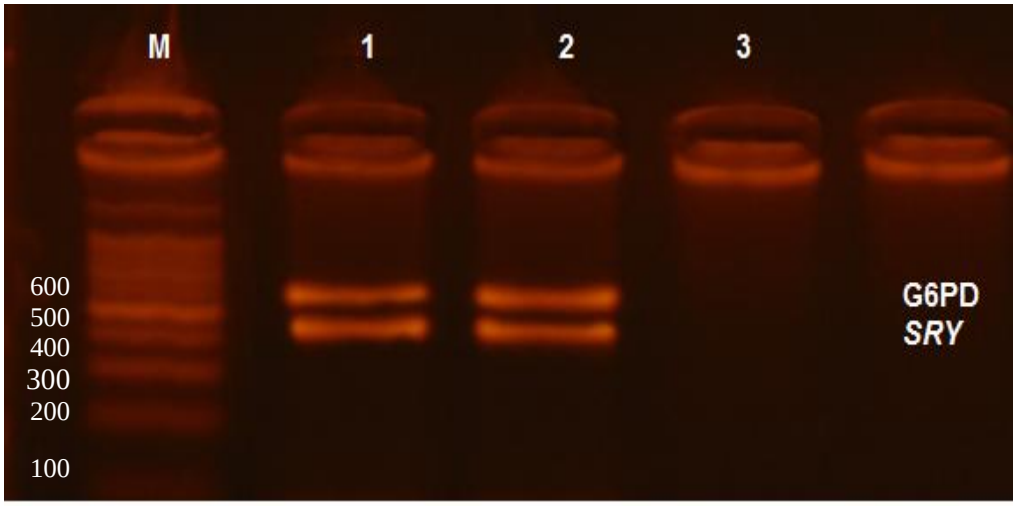

Figure 2. Ethidium bromide stained 1.5% agarose gel for SRY and G6PD amplicons. M: 100 bp DNA ladder; Lane1: positive control, Lane 2: SRY amplicon (472 bp) and G6PD amplicon (583 bp) obtained from WGA-PCR of a single blastomere , Lane 3:negative control. **Note**: for the sake of illustration, SRY and G6PD PCR products were combined before loading into the gel.

Amplification of X- and Y-Chromosome-Specific Regions

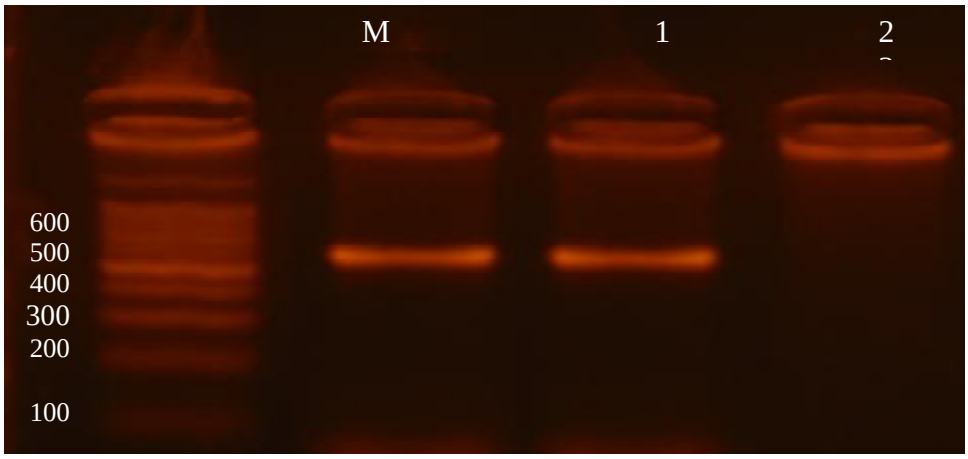

Figure 3. Ethidium bromide stained 1.5% agarose gel for G6PD amplicon. M: 100 bp DNA ladder; Lane1: positive control, Lane 2: G6PD amplicon (583 bp) obtained from single cell WGA-PCR of a single blastomere, Lane 3: negative control.