Zygote

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Development of a PCR-based method to identify fetal sex during IVF cycles

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Research Article

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Abstract

One of the most recognizable cases of preimplantation genetic diagnosis (PGD) is X-linked diseases. Diagnosis of fetal sex is essential for couples who are known to be at risk of some X-linked disorders. The objective of this study was to discriminate between female (XX) and male (XY) embryos by detecting sex chromosomes-specific sequences in spent culture medium and comparing these results to PGD/CGH array results. It may open new window for the development of a non-invasive PGD method. 120 Embryo's spent media from Day 3 and Day 5 embryos were collected. Modified phenol-chloroform solution was used for DNA extraction from spent media. Sex determination was performed using SRY, TSPY and AMELOGENIN evaluation through quantitative polymerase chain reaction (q-PCR) method. IBM SPSS and MedCalc were used for statistical analyses to compare sex determination of embryos by spent medium with PGD/CGH array results. Culture time was demonstrated to increase the DNA amount among day 5 embryos culture medium samples. Non-invasive PGD by means of spent culture medium gave a sensitivity, specificity, positive predictive value and negative predictive value of 100% for sex determination. Results of sex determination using spent medium by q-PCR were consistent with the results of PGD/CGH array. Improvements in cell-free DNA extraction and PCR amplification procedures provide us an effective method to perform a PGD test without biopsy in the future, especially about X-linked diseases.

Introduction

Preimplantation genetic diagnosis (PGD) as a routine clinical tool is combined with invasive procedures such as biopsy of cleavage-stage embryos or trophectoderm cells which can give couples a chance to avoid having a baby with genetic defects during in vitro fertilization (IVF) cycles (Magli *et al.*, 2016, McReynolds *et al.*, 2011, Meseguer *et al.*, 2011, Palini *et al.*, 2013). Embryo biopsy procedure for genetic analysis is a traumatic manipulation that may impair embryo development (Shahine and Lathi, 2014). This procedure is also expensive and time consuming (Yang *et al.*, 2017). Thus, a safer and cheaper approach is needed to screen preimplantation embryos for genetic disease (Shamonki *et al.*, 2016). One of the most recognizable cases of PGD is X-linked diseases. Diagnosis of fetal sex is essential for couples who are known to be at risk of some X-linked disorders (Assou *et al.*, 2014). If parents have these types of disorders, male fetuses will have the defect, however, the female fetuses will be healthy but the carrier (Wu *et al.*, 2015). According to previous studies, embryonic genomic DNA has been reported to be found in spent embryo culture medium (Xu *et al.*, 2016, Feichtinger *et al.*, 2017, Galluzzi *et al.*, 2015, Shamonki *et al.*, 2016). Proposing the spent culture medium of embryos obtained by IVF as a source of embryonic DNA has opened novel perspectives for the development of the non-invasive screening preimplantation embryos method (Stigliani *et al.*, 2013). The reliability of this new technique for clinical use, however, needs to be determined.

A number of Y chromosome-specific sequences such as SRY and TSPY have been investigated for sex determination. The highly conserved TSPY gene, repeated 27–40 times, is one of the widely used genes for this purpose. In the testis, exclusively, a 1.3 kb transcript is produced which is translated to a 33 kDa protein. This protein is homologous to the nucleosome assembly protein 1 (NAP-1) and the proto-oncogene SET (Schnieders *et al.*, 1996), both of which are able to bind with B-type cyclins to activate the cyclin B1-CDK1 kinase during the cell cycle (Kellogg *et al.*, 1995). Detection of TSPY protein in testis sections using in situ hybridization technique shows that the TSPY protein is located primarily in the cytoplasm of the mitotic spermatogonia. It is suggested

that TSPY plays a role in spermatogonial differentiation and/or proliferation (Schnieders *et al.*, 1996).

The SRY gene is located on the nonrecombining region of the Y chromosome and consists of a single exon encoding amino acids. SRY gene contains a region termed the high mobility group (HMG) box which is located in the center. The SRY-HMG box region has special characteristics, which makes it a perfect target for sex determination tests that are DNA based. For instance, the sequence of this region is highly conserved between different species (Shahid *et al.*, 2010).

The male genotype can be indicated using these targets, by amplification of the product from the TSPY or SRY gene, but a female genotype cannot be indicated accurately. This limitation may be caused by DNA lack/degeneration in the examined sample. In this case, the amplicon may be absent that limits the test results. Another reason could be a false-negative result, caused by a technical error during the process of examination. Recently, the sex determination test used by various PCR kits for short tandem repeats (STRs) has become a standardized method. In the test, the AMELX and AMELY sequences (homologous sequences) of amelogenin genes in the X and Y chromosomes are simultaneously amplified (Nakahori *et al.*, 1991). On the electropherogram, the single peak caused by the X chromosome can be used to discriminate the female sample from a false negative (Sullivan *et al.*, 1993). The amelogenin gene, which has been conserved during the evolution of vertebrates, is translated to a vital protein in the mammalian tooth and enamel matrix. The AMEL amplification by PCR has been used by several studies for sex determination (Sullivan *et al.*, 1993). However, in some populations, male subjects might be misidentified as female, due to interstitial deletion in the Yp involving AMELY (Steinlechner *et al.*, 2002, Thangaraj *et al.*, 2002, Roffey *et al.*, 2000, Jobling *et al.*, 2007, Yong *et al.*, 2007).

In this study, we developed a new method for sex determination using a multiplex PCR of the samples derived from blastocyst culture media.

Materials and methods

Fertilized oocytes obtained from patients, candidates for PGD, were cultured individually until day 3 in 20 µl of G-1 PLUS sequential medium (Vitrolife, Gothenburg, Sweden) and from day 3 to day 5 in 20 µl of G-2 PLUS sequential medium (Vitrolife, Gothenburg, Sweden). Medium droplets were overlaid with paraffin oil and incubated at 37 °C in a humidified atmosphere of 6% CO_2. Trophectoderm biopsy was performed on Day 5 after ICSI as a part of the clinical routine. After embryo removal, embryologist collected spent embryo culture medium in RNAase-DNAase-free tubes and promptly frozen at −80 °C for cell-free DNA analysis. During sample collection, appropriate precautions were followed to avoid contamination. In this study, two groups were evaluated, in the first group, spent media was collected on day 3 and in the second group spent media was collected on day 5.

120 spent culture media samples (64 spent media from day 3 and 56 from day 5) were used in the present study. 30 medium droplets incubated in parallel under the same conditions, without previous exposure with embryo, were also processed for PCR negative controls, to exclude contamination.

Standard group and reference DNA

Male whole blood cells and uterine endometrial cells were employed as standard groups for men and females, respectively, in this investigation. An equivalent volume of embryo culture media cultured under the same conditions without prior contact with the embryo was collected as a negative control. After DNA extraction using a DNA extraction kit (QIAGEN) according to the protocol, the concentration of DNA was measured by Nano drop (Thermo Scientific). The elution buffer from the DNA extraction kit was used as blank. Measured DNA was then serially diluted to calculate the range, linearity and limit of detection (LOD) of the test. Standard curves were plotted with 1 to 10000 copy number/µl concentrations, and serial dilutions of total DNA obtained from DNA were made.

To make a standard solution with a certain quantity of genomic DNA, Nano drop was used to assess the concentration of total extracted DNA. After that, the concentration was divided by the weight of a diploid cell's DNA (6.5pg). Finally, concentrations of 10,000, 1000, 100, 10 and 1 copy number/l were generated by serial dilution. Finally, we also used this serials dilution as control groups to compare with examined samples.

Cell-free DNA quantification from spent culture media

According to previous studies (Yang *et al.*, 2017), we first used the boiling method and then the ethanol precipitation of DNA to extract DNA from the culture medium, but no acceptable results were obtained from these methods. The final and efficient method used in this study to extract DNA was a modified phenol-chloroform solution. In this procedure, first, TE (Tris-EDTA) buffer and PK (proteinase k) were added to the culture medium, equivalent and 0.1 volume of culture medium, respectively. Second, this solution was incubated in a water bath for 3 hours at 56 degrees Celsius. The culture medium solution was then mixed with the same amount of phenol-chloroform (1:4) solution and centrifuged for 10 minutes at 12000 RCF. The supernatant was then placed into other tubes, avoiding contact with the tube's bottom. This step was repeated twice. Afterwards, we added absolute ethanol 96%, glycogen and ammonium acetate, the double volume of Supernatant, 0.2 of Supernatant and the same volume of Supernatant, respectively. This solution was incubated at −20 °C overnight and centrifuged for 15 min in 12000 RCF and then washed pellet with ethanol 75% and centrifuged for 10 min in 10000 RCF twice.

Quantitative polymerase chain reaction (qPCR)

The present level of SRY, TSPY and AMELOGENIN genes in the culture media sample and control group was evaluated by real-time PCR (Corbett, Australia). The PCR reactions were done in a total volume of 10 µl containing, 1 µl gene-specific primers, 2 µl master mix (5X EVA Green qPCR Master Mix, Bioneer), 5 µl extracted DNA and 2 µl DEPSE water. The thermal cycle profile was one cycle at 95° for 15 min, followed by 55 cycles at, 95°for 15 s, 60°for 20 s and 72° for 20 s, and finally, a melting curve program was from 60 to 95 °C. All assays were performed in triplicate. No template controls were included in each PCR run. Melting curves of PCR reactions were monitored to ensure that there was one single PCR product and no primer dimmer. Standard curves were obtained for each gene using the logarithmic dilution series of samples. Standard curves were used to assess primers efficiency. The gene-specific primers used are presented in Table 1.

Data were analysed using MedCalc software based on this method ability for accurate diagnosis of males and females as well as its specificity, sensitivity and accuracy.

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Table 1. The primer sequences (5′-3′) used in quantitative real-time polymerase chain reaction

Genes	Forward primers	Reverse primers	Annealing temperature (˚C)	Product size (bp)
SRY	GCGAGACCACACGATGAATG	TGAAACGGGAGAAAACAGTAAAGG	59.35	79
AMELOGENIN	CCCTGGGCTCTGTAAAGAATAGTG	ATCAGAGCTTAAACTGGGAAGCT	60/68	112/106
TSPY	TGTAAGTGACCGATGGGCAG	AACTCCCCTTTGTTCCCCAA	60	60

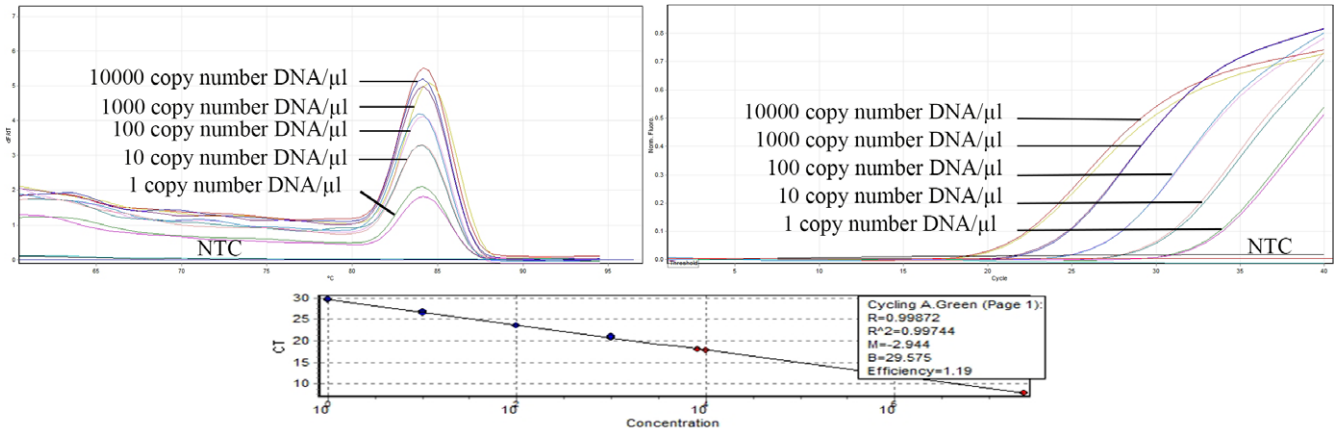

Figure 1. Melting curve, quantitation and standard curve analysis of Amplification SRY gene of whole blood cell of men.

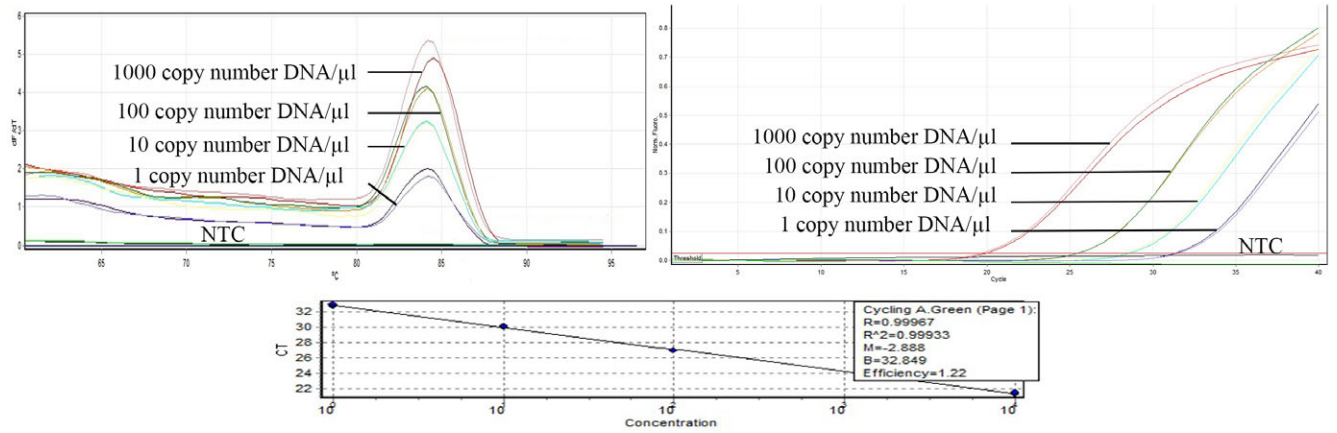

Figure 2. Melting curve, quantitation and standard curve analysis of Amplification TSPY gene of whole blood cell of men.

Results

Analytical validation of qPCR method

The gDNA in spent medium samples was detected and quantified by real-time PCR using the SRY, TSPY, AMELOGENIN genes amplification as a target. The gDNA content in the media was estimated using a standard curve plotted with purified human gDNA as serial dilution. The amount of endometrial and men's whole blood cell gDNA was 86000 and 52000 pg/µl, respectively. According to the weight of haploid DNA is approximately 6.5 pg/µl, we calculated the amount of DNA extracted approximately and used the values obtained for dilution.

Hence, as shown in Figures 1–4, the LOD of this examination was 1copy number/RXN which means if only 1copy of target DNA has existed in reaction, it will be detected. This finding was corroborated by the same melting temperature of all concentrations of standard DNA and samples. The melting peak of SRY and TSPY genes was 83.27 °C (Figure 1), 83.03 °C (Figure 2), respectively. AMLOGENIN gene showed a single melting peak at 80.33 °C for females and males (Figures 3 and 4).

Discovering and quantifying the SRY, TSPY and AMELOGENIN genes in embryo culture media

We evaluated spent culture media samples from 120 individual human embryos that were cultured for Days 3 and 5 Days. Out of 64 spent media collected on day 3, AMELOGENIN and TSPY genes were amplified in none of the samples, whereas amplification of the SRY gene was observed in only 10 samples of them. Among 56 samples which collected on day 5, 33 and 23 of

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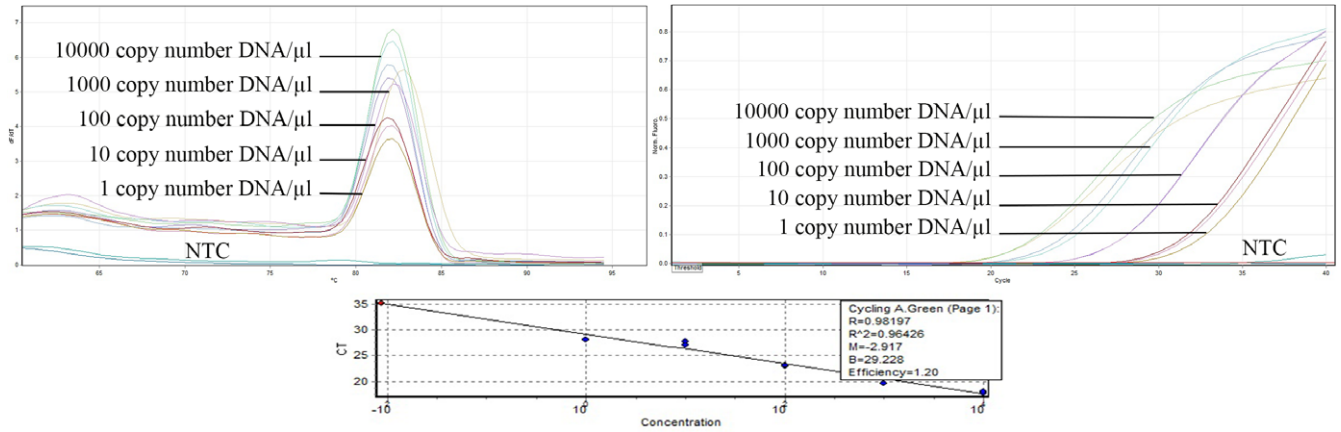

Figure 3. Melting curve, quantitation and standard curve analysis of Amplification AMELOGENIN gene of whole blood cell of men.

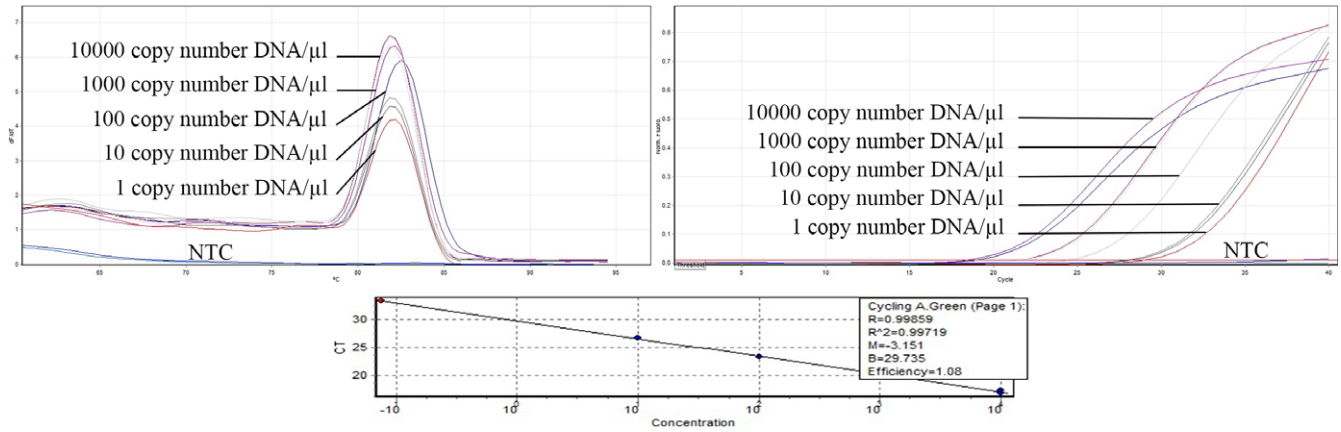

Figure 4. Melting curve, quantitation and standard curve analysis of Amplification AMELOGENIN gene of endometrial cells of women.

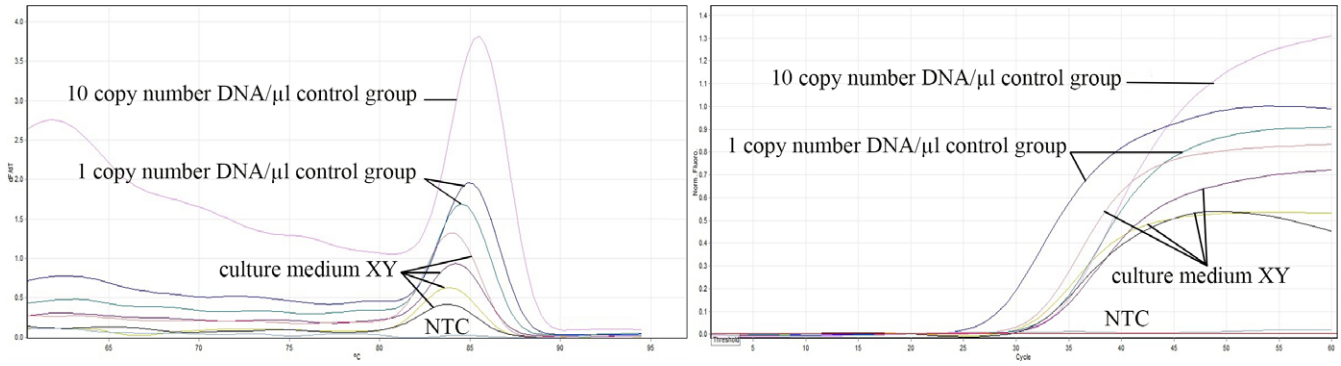

Figure 5. Melting curve and quantitation analysis of amplification SRY gene of culture medium.

them were male and female, respectively, according to PGD/CGH array results.

Sex determination in all medium samples collected on day 5 was confirmed by comparing with PGD/CGH array results (Figures 5–8). There was at least one of the mentioned genes compatible (Table 2). Among 33 male samples, 4 samples were SRY and AMELOGENIN positive and TSPY negative. However, in 4 of 33 samples, SRY and TSPY positive and AMELOGENIN negative. In 5 out of 33 male samples, only SRY gene was amplified. In 3 out of 33 male samples, SRY was negative and TSPY or AMELOGENIN positive. Moreover, all three genes, SRY, AMELOGENIN and TSPY, were amplified in 17 of 33 male samples. SRY, AMELOGENIN and TSPY did not amplify in any of the 23 female samples (Figure 9).

According to the study results, the test's sensitivity, specificity, positive predictive value and negative predictive value was 90.91 %, 100%, 100% and 88.46 %, respectively, by considering SRY gene alone. These results were 72.7%, 100%, 100% and 71.88 %,

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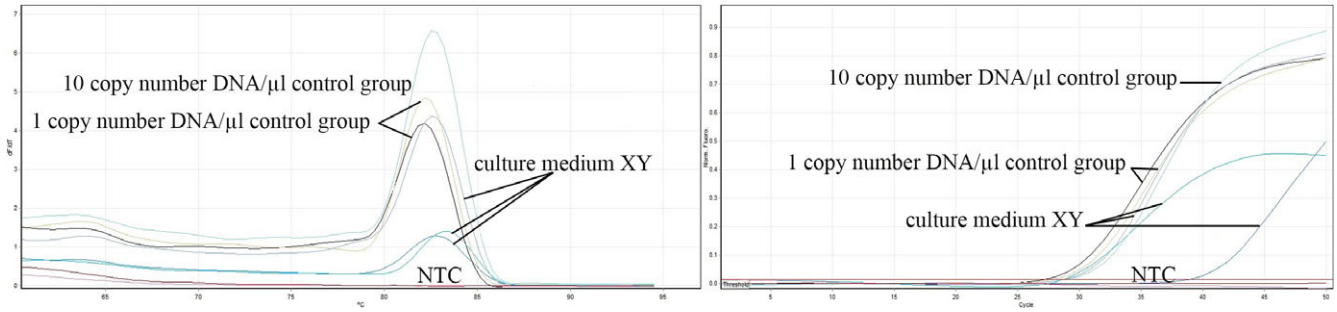

Figure 6. Melting curve amplification and quantitation analysis_cycling A. Green of AMELOGENIN XY gene of culture medium.

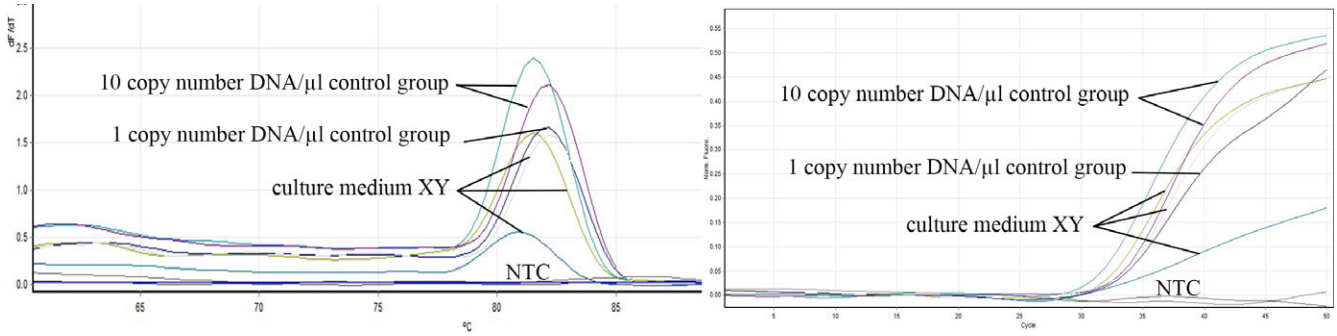

Figure 7. Melting curve of amplification and quantitation analysis_cycling A. Green of AMELOGENIN XX gene of culture medium.

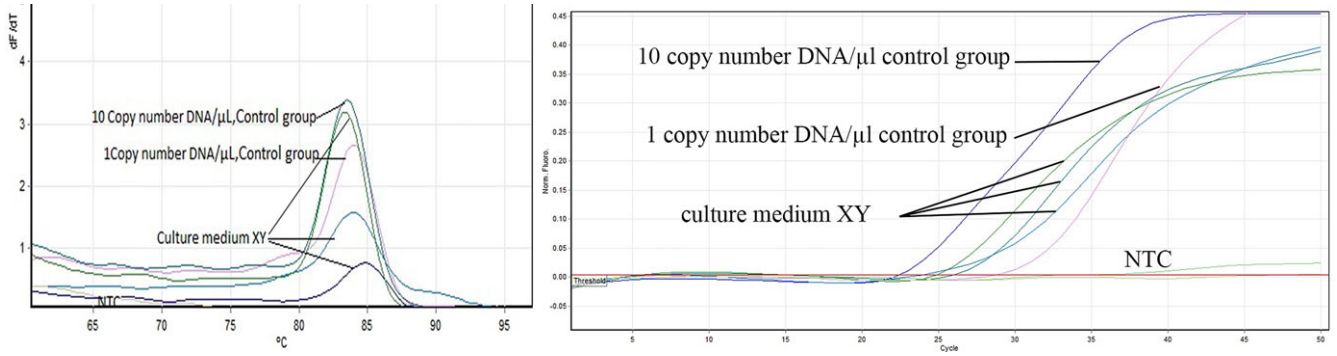

Figure 8. Melting curve of amplification and quantitation analysis_cycling A. Green of TSPY gene of culture medium.

respectively, by considering AMELOGENIN or TSPY gene alone. All of these items increase to 100% by considering all three genes. Furthermore, the accuracy of SRY, AMELOGENIN or TSPY gene to determine the sex of embryo was 94.64 %, 83.93% and 83.93%, respectively, that increase to 100% by considering all three genes.

Discussion

Sex-linked diseases are a type of genetic defect which affects the male fetus; therefore, sex determination is essential in these cases (Esmaeili *et al.*, 2020). More than 200 X-linked recessive disorders which affect hemizygous male could be easily prevented by gender selection (Esmaeili *et al.*, 2020). Currently, genetic screening of embryos before implantation relies on an invasive biopsy process to obtain DNA from embryo generated by IVF (Esmaeili *et al.*, 2020, Feichtinger *et al.*, 2017). Although the biopsy procedure is the preferred method worldwide and highly reproducible among clinics, it can be detrimental to the embryo development and implantation potential. It is also expensive and time-consuming (Feichtinger *et al.*, 2017). It seems this invasive procedure should be replaced by a non-invasive and more cost-effective PGD technique (Yang *et al.*, 2017).

Recently, the detection of cell-free DNA in the spent culture medium of embryos proposes the possibility of a non-invasive PGS approach. The probability of amplifying cell-free DNA from the culture medium has been previously investigated for diagnosis of X-linked diseases, cystic fibrosis and alpha thalassemia (Capalbo *et al.*, 2016). The result of our study indicates that the amount of

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Table 2. Result of sex determination in all medium samples collected on day 5 comparing with PGD/CGH array results

Number	Dev.	PGD	SRY	AMEL	TSPY
1.	5	M	+	+	+
2.	5	M	+	–	+
3.	5	F	–	+	–
4.	5	M	+	+	–
5.	5	M	+	+	+
6.	5	M	+	+	+
7.	5	M	+	–	–
8.	5	F	–	+	–
9.	5	F	–	+	–
10.	5	F	–	+	–
11.	5	M	–	+	+
12.	5	F	–	+	–
13.	5	M	+	+	+
14.	5	M	+	–	+
15.	5	F	–	+	–
16.	5	M	–	+	+
17.	5	M	+	+	–
18.	5	M	+	+	+
19.	5	F	–	+	–
20.	5	M	+	+	+
21.	5	F	–	+	–
22.	5	F	–	+	–
23.	5	M	+	+	–
24.	5	F	–	+	–
25.	5	M	+	+	–
26.	5	M	+	+	+
27.	5	F	–	+	–
28.	5	M	+	+	+
29.	5	M	+	–	–
30.	5	F	–	+	–
31.	5	F	–	+	–
32.	5	M	–	+	+
33.	5	F	–	+	–
34.	5	M	+	+	+
35.	5	M	+	+	+
36.	5	F	–	+	–
37.	5	F	–	+	–
38.	5	F	–	+	–
39.	5	M	+	+	+
40.	5	M	+	+	+
41.	5	M	+	–	–
42.	5	M	+	+	+
43.	5	F	–	+	–
44.	5	F	–	+	–
45.	5	M	+	–	+
46.	5	M	+	–	–
47.	5	F	–	+	–
48.	5	F	–	+	–
49.	5	M	+	+	+
50.	5	M	+	+	+
51.	5	M	+	–	+
52.	5	F	–	+	–
53.	5	M	+	+	+
54.	5	M	+	–	–
55.	5	M	+	+	+
56.	5	F	–	+	–

cell-free DNA in human embryo spent media is significantly more than in medium that has not been exposed to embryos. It reveals that the embryo releases genetic substances into the culture media.

We investigated and compared the amount of the gDNA using TSPY, SRY and AMELOGENIN expression by qPCR on day 3 and day 5. The results of our experiment represent that expression of these genes was significantly higher on day 5 compared to day 3, suggesting that embryo stage and collection time significantly influenced DNA release in culture medium. However, we could not quantify the amount of extracted DNA because we did not access Bioanalyser or other devices for quantification of very low amount of DNA.

Inconsistent with the results of our experiments, it was reported a high correlation between sex chromosomes determination and a lower rate of false-negative results in culture media from day 5 against samples from day 3 (Feichtinger *et al.*, 2017). Galluzzi *et al.* suggested that the TSPY gene appears poorly amplifiable before day 5/6, these results were also consistent with the results of our experiments (Capalbo *et al.*, 2016).

The present study removed cumulus cells, ICSI fertilization and single embryo culture to avoid contamination and false-negative results (Shamonki *et al.*, 2016). We compared the results of medium-based sexing with those obtained by aCGH to assess the accuracy and sensitivity of the test. The sex of embryos was diagnosed correctly from the spent medium samples using TSPY, SRY and AMELOGENIN amplification in all of 56 samples from day 5. Linlin yang investigated the presence of gDNA in culture medium only by amplification of SRY gene (Yang *et al.*, 2017), while in the current study, TSPY, SRY and AMELOGENIN genes were used to detect the X and Y-chromosomal DNA fragment. Sex determination was not approved in some samples by all three genes amplification together, it means if we used just SRY gene, some samples would be mistakenly considered female and false negative, while evaluation of AMELOGENIN and TSPY genes simultaneously causes the results of the test to become real positive. The choice of SRY and TSPY genes is due to male embryos' definitive involvement in X-linked diseases. The AMELOGENIN gene was used to detect X, Y chromosomal DNA fragments in spent media.

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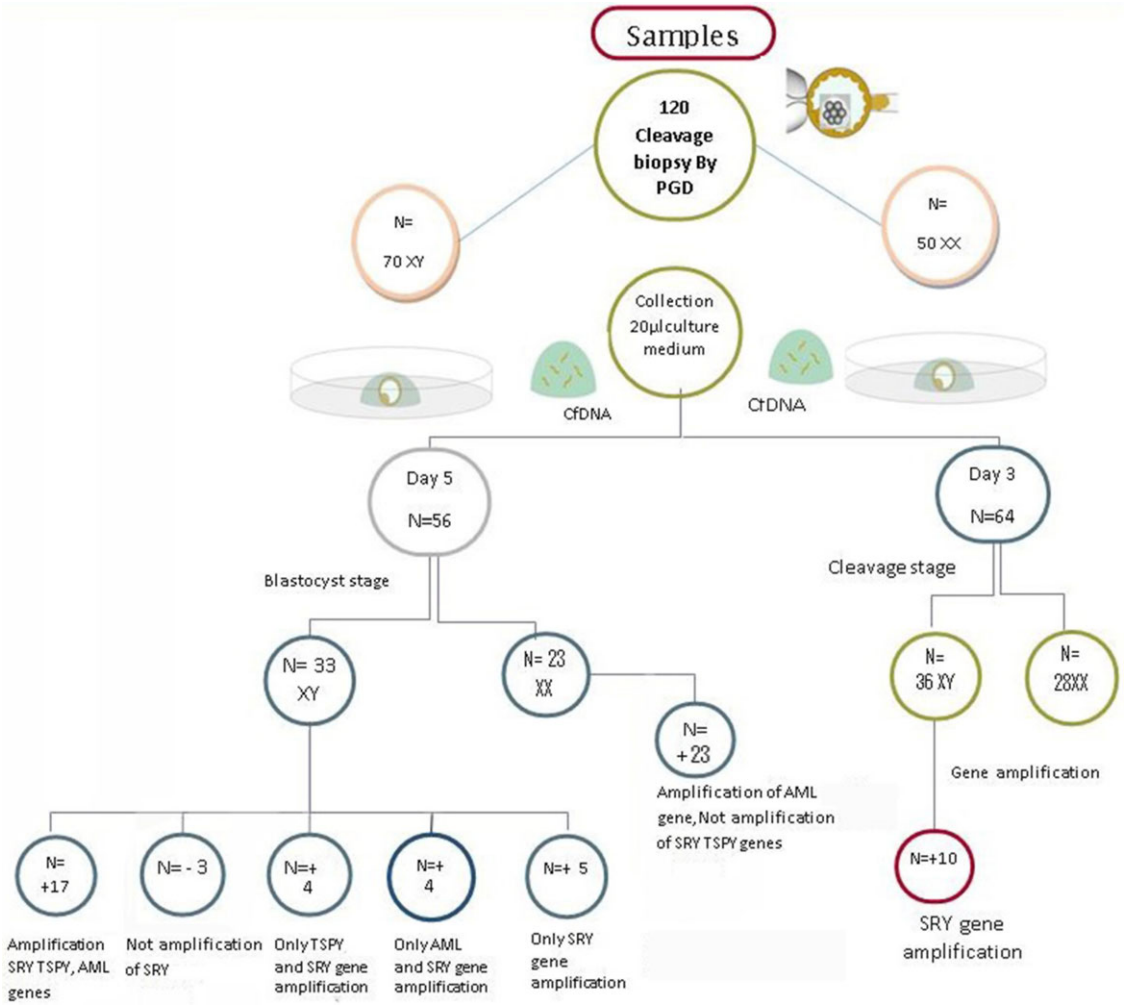

Figure 9. Comparing PGD/CGH array results to cell-free DNA analysis. Sixty-four spent culture media were collected on day 3. Fifty-six spent culture media were collected on day 5. The results from trophectoderm biopsy and cell-free DNA analysis were compared and classified according to the observed patterns.

Genetic screening and sex determination using both sex chromosomes (X and Y) are more reliable. However, we could not discriminate between AMELOGEGNIN gene on X and Y chromosomes in real-time PCR results, due to the close melting temperature, but we suggest that this determination should be done on both sex chromosome (Yang *et al.*, 2017). Three different DNA extraction methods were compared, boiling, ethanol precipitation and phenol-chloroform solution. At first, boiling, which was previously investigated to extract cell-free DNA, was attempted to use (Yang *et al.*, 2017). According to the results of real-time PCR for sexing, phenol-chloroform solution was a more sensitive, accurate and affordable method among these extraction methods.

Since the sensitivity and accuracy of this method in comparison with CGH array-PGS, as a reliable technique were 100.00%, this method is probably can replace the current invasive PGS. Hence, it seems embryo stage of development, collecting time and optimized DNA extraction method are important factors in spent culture medium-PGS.

Conclusion

Preimplantation non-invasive sexing on culture medium is an alternative approach for the prevention of transmission of sex-linked genetic diseases. However, before its clinical use, further studies with a larger sample size must investigate PGS reliability using spent culture medium.

Data availability. Not applicable.

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Author contributions. AS.M: acquisition of data, data analysis and drafting the manuscript, S.A: design of study and acquisition of data, M.M: revising manuscript critically for important intellectual content, M. B: data analysis, J.SM: acquisition of data, F.N: data analysis, M.B: conception and design and approved final version of article, FS.A: conception and design and approved final version of article.

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Competing interests. The authors declare no conflicts of interest.

Ethical standard. All participants signed written informed consent before being enrolled in the study and the study protocol was approved by the ethics committee of Iran University of Medical Sciences, Tehran, Iran (IR.IUMS.FMD.REC.1398.341).

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