Short Communication

Evaluation of a Modified DNA Extraction Method for Isolation of Cell-Free Fetal DNA from Maternal Serum

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Abstract

Background: Discovery of short cell free fetal DNA (cffDNA) fragments in maternal plasma has created major changes in the field of prenatal diagnosis. The use of cffDNA to set up noninvasive prenatal test is limited due to the low concentration of fetal DNA in maternal plasma therefore, employing a high efficiency extraction method leads to more accurate results. The aim of this study was to evaluate the efficiency of Triton/Heat/Phenol (THP) protocol in comparison with the QIAamp DNA Blood mini Kit for cffDNA purification.

Methods: In order to evaluate the efficiency of THP protocol, DNA of Rhesus D (RhD) negative pregnant women's plasma was collected, then real-time PCR for *RHD* exon 7 was performed. The Ct value data of real time PCR obtained by two different methods were compared and after delivery serology test on cord blood was done to validate the real time PCR results.

Results: The results indicated significant differences between two extraction methods (p=0.001). The mean±SD of Ct-value using THP protocol was 33.8±1.6 and 36.1±2.47 using QIAamp DNA Blood mini Kit.

Conclusion: our finding demonstrated that THP protocol was more effective than the QIAamp DNA Blood mini Kits for cffDNA extraction and lead to decrease the false negative results.

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Keywords: Fetus, Prenatal diagnosis, Real time polymerase chain reaction, THP protocol

Introduction

Molecular analysis of cffDNA in maternal plasma has emerged since 1997 for prenatal diagnosis like fetal *RHD* genotyping, fetal sexing for X-linked disorders [1], paternally inherited genetic diseases and pregnancy-associated conditions such as preeclampsia [2]. cffDNA is a naked molecule and short DNA fragments, 193 base pairs in length, which circulate in the peripheral maternal blood during pregnancy and disappears 2 *hr* after delivery [3,4]. The placenta is most likely the origin of the cffDNA although other sources with minor roles such as fetal hematopoietic cells and direct transfer of fetal DNA molecules in maternal plasma have been proposed [5-7].

Access to amniotic fluid for prenatal screening need to employ invasive procedures [8,9]. In fact the most important advantage of Non-Invasive Prenatal Diagnostic tests (NIPD) is decreasing the risk of miscarriage, which is around 1-2% in invasive methods. NIPD eliminate problems related to the analysis of chorionic and amniotic cell culture results. Also, it can be used earlier (5-7 week gestations) than routine procedures like amniocentesis, cordocentesis and chorionic villus sampling [3,5,10,11].

Despite the significant advantages of non-invasive prenatal screening, unequal total amount of cffDNA in different cases is an important challenge and the greatest difficulty is that just 3-6% of the total DNA in maternal plasma is originated in fetal, so the extraction of cffDNA is a crucial step and high DNA yield results in the reliable detection [12,13].

There is no agreement on a standard method for cffDNA isolation from maternal plasma, therefore we decided to compare two extraction systems, a modified Phenol-chloroform method and a column-based DNA extraction method. *RHD* gene (BN000065) is a part of *RH* gene located on chromosome 1, and consisting of 10 exons and 10 introns. Exon 7 of *RHD* gene was subjected to qPCR as target gene for amplification.

Materials and Methods

Twenty five RhD negative pregnant women without any pregnancy complications were enrolled during prenatal medical visits at Hafez Hospital, Shiraz, Iran. Gestational ages were ranged from 17 to 28 weeks. Their husbands had to be RhD positive.

Sample preparation

Peripheral blood of 10 non-pregnant RhD positive women were collected in EDTA tube and used as positive control. Ten min Centrifugation at 2000 *g* within 6 *hr*, followed by second centrifugation at 3000 *g* for 10 *min* was done and separated plasma was stored at -80 ℃.

DNA extraction

In the first method, QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), DNA was isolated from 200 *µl* of plasma according to manufacturer's instruction, then eluted in 30 *µl* of ddH_2O in the final step. To employ the second, THP method, 500 *µl* of plasma was incubated with 5 *µl* triton x-100 (Sigma-Aldrich-UK) at 98 ℃ for 5 *min* and was made cold for 5 *min*, then equal volume of Phenol-Chloroform-Isoamylalcohol (25:24:1, V: V: V) (Sigma-Aldrich-UK) was added and centrifuged for 10 *min* at 14000 *g*. Precipitation was done overnight with 2.5 volume of 100% ethanol at -20 ℃. DNA pellet was eluted in ethanol, dried at room temperature and finally suspend in 50 *µl* of ddH_2O. This method is based on the study of Xue and Colleagues with a small modification. They precipitated DNA in 1/10 volume of 3 *M* Sodium Acetate (NaOAc) and 2.5 volumes of 100% ethanol, but we use only 2.5 volume of 100% ethanol for precipitation [14].

Quantitative analysis of DNA

Quantitative analysis of DNA was performed using SYBR Green (Maxima SYBR Green/ROX qPCR Master Mix (2X), Thermo Scientific, Lithuania) fluorescence real-time PCR with a Rotor-Gene Q (Qiagen, Hilden, USA) instrument. The presence of cffDNA was identified using *RHD* exon 7. The β-globin gene was used to evaluate the quality of the total DNA. Primers were selected based on previous study (Table 1) [15]. The concentration of reagents, temperature and time of cycling for amplification of two genes were identical. Final PCR reaction volume was 25 *µl* including 5 *µl* DNA and 300 $nmolL^{-1}$ primer concentration. The protocol was included two steps of hold temperature: 50 ℃ for 2 *min*, 95 ℃ for 10 *min* continued by 50 cycles of 94 ℃ for 60 *s*, 55 ℃ for 60 *s* and 72 ℃ for 60 *s*.

To determine the efficiency of qPCR, ten dilution series of pooled RhD positive DNA were prepared (Figure 1). Ten non pregnant Rh+and Rh-women were used as positive and negative control respectively. To ensure there was no contamination, no template control (sterile H_20) was used in each PCR run. The quality of total extracted DNA was tested using β-globin sequences. Serology test of cord blood was used as a gold standard to define the accuracy of qPCR results for determining RhD status.

Statistical analysis

Two extraction methods were assessed by paired Student's t-test (p-value) to analyze cffDNA significant differences in pregnant women, and Wilcoxon Signed Ranks test was used to present cfDNA significant differences in non-pregnant women. To perform all the statistical analyses the SPSS package was used.

Ethical considerations

This study was approved by the ethics committee of the Shiraz University of Medical Sciences, Shiraz, Iran. Pregnant women who participated in this research filled out the consent form consciously.

Results

Two extraction methods, THP and QIAamp DNA Blood Mini Kit, were employed in order to compare cffDNA concentration (quality and quantity) and in non-pregnant women to compare the amount of cfDNA in their plasma. For analyzing, DNA was subjected to Real-time PCR to detect exon7 in plasma of RhD negative pregnant women. To measure quality of cffDNA, obtained prenatal results was compared with the serology results of cord blood that indicated 100% accuracy for both cffDNA extraction methods. False negative and false positive results were not observed. Quantification of qPCR data was fulfilled using Ct values as index for cffDNA concentration. The Ct is inversely proportional to the amount of target DNA (Figure 2). A strong linear relationship between the Ct values and the log of the concentrations was observed (R2 >0.99). In pregnant group the mean±SD of Ct-value from 2 to 3 replicates gained by THP protocol was 33.8±1.6 (range: 30.2-36.1), while for QIAamp DNA Blood Mini Kit

Table 1. Sequences of PCR primers for real time PCR assays

Primer name	Sequence 5 to 3	Product length
RHD (exon7) Forward	CTCCATCATGGGCTACAA	90
RHD (exon7) Reverse	CCGGCTCCGACGGTATC	
β-globin Forward	GTGCACCTGACTCCTGAGGAGA	102
β-globin Forward	CCTTGATACCAACCTGCCCAG	

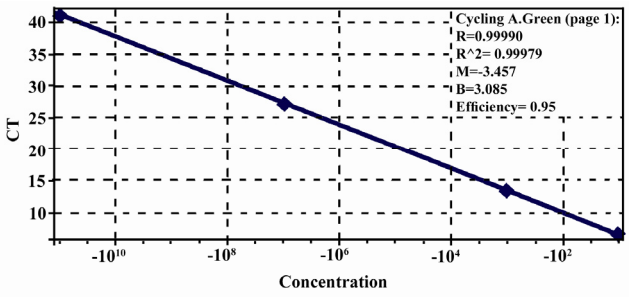

Figure 1. Q-PCR standard curve of RHD exon 7 using 10-fold serially diluted RHD positive samples. The plot indicates the relationship between Ct value and DNA concentration.

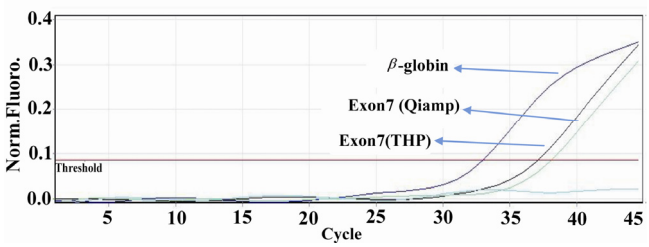

Figure 2. Real-time quantitative RCR. Amplification plots of cffDNA using q-PCR for the RHD (exon7)gene (THP protocol, QIAamp), β-globin and NTC (non-template DNA).

was 36.1±2.47 (range: 33.71-40.81). In the non-pregnant group, which was used as a positive control for *RHD* exon 7, Ct-value for QIAamp DNA Blood Mini Kit and THP protocol obtained 32.2 (range: 27.5-35) and 30.9 (range: 26.83-34.6) respectively. Evaluation the results of two extraction methods showed the higher efficiency of the THP protocol for cffDNA extraction (p=0.001), but comparing the two methods for isolating cfDNA in the positive control group (non-pregnant RhD positive women) showed no significant difference (p=0.241).

Discussion

However the use of free fetal DNA caused a major impact on prenatal diagnosis, the low concentration of fetal DNA and extraction problems have been a major constraint on its use in clinical settings. The fundamental problem for extracting DNA from plasma is related to the size of nucleic acid fragments [16]. According to Chan and coworkers, cfDNA in non-pregnant women were ranged from 145 to 201 *bp*, while they are longer in pregnant woman. Using specific fetal gene sequence (SRY in male fetuses) indicated that the fetal DNA fragment size was shorter than free maternal DNA (<313 *bp*) [4].

Our results indicated the quantity of DNA yield was improved when we used THP protocol in comparison with QIAamp DNA Blood Mini Kit as the most common method for cffDNA extraction [17-20]. In order to cffDNA detection, real time data of the two methods were compared with cord blood serology result and 100% concordance was reported.

According to the workshop on cell free fetal DNA extraction methods that was held by several laboratories, the higher results were gained with the QIAamp DSP Virus Kit and the results of QIAmp DNA Blood Mini Kit were close to DSP Virus Kit when the plasma volume was used more than 500 *ul* for initial DNA extraction [21]. A possible reason for poor efficiency of QIAamp DNA Blood Mini Kit is that it was developed for extraction of large fragments while cffDNA size is <300 *bp* [13].

Xue and Colleagues declared that THP protocol (modified Phenol-chloroform extraction method) has the ability to isolate nucleotide fragments as small as 100 *bp*. They asserted the use of THP protocol gives better results than QIAamp Blood DNA Midi Kit (Qiagen, UK), but evaluation of the cfDNA in non-pregnant women in our study displayed that the concentration difference between the THP protocol and QIAamp DNA Blood Mini Kit was not statistically significant. Although the small sample size makes it difficult to draw conclusions, previous studies have shown that the QIAamp DNA Blood Mini Kit can extract cffDNA more efficiently than the QIAamp Blood DNA Midi Kit [14,21].

Conclusion

Although THP protocol is cheap, modifiable and more effective for isolating of cffDNA from limited clinical samples, it is not the perfect choice for use in large scale. On the other hand, using QIAamp DNA Blood Mini Kit due to lower yields increases the false negative cases. In conclusion, we suggested that laboratories employ QIAamp DNA Blood Mini Kit for all fetuses, but to avoid false negative results, samples that have been predicted as negative (*e.g*. *RHD* gene or SRY sequence) should be evaluated again by THP protocol.

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