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

Zeinab Keshavarz 1,2, Leili Moezzi 1, Reza Ranjbaran 1, Farzaneh Aboualizadeh 1, Abbas Behzad-Beihabani 1, Masooma Abdullahi 1, and Sedigheh Sharifzadeh 1*

1. Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
2. Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran

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.

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 3,8. 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 5,9.

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 ml 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°C.

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 ddH2O 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°C 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°C. DNA pellet was eluted in ethanol, dried at room temperature and finally suspend in 50 μl of ddH2O. 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, Germany) 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°C for 2 min, 95°C for 10 min continued by 50 cycles of 94°C for 60 s, 55°C for 60 s and 72°C 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 H2O) 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 cffDNA 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 cffDNA 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 | CCTCACTATGGGCTACAA | 90              |
| RHD (exon7) Reverse | CCGGCCTCGGAGGTATC |                 |
| β-globin Forward  | GTGCACCTGACTCCTGAGGA |               |
| β-globin Forward  | CCTTGATACCAACCTGCGCAG | 102            |

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.
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 of 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 cffDNA 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, cffDNA 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 cffDNA 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.

**Acknowledgement**

This article was extracted from the biotechnology MS thesis written by Zeinab Keshavarz, and was founded by Shiraz University of Medical Sciences grants, grant No. 90-10-45-3311. We would like to thank laboratory and midwifery staffs of the Hafez Hospital for their eager cooperation.

References

1. Costa JM, Benachi A, Gautier E. New strategy for prenatal diagnosis of X-linked disorders. N Engl J Med 2002; 346(19):1502.
2. Zhong XY, Holzgreve W, Hahn S. Circulatory fetal and maternal DNA in pregnancies at risk and those affected by preeclampsia. Ann NY Acad Sci 2001;945(1):138-140.
3. Schmidt B, Weickmann S, Witt C, Fleischhacker M. Improved method for isolating cell-free DNA. Clin Chem 2005;51(8):1561-1563.
4. Chan KA, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, et al. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem 2004; 50(1):88-92.
5. Bianchi DW. Circulating fetal DNA: its origin and diagnostic potential-a review. Placenta 2004;25(Suppl A): S93-S101.
6. Edlow AG, Bianchi DW. Tracking fetal development through molecular analysis of maternal biofluids. Biochim Biophys Acta 2012;1822(12):1970-1980.
7. Anker P, Stroun M. Immunological aspects of circulating DNA. Ann NY Acad Sci 2006;1075(1):34-39.
8. Bianchi DW, LeShane ES, Cowan JM. Large amounts of cell-free fetal DNA are present in amniotic fluid. Clin Chem 2001;47(10):1867-1869.
9. Hui L, Bianchi DW. Cell-free fetal nucleic acids in amniotic fluid. Hum Reprod Update 2011;17(3):362-371.
10. Swanson A, Sehnert AJ, Bhatt S. Non-invasive prenatal testing: technologies, clinical assays and implementation strategies for women’s healthcare practitioners. Curr Genet Med Rep 2013;1(2):113-121.
11. Yang YH, Han SH, Lee KR, Kim SY, Ryu HM. Noninvasive prenatal diagnosis using cell-free fetal DNA in maternal plasma: clinical applications. J Genet Med 2011;8(1):10.
12. Clausen FB, Krog GR, Rieneck K, Dziegiel MH. Improvement in fetal DNA extraction from maternal plasma. Evaluation of the NucliSens Magnetic Extraction system and the QIAamp DSP virus kit in comparison with the QIAamp DNA blood mini kit. Prenatal Diag 2007;27(1):6-10.
13. Lo Y, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350(9076):485-487.
14. Xue X, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. Clin Chim Acta 2009;404(2):100-104.
15. Wang XD, Wang BL, Ye SL, Liao YQ, Wang LF, He ZM. Non-invasive foetal RHD genotyping via real-time PCR of fetal DNA from Chinese RhD-negative maternal plasma. Eur J Clin Invest 2009;39(7):607-617.
16. Kimura M, Har a M, Itakura A, Sato C, Ikebuchi K, Ishihara O. Fragment size analysis of free fetal DNA in maternal plasma using Y-STR loci and SRY gene amplification. Nagoya J Med Sci 2011;73(3-4):129-135.
17. Legler TJ, Liu Z, Mavrou A, Finning K, Hromadnikova I, Galbiati S, et al. Workshop report on the extraction of foetal DNA from maternal plasma. Prenat Diagn 2007;27(9):824-829.
18. Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. Transfusion 2002;42(8):1079-1085.
19. Hromadnikova I, Vechetova L, Vesela K, Benesova B, Doucha J, Kulovany E, et al. Non-invasive fetal RHD exon 7 and exon 10 genotyping using real-time PCR testing of fetal DNA in maternal plasma. Fetal Diagn Ther 2005;20(4):275-280.
20. Hromadnikova I, Vechetova L, Vesela K, Benesova B, Doucha J, Vlk R. Non-invasive fetal RHD and RHCE genotyping using real-time PCR testing of maternal plasma in RhD-negative pregnancies. J Histochem Cytochem 2005;53(3):301-305.
21. Johnson KL, Dukes KA, Vidaver J, LeShane ES, Ramirez I, Weber WD, et al. Interlaboratory comparison of fetal male DNA detection from common maternal plasma samples by real-time PCR. Clin Chem 2004;50(3):516-521.