THE USE OF FETAL FREE DNA AS NON-INVASIVE TECHNIQUES ON SEX IDENTIFICATION

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ABSTRAK

Urgensi penggunaan sampel berdasarkan cell free fetal pada kasus forensik di masa depan tidak dapat dihindarkan. Janin mengingat kompleksitas kasus yang berkaitan dengan identifikasi abortus provokatus atau kasus kriminal lainnya diperkirakan akan terus meningkat. Untuk mengatasi masalah ini, solusi praktis bagi para ahli DNA forensik adalah penggunaan teknik non-invasif dengan sampel yang berasal dari fetus cell-free. penggunaan bahan ini diharapkan dapat membantu proses identifikasi pribadi forensik untuk membantu penegakan hukum di Indonesia. Penelitian ini menggunakan cell free fetal DNA untuk mendeteksi jenis kelamin janin pada kehamilan 8 minggu atau lebih, di mana penggunaan USG masih tidak memungkinkan. Hasil dari penelitian ini adalah cell free fetal DNA dalam sirkulasi ibu menjanjikan harapan baru untuk kepentingan identifikasi forensik karena memungkinkan pemeriksaan forensik dalam kasus paternitas dapat dilakukan di masa depan. ***(FMI 2015;51:53-58)***

Kata kunci: *fetal cell free, teknik non-invasif, unborn child identification*

ABSTRACT

Urgent use of samples based on cell free fetal on forensic cases in the future will be inevitable need. Unborn child given the complexity of the cases related to abortus provocatus identification or other criminal cases that are predicted to continue to rise. To overcome these problems, a practical solution for forensic DNA experts is the use non-invasive techniques with samples derived from cell free fetus. the use of this material is expected to help the process of forensic personal identification to assist law enforcement in Indonesia. This study using free fetal DNA to detect the sex of a fetus at 8 weeks gestation or more, where the use of ultrasound is still not possible. The result of this study is cell free fetal DNA in the maternal circulation promising new hope for the benefit forensic identification. It enable forensic examination in cases of paternity can be done in the future. ***(FMI 2015;51:53-58)***

Keywords: *fetal cell free, non-invasive technique, unborn child identification*

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INTRODUCTION

One of the controversial problems for forensic molecular expert is the use of cell free fetal sample in forensic analysis, as in the case of "Unborn child disputed". The main concern for this controversy lays on the possibility that the quantity of cell free fetal DNA obtained from pregnancy is not adequate enough to be used in paternity examination in forensic case. On the other hand, the urgency of using sample based on cell free fetus in forensic case will be undeniable need, as determined by the complex cases of unborn child identification on rape or other criminals. Evidence of cell free fetus from previous pregnancies is feared to intercept quick justification.

The problem mentioned above requires quick break-through method to provide practical solution for an forensic DNA expert to implement non-invasive technique using sample from cell free fetal DNA. The objective of this study was to explore non-invasive technique in order to enhance justification response to identify unborn child in forensic case using cell free fetal.

Invasive tests with chorionic villus sampling and amniocentesis techniques have been used as a specimen examination in cases involving the fetus by a mother, while it increases the risk of miscarriage. Fetal DNA sampling technique as described above is obtained via biopsy of chorionic villi lining the walls of the placenta or by amniocentesis (taking approximately 20 ml of amniotic fluid) and performed by an obstetrician. However, both of these techniques carry certain risks, in which the worst is miscarriage. To prevent miscarriages, the experts use a method called non-invasive DNA sampling method based on cell free fetal (Sarasola et al 2006). The prenatal DNA test with a non-invasive technique has been using fetal cells in maternal blood free at 8-week gestational age or more (Guo et al 2012). Sex typing in forensic paternity is performed in conjunction with available short tandem repeat (STR) to

provide the sex of individual who is the source of DNA sample in question (Butler 2001). According to Colmant et al (2013), detection using cell free fetal DNA is able to determine the sex of a fetus after 8 weeks of pregnancy. This method is related to the blood circulation of the fetus or the mother's circulating blood which turned out to contain "waste" of the fetus from the simultaneous uptake of oxygen in the placenta wall, which is accompanied by the release of "waste" of a fetus that is transferred into the mother's blood. The mother's blood supply would then contain the DNA of fetal cells, which are the result of fragmentation of free and nucleated cells, so it can be used as material inspection is non-invasive fetal DNA. However, the study of free fetal DNA becomes very crucial to do (Barrett et al 2011) because of the amount of fetal DNA in early pregnancy is usually less than 10% of the total free circulating DNA (Barrett et al 2011, Lo et al 1998). Although circulating maternal blood contains DNA from fetal cells, but until now it remained uncertain whether this cell free fetal DNA has a high success rate as it does with USG.

MATERIALS AND METHODS

This research was conducted through laboratory experiment using forensic DNA testing paternity with gene amelogen into determine sex identity, using a non-invasive technique of cell free fetal DNA from the blood of ten pregnant women in the first trimester as volunteers. Selection of study subjects was random without any specific criteria and the participants have signed the informed consent. Seven until eight ml maternal blood samples were collected in Vacutainer EDTA containing tubes and centrifuged at 2000 rpm for 10 minutes in Himac, Hitachi and aliquoted in 2-ml Eppendorf tubes, and the DNA was extracted with DNA-zol (Invitrogen). DNA concentrations were then calculated using the UV Specthrophotometer (UV-Visible Spectrophotometer, Shimatzu) and followed by PCR amplification using PCR mix from Promega as follows: PCR Mix-dNTP (ATP, CTP, TTP, GTP), MgCl2, Taq Polymerase and buffer as much as 12.5 mL was added with l2.5 mL amelogenin gene oligonucleotide and 2.5 mL DYS19 oligonucleotide. Thereafter, it was added with nuclease free water as much as 6.5 mL and added the results of the DNA sample as much as 1 mL. The end result of reaction volume of 5µl PCR optimization could be done immediately. Optimization cycle was done based on instructions from Promega with repetition cycle denaturation, annealing and extension up to approximately 30 cycles. The results of PCR amplification (Gene Amp PCR System 2400, Perkin Elmer) was then visualized by gel electrophoresis using acrylamide gel (Promega corp, 2010) and agarose gel 2 % from Sigma.

RESULTS

The quantification results and purity levels of DNA derived from cell free fetal DNA and the DNA from the mother can be seen in Table 1 and 2

Table 1. Quantification result of fetal free cell DNA form blood sample

Sampel ID	Blood (ng/ul)	Fetal Free (ng/ul)	(%)
MP1	544.94	20.61	3.8
MP 2	254.81	56.36	22.1
MP 3	220.8	53.7	24.3
MP 4	291.32	49.9	17.1
MP 5	187.89	59.59	31.7
MP 6	62.09	24.13	38.9
MP 7	207.08	43	20.8
MP 8	325.54	64.5	19.8
MP 9	728.66	42.53	5.8
MP10	227.02	38.35	16.9
Average	305.02	45.27	14.8

Table 1 shows that the mean percentage of cell free fetal DNA concentration compared with the DNA concentration from maternal blood in this sudy was 14.8%. This value, according to Barrett et al (2011), is enough to be used in the analysis or molecular detection using cell free fetal DNA as well as on the molecular detection of trisomy or other molecular analysis. This opinion is the same as that of Norton et al (2012) which stated that the threshold(s) in the use of cell free fetal DNA fraction is 4% or more.

Table 2. Quantification result of fetal free cell DNA form blood sample

Sample ID	ng/ul	A260	A280	260/280
MP d1	544.94	10.899	6.057	1.8
MP d2	254.81	5.096	2.793	1.82
MP d3	220.8	4.416	2.584	1.71
MP d4	291.32	5.826	3.206	1.82
MP d5	187.89	3.758	2.112	1.78
MP d6	62.09	1.242	0.713	1.74
MP d7	207.08	4.142	2.207	1.88
MP d8	325.54	6.511	3.43	1.9
MP d9	728.66	14.573	8.031	1.81
MP d10	227.02	4.54	2.687	1.68
	Average of purify			1.79
Sample ID	ng/ul	A260	A280	260/280
MP cf1	20.61	0.412	0.281	1.47
MP cf2	56.36	1.127	0.721	1.56

MP cf3	53.7	1.074	0.996	1.08
MP cf4	49.9	0.998	0.677	1.47
MP cf5	59.59	1.192	1.093	1.09
MP cf6	24.13	0.483	0.391	1.4
MP cf7	43	0.86	0.524	1.64
MP cf8	64.5	1.29	0.76	1.7
MP cf9	42.53	0.851	0.709	1.2
MP cf10	38.35	0.767	0.672	1.14
Average of purify				1.41

Table 2 illustrates the level of purity of DNA from fetal cell free DNA compared to that from maternal blood. This table shows that the purity of DNA from the mother is relatively not much "contaminated" by the protein, which had an average purity of 1.79. This value is higher than the average purity of DNA from cell free fetal DNA, which was only 1.41. Even specifically found DNA from cell free fetal has relatively small degree of purity, which was about 1.08 (MP3) and 1.09 (MP5).

Table 3. Result of PCR-amelogenin

Sample ID	*Allele*	
	Blood	*Fetal Cell Free*
MP1	XX	XX
MP2	XX	XX
MP3	XX	XY
MP4	XX	XX
MP5	XX	XX
MP6	XX	XX
MP7	XX	XY
MP8	XX	XY
MP9	XX	XX
MP10	XX	XX

Table 3 illustrates the success of PCR amplification in the amelogenin gene for sex detection. Based on the results of sex identification by amelogenin gene, it obtained 7 cell free fetal DNA detected as female. to ensure that the results really accurate or exactly detected as positive female (XX) with amelogenin gene, we checked using Y chromosome locus (DYS19), where this examination represent a male gene.

Test results obtained DYS19 locus examination of the sample (MP3 sample) and DYS19 positive on cell free fetal. From these Figures 1 and 2 it can be seen that the sample detected had male sex as the sample MP3s, then it was checked with DYS19 locus, which detected band or bands at positions 232-268 bp. Vice versa, if the sample detected by amelogenin examination was found to be positively women, DYS19 examination would detect no band or bands at positions 232-268 bp.

This is in contrast to the results on sample MP6 (Figure 3), where the samples detected positive women, but it turned out, after confirmation by DYS19 examination, the locus detected band or bands at positions 232-268 bp. This phenomenon is probably caused by the dominance of the primary binding by maternal DNA, where as "high" quantity of maternal DNA, then are amplified by gender determination using the amelogenin gene is derived from maternal DNA.

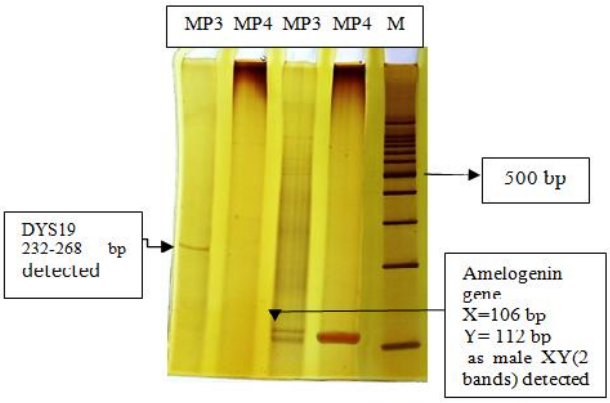

Figure 1. Results from MP3-MP4 samples

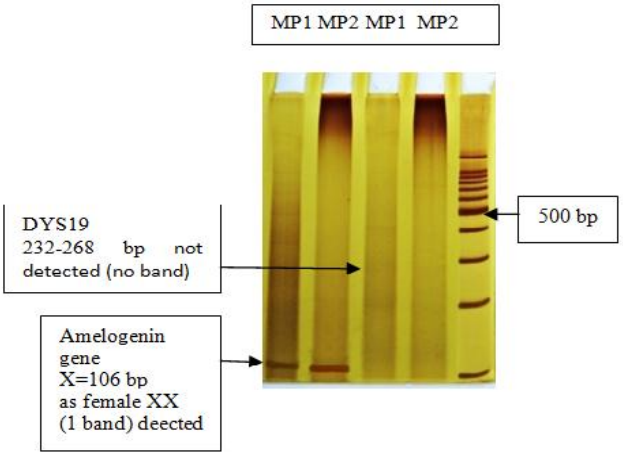

Figure 2. Results from MP1-MP2 samples

DISCUSSION

Development of a noninvasive technique using cell free fetal DNA in the maternal circulation is promising new hope for the benefit of prenatal diagnosis (Lo et al 1999). The use of cell free fetal DNA in maternal plasma allows forensic examination in cases of paternity (Rong et al 2012, Sarasola et al 2006). It is based on several successful studies conducted by using cell free fetal DNA in maternal circulation in determining the sex of the fetus. Even within a particular condition, according to Colmant et al (2013), detection using cell free fetal DNA is able to determine the sex of a fetus after 8 weeks of pregnancy.

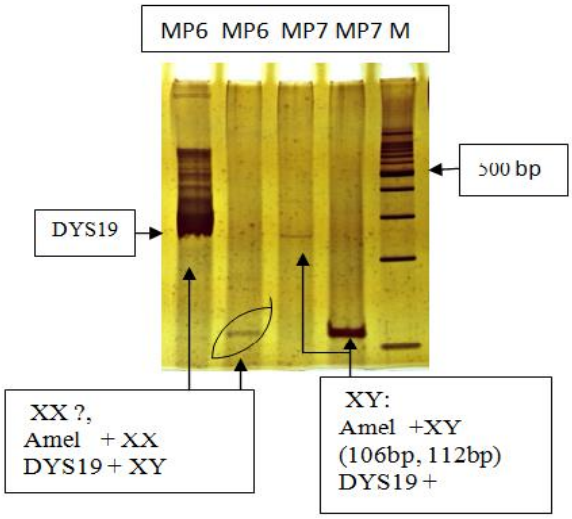

Figure 3. Results on sample MP3

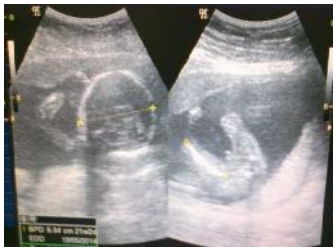

Figure 4. USG confirmation showing male sex

The results of this study showed that the average quantity of DNA derived from free fetal DNA has a smaller amount (45.27 ng/ul) rather than the quantity of DNA derived from maternal blood (305.02 ng/ul) to the ratio of 9.05%. This percentage indicates that the fraction of the quantity of DNA derived from fetal free, as stated by Brar et al (2013), is low or low fraction. However, a low fraction of the quantity of DNA, which does not mean that the DNA came from free fetal DNA, does not "have" the ability to be used as a specimen examination of DNA by PCR (Polymerase Chain Reaction). This is because the condition of the specimen DNA that can be used in DNA profiling in forensic science have a number or DNA content of approximately 20 ng/ml (Notosoehardjo 1999), with a purity of DNA ranging from 1-2 (mean purity of DNA from blood was 1.79 and the purity of maternal fetal free DNA is 1.41). These findings indicate that there is a suspicion of the presence of protein contamination on the isolated DNA in the sample. However, it is not a constraint because pure value in a range of 1-2 can still be used for DNA typing by PCR (Muladno 2002). The use of PCR as a method of DNA amplification in this study, a highly sensitive method is used as a DNA amplification technique, especially in the field of forensic DNA typing, which are often found in relatively small quantities (Prinz et al 2006). So that the least amount of target DNA, in certain circumstances does not affect the success of PCR in the field of forensics.

In this study we used amelogenin gene as starting point before using loci contained on short tandem repeat (STR). Rong et al (2012) states that the doubling of DNA amplification products at amelogenin gene, such as the 106 and 112 bp, basically can be performed. Nevertheless, cell free fetal DNA in maternal plasma contained in general shorter than maternal DNA. This has led to a failure in DNA amplification process, including the "false negatives" occurrence in fetal sex determination. Another reason is that the average quantity of cell free fetal DNA from the fetus or fetal origin in maternal plasma is only 3-6%. Such few quantity may result in the suppression of allele, ie the primary dominance of DNA binding by maternal as "high" quantity of maternal DNA. Amplification failure may increase if there is a different procedure with several methods in different individuals.

In the opinion of Burger et al (1999), the levels of DNA is an important factor in forensic DNA testing. This is because DNA level affects the success of genotyping in DNA samples. The potential reduction in the levels of DNA to 1 ng may decrease the detection capability Short Tandem Repeat (STR) to 95%. With regard to the levels of human DNA, there are several different opinions on the amount of the minimum levels of DNA that can be used for the purpose of forensic DNA analysis in the field. According Mandrekar et al (2001), the minimum required levels of DNA for forensic DNA typing in the field ranged from 0.1 to 50 ng. Meanwhile, according to Kline et al (2003), that the DNA template is recommended for use in DNA typing is 100-1000 ng. Notosoehardjo (1999) argues that the minimum required levels of DNA in forensic DNA examination, each for 50 ng and 20 ng. As according to Butler (2001), examination of DNA genotyping can still be carried out with good results, with minimal DNA concentration ranged from 0.5 ng-2.5 ng (measured using QuantiBlot).

Although there are differences in the levels of the minimum amount of DNA that can be used in DNA analysis, in principle the amount of DNA required levels in forensic DNA analysis depends on the needs and the type of examination performed. In forensic DNA examination based RFLP or restriction fragment length polymorphism, DNA concentration required is relatively large, about 100-1000 ng (Kline et al 2003). In addition to the amount or concentration of DNA from material examination, PCR-based DNA testing also

required sufficient DNA quality. Adequacy of qualified DNA can be degraded to minimum condition. If DNA is degraded in severe conditions this can make the primer unable to anneal the DNA targets to be duplicated. So that good quality DNA is a fundamental prerequisite for the success of the overall PCR reaction. This opinion is in line with Chung et al (2004), which states that the sensitivity of PCR is a function of the number of cycles, as well as the levels of DNA integrity. So that the reduced levels of DNA in forensic samples, up to a certain level will not affect too much to the success of DNA profiling in forensic science, especially in the examination of STR. This is because the levels are necessary in the evaluation of DNA STR tend to be less compared with other DNA test, so the risk of failure in the amplification of DNA with this technique is very small.

CONCLUSION

The average quantity of DNA derived from free fetal DNA has a smaller amount of the quantity of DNA from maternal blood to the ratio of 9.05%. This percentage indicates that the fraction of the quantity of DNA derived from fetal cell free fraction is low. Despite low, cell free fetal DNA has the ability to be used as a DNA specimen at gender determination by amelogenin gene and Y chromosome (DYS19). Further studies are needed to analyze the use of cell free fetal DNA in other loci that have not been used in this research in the field of forensic DNA testing in the future.

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