Discrimination between Human and Animal DNA by Loop-mediated Isothermal Amplification of *APOL1* Gene in Forensic Analysis

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Abstract

A method has been developed for discrimination between human and animal in forensic study by loop-mediated isothermal amplification (LAMP) analysis of the human (*Homo sapiens sapiens*) apolipoprotein L1 (*APOL1*) gene. DNA from blood of 8 sample species *Homo sapiens sapiens* (human), *Pan* sp. (Chimpanzee), *Mus musculus* ssp. (house mouse), *Canis lupus familiaris* (domestic dog), *Felis catus* (domestic cat), *Sus scrofa* (domestic pig) *Bos* sp. (cow) and *Gallus gallus* (domestic chicken) was extracted and subjected to LAMP for *ApoL1* amplification to test specificity and sensitivity. After DNA amplification, gel electrophoresis process was performed to detect LAMP product. Only LAMP products of human DNA showed a smear band. The limitation of detection was performed with reproducibility to be as low as 10 pg of genomic DNA. Therefore, the 10 pg of genomic DNA was equivalent to about 3 human diploid cell. This results showed the limitation of detection at least 3 cell by using this primer set and specific human DNA. Our finding showed this *APOL1* primer set for LAMP technique to be specific and reliable for detection of human DNA.

***Keywords**: loop-mediated isothermal amplification, LAMP, APOL1, human identification*

บทคัดย่อ

วัตถุประสงค์การศึกษาในครั้งนี้เพื่อเป็นการพัฒนาเทคนิค loop-mediated isothermal amplification (LAMP) เพื่อใช้ในการ แยกความแตกต่างระหว่างตัวอย่าง DNAของมนุษย์กับตัวอย่าง DNAของสัตว์ชนิดต่างๆซึ่งจะมีประโยชน์เป็นอย่างมากในงานทาง ด้านนิติวิทยาศาสตร์ โดยทำการสกัด DNAจากตัวอย่างเลือดของ *Homo sapiens sapiens* (มนุษย์), *Pan* sp. (ลิงชิมแปนซี), *Mus musculus* ssp. (หนูบ้าน), *Canis lupus familiaris* (สุนัขบ้าน), *Felis catus* (แมวบ้าน), *Sus scrofa* (หมูบ้าน) *Bos* sp. (วัว) และ *Gallus gallus* (ไก่บ้าน) หลังจากนั้นนำ DNA ที่ได้ไปเพิ่มปริมาณ *APOL1* ยีน โดยปฏิกริยา LAMP เพื่อทำการทดสอบหาความจำเพาะ (specificity) และความไว (sensitivity) ของเทคนิค LAMP และใช้เทคนิค gel electrophoresis ในการวิเคราะห์หา LAMP product ผลการวิจัยพบแถบปื้น DNA ในตัวอย่าง LAMP product ของมนุษย์เท่านั้น ข้อจำกัดในการตรวจโดยวิธีนี้สามารถตรวจ ซ้ำหาปริมาณ DNA ของมนุษย์ได้น้อยที่สุดอยู่ ที่ 10 pg โดยที่ 10 pg ของ DNA เทียบเท่ากับ 3 เซลล์ของมนุษย์ จากผลการทดลองแสดง ให้เห็นว่าข้อจำกัดของการตรวจปริมาณได้อย่างน้อยที่ 3 เซลล์โดยใช้ primer ที่จำเพาะกับ DNA ของมนุษย์ ข้อสรุปจากผลการทดลองที่ได้ ทำให้เห็นว่าการใช้ primeที่จำเพาะต่อยีน APOL1 ในเทคนิค LAMP มีความแม่นยำสูงในการพิสูจน์ความเป็นมนุษย์

***คำสำคัญ:** loop-mediated isothermal amplification, LAMP, APOL1, การพิสูจน์ความเป็นมนุษย์*

1. Introduction

Human identification is one of the most commonly important problems in forensic investigations. If the biological evidence come from human origin, it should be predetermined. At present, PCR of short tandem repeats (STR) which produces human-specific results (Sparkes et al., 1996; van Oorschot et al., 1994) is routinely used as an established method for forensic practice. However, STR typing fails in samples of poor quality so PCR - based human mitochondria DNA such as mitochondria D-loop region, cytochrome *b* and 16S rRNA genes, were tested and reported for forensic proposes (Bataille et al.,1999; Parson, Pegoraro et al.,2000; Tozzo et al., 2011). Morever, Apolipoprotein L1 gene (*ApoL1*) is an interesting gene found in human was lost from other primates such as chimpanzees genome (Cooper et al., 2011).

ApoL1 was initially discovered as one of the lipoprotein bound to high density lipoprotein (HDL) particle from human serum (Duchateau et al., 1997). ApoL1 may play a role in the inflammatory response (Wan et al., 2008) and has a role in innate immunity by protecting against *Trypanosoma brucei* infection (Perez-Morga et al., 2005; Poelvoorde et al.,2004). ApoL1 forms pore on the lysosomal membranes of

trypanosomes which causes in flux of chloride, swelling of the lysosome and lysis of the trypanosome (Perez-Morga et al., 2005; Vanhamme et al., 2003).

Nowadays, LAMP has become a novel method to replace PCR due to its more rapid and specific reaction. In this present time, LAMP has been developed and used in many applications , such as sex determination in human (Kanchanaphum et al., 2013) detection of pork meat in Halal food (Kanchanaphum et al., 2014) and detection of *Yersinia enterocolitica* in pork meat (Gao et al., 2009). This method was performed under isothermal conditions in the temperature range 60°C-65°C for 60 minutes (Notomi et al., 2000). There are two sets of primer. Inner primer and outer primer sets used in LAMP were specific at six different DNA sequences within the target DNA and primary DNA amplification began by the inner primer set. The characteristic intermediary DNA structure formed by LAMP, called a stem-loop DNA fragment, was generated and large amounts of DNA products were produced by an auto-cycle reaction (Zhang et al., 2009).

2. Objective

This study describes the development method for human DNA identification using LAMP technique. The strong point of the method is more faster and less complicated equipment than PCR for human identification.

3. Materials and methods

3.1 DNA template preparation

Genomic DNA was extracted from 8 sample species *Homo sapiens sapiens* (human), *Pan* sp. (Chimpanzee), *Mus musculus* ssp. (house mouse), *Canis lupus familiaris* (domestic dog), *Felis catus* (domestic cat), *Sus scrofa* (domestic pig) *Bos* sp. (cow) and *Gallus gallus* (domestic chicken) by Thermo Scientific Genomic DNA Purification Kit. Human blood samples were obtained from male and female volunteer aged between 18-25 years old.

3.2 LAMP primer design

The design of the LAMP primers was based on a Human Apolipoprotein L1 (GenBank accession No. GCA_000001405.15). The PrimerExplorer V4 software available on the Eiken Chemical Co.Ltd., website (http://primerexplorer.jp/e/) was used to design F3, B3, FIP and BIP primers, as shown in Table 1.

Table 1 Primer sequences used for LAMP amplification

Primer	Sequence (5'-3')
F3	TCAGAGGTCATCTCACCCAC
B3	TTCTTCTCCATCACCCCAGA
FIP	CCAACTTGTGCCAGGCCCTGGGAATGACTTGCCCAGGGT
BIP	AACAGAGGGACAGAGGTGAGTCCTATGATCTACCTGCTC

3.3 LAMP reaction and analysis

In LAMP amplification, all reactions were carried out in 25 µl of 1x*Bst* DNA polymerase buffer containing 5 mM $MgSO_4$, 400 mM betaine, 1.2 mM dNTPs, 0.8 µM F3 and B3 primers, 2 µM FIP and BIP primers, 8 U *Bst* DNA polymerase (New England Biolabs) and 10 ng of each DNA extracts as a template. Reactions were incubated at 65°C for 45 min and followed by enzyme inactivated at 80°C for 5 min.

For Specificity test, DNA samples of 7 animal species were tested. These included genomic DNA of primate animal with high similarity to human, i.e,. *Pan* sp. (chimpanzee) and domestic animals, i.e., *Mus musculus* ssp (house mouse), *Canis lupus* (domestic dog), *Felis catus* (domestic cat), *Sus scrofa* (pig), *Bos* sp. (cow) and *Gallus gallus* (chicken). β-actin gene was amplified by conventional PCR (Glare et al., 2002) for to demonstrate that all DNA tested were amplifiable prior to LAMP amplification. Total PCR reaction included 0.4 µM of each forward primer (5'-CTCCCTGATGGTCAGGTCAT-3') and reverse primer (5'-ATGCCAGG GTACATTGTGGT-3'), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1xPCR buffer (50 mM KCl, 10 mM Tris-HCl), and 1.25 unit of *Taq* DNA polymerase (New England Biolabs). PCR was carried out in a BIO RAD MJ Mini Personal Thermal Cycler. The cycle condition included a single initial denaturation at 94 °C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension step at 72°C for 5 min. The expected PCR product size was 203 bp.

Sensitivity of LAMP reaction was tested using 3 replicates with human genomic DNA at 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg, respectively.

After PCR and LAMP reaction, the products were analyzed by loading 10 µl of reaction products on 1.5% agarose gel. After gel electrophoresis, the gel was stained with ethidium bromide and visualized under an ultraviolet light

4. Results and Discussion

Nowadays, the main molecular biology technique used to determine human in forensic study are conventional PCR (Matsuda et al., 2005) and Real time PCR (Thompson et al., 2014). However, PCR requires sophisticated equipment and is time consuming. Therefore, a novel LAMP technique has been developed to identify human, which was initiated by Notomi and colleagues (2000) In this study, the specificity of LAMP reaction against DNA samples of 8 sample species was shown in Figure.1. Figure.1A indicated that the LAMP product of *ApoL* 1 gene was shown in only human DNA in lane 1. No LAMP product was detected in other lanes. Whereas , the 203 base pair of PCR product of β-actin gene from all DNA samples in the test were shown in every lane (Figure. 1B). These amplification of this PCR product revealed that there were genomic DNA in all DNA samples in the test. So this specificity test suggested that this LAMP primer set was only specific in human.

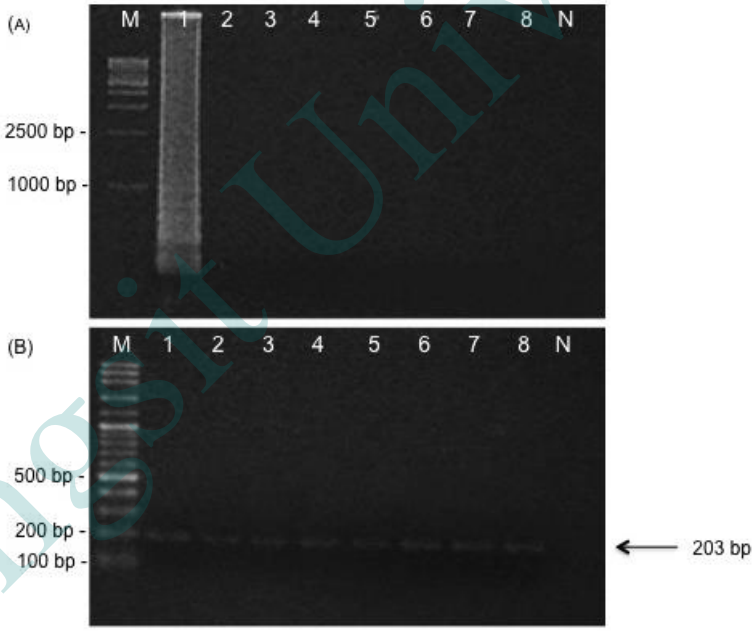

Figure 1 Specificity test. DNA samples included in the test were in Figure 1 (A). It shows that the LAMP primer set is specific to human DNA, as the LAMP product was only detected in lane 1. DNA samples included in the test were *Homo sapiens sapiens* (human) in lane 1, *Pan* sp. (Chimpanzee) in lane 2, *Mus musculus* ssp. (house mouse) in lane 3, *Canis lupus familiaris* (domestic dog) in lane 4, *Felis catus* (domestic cat) in lane 5, *Sus scrofa* (domestic pig) in lane 6, and, *Bos* sp. (cow) in lane 7, *Gallus gallus* (domestic chicken) in lane N and negative control in lane 9. Figure 1 (B) shows the presence of 203 bp PCR product of β-actin gene in 8 DNA samples.

Sensitivity and reproducibility test of human specific gene by *ApoL* 1 gene LAMP primer set was tested by using approximately 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg of human DNA, respectively in three replicates. Results showed that the limitation of human DNA detection of this LAMP was 10 pg. (Figure 2) Human diploid cell was approximately 3.59 pg (Butler, 2009), therefore, 10 pg of genomic DNA equivalent to about 3 human diploid cells. This results showed the limitation of detection at 3 cells by using this primer set.

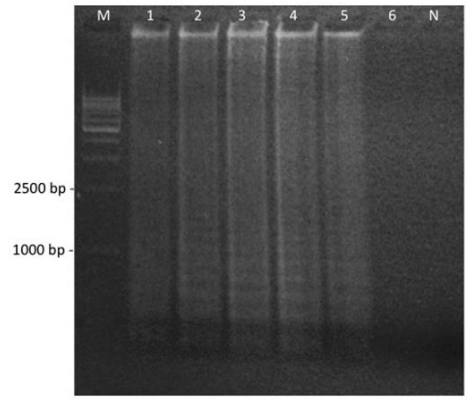

Figure 2 Sensitivity test. Sensitivity of LAMP primer set were tested with human genomic DNA at 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg in lane 1-6, respectively. Lane N is negative control.

Farrall (Farrall, 2014) reports that *ApoL* 1 gene has genetic variants that encode nonsynonymous (p.S342G and p.I384M) or in-frame deletion (p.NYK388K) alleles in Africans but absent in Asian and European population. In this study, all volunteers are Asian population. Then the variations of *ApoL* 1 gene was not detected. Although there is nonsynonymous (p.S342G and p.I384M) or in-frame deletion (p.NYK388K) alleles in Africans population, it may not effect this LAMP primer set. Because the in-frame deletion of this gene was only 6 base pairs and did not involve in the binding site of this LAMP primer set. However it is very interesting to test in more range population in further study.

LAMP does not require sophisticate instrument such as thermal cycle machine. It is fast and specific, involving 4 primers base on 6 specific sequence on the target gene to generate the ladder band. The reaction time of LAMP was about 1 hr because LAMP used one temperature (60-65°C) for DNA amplification. Another important advantage of LAMP is that the amplified product can be observed directly with the naked eye because of the white precipitation of magnesium pyrophosphate ($Mg_2P_2O_7$) in positive LAMP product (Notomi et al., 2000) and as fluorescence in the presence of either ethidium bromide or PicoGreen during UV illumination . This result is conformed with Watthanapanpituck and colleague (Watthanapanpituck et al., 2014). They used LAMP combined with a colorimetric gold nanoparticle hybridization probe to identify human DNA in forensic evidence.

5. Conclusion

This LAMP technique is very useful for human identification in forensic evidence because of its accuracy and convenience. It can be conducted rapidly and without complex instrumentation.

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