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Original Article

Identification of human DNA by loop-mediated isothermal amplification (LAMP) technique combined with white ring precipitation of $Cu(OH)_2$

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Abstract

Loop-mediated isothermal amplification (LAMP) analysis has been developed for human identification. DNA templates of 8 sample species including *Homo sapiens* (human) were subjected to LAMP for apolipoprotein L1 gene (*ApoL1)* amplification. After DNA amplification, gel electrophoresis was performed. Furthermore, $CuSO_4$ was added in to the LAMP product for staining visible to the naked eye. The LAMP products were only present with human DNA. The limit of detection was reproducibly as low as 10pg of genomic DNA. Moreover, by adding $CuSO_4$ to the LAMP product, the $Cu(OH)_2$ precipitate, which formed a ring-shaped deposit, was not seen with the human DNA, but was seen with the LAMP negative samples. Our findings show the LAMP technique to be a powerful method for identifying the presence of human DNA. The addition of $CuSO_4$ to the LAMP product is an alternative that can be used in field studies, and does not require access to gel-electrophoresis.

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

1. Introduction

Identifying the presence of human DNA is a common important problem in forensic investigations. Its purpose is to determine if biological evidence is of human origin. Currently, PCR of short tandem repeats (STR) usually produces human-specific results (Sparkes *et al.*, 1996; van Oorschot *et al.*, 1994) and is routinely used as an established method in forensic practices. However, STR typing fails in samples of poor quality, so PCR amplification of human mitochondrial DNA, such as mitochondrial D-loop region, cytochrome *b* and 16S rRNA genes, is reported for forensic proposes (Batailleet *al.*, 1999; Parson *et al.*, 2000; Tozzo *et al.*, 2011). Moreover, the apolipoprotein L1 gene (*ApoL1*) is quite interesting as it is found in human but is absent from other primates, such as chimpanzees (Cooper *et al.*, 2011). So, this is a good candidate gene for discriminating between humans and animals.

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ApoL1was initially discovered as one of the lipoproteins bound to high density lipoprotein (HDL) particles in human serum (Duchateau *et al.*, 1997). ApoL1 may play a role in the inflammatory responses (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 pores on the lysosomal membranes of trypanosomes, which causes an influx 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 alternative to replace PCR due to its more rapid and specific reactions. Currently LAMP has been developed for use in many applications, such as sex determination of humans (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). It is performed under isothermal conditions in the temperature range 60-65°C for 60 minutes (Notomi *et al.*, 2000). There are two sets of primers, inner and outer primer sets, used in LAMP specific to six segments within the target DNA, and the primary DNA amplification is begun by the inner primer set. The cha-

racteristic intermediary DNA structures formed by LAMP, known as stem-loop DNA fragments, are generated along with large amounts of DNA products from an auto-cycle reaction (Zhang *et al*., 2009).

This report describes the development of LAMP testing to identify the presence of human DNA. The strong points of our method include higher speed as well as less complicated equipment than with the PCR identification of human DNA; this technique is particularly appropriate for field studies.

2. Materials and Methods

2.1 DNA template preparation

Genomic DNA from the 8sample species, *Homo sapiens sapiens* (human), *Pan* sp. (Chimpanzee), *Mus musculus* ssp. (house mouse), *Canis lupus familiaris* (domestic dog), *Feliscatus* (domestic cat), *Susscrofa* (domestic pig) *Bos* sp. (cow), and *Gallus gallus* (domestic chicken) were supplied by Asst. Prof. Dr. Sasimanas Unajak.

2.2 LAMP primer design

The design of the LAMP primers was based on Human Apolipoprotein L1 (GenBank accession No. GCA_ 000001405.15). The Primer Explorer V4 software available on the Eiken Chemical Co. Ltd. website (http://primerex plorer.jp/e/) was used to design the F3, B3, FIP and BIP primers shown in Table 1, and their positions are shown in Figure 1.

2.3 LAMP reaction and analysis

In LAMP amplification, all the 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 extract as a template. Reactions were incurred by incubation at 65°C for 45 min, followed by enzyme inactivation at 80°C for 5 min.

To assess specificity, DNA samples of the 7 animal species were tested. These included genomic DNA of a primate animal which high similarity to human, i.e, .*Pan* sp. (chimpanzee), and of domestic animals, i.e., *Mus musculus* ssp (house mouse), *Canis lupus* (domestic dog), *Feliscatus* (domestic cat), *Susscrofa* (pig), *Bos* sp. (cow) and *Gallus gallus* (chicken).The β-actin gene was amplified by conventional PCR (Glare, Divjak, Bailey, & Walters, 2002) for demonstrating that all the DNA types tested were amplifiable prior to LAMP amplification. Total PCR reaction included 0.4 µM of each forward primer (5'-CTCCCT GATGGTCAGGTCAT-3') and reverse primer (5'-ATGCC AGG GTACATTGTGGT-3'), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1xPCR buffer (50 mMKCl, 10 mMTris-HCl), and 1.25 units of *Taq* DNA polymerase (New England Biolabs). PCR was carried out in a BIO RAD MJ Mini Personal Thermal Cycler. The cycle conditions 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, and 72°C for 30 sec; and a final extension step at 72°C for 5 min. The expected PCR product size was 203 bp.

Table 1. The primer sequences used for LAMP amplification

Primer	Sequence (5'-3')
F3	TCAGAGGTCATCTCACCCAC
B3	TTCTTCTCCATCACCCCAGA
FIP	CCAACTTGTGCCAGGCCCTGGGAATGACTTGC CCAGGGT
BIP	AACAGAGGGACAGAGGTGAGTCCTATGATCTA CCTGCTC

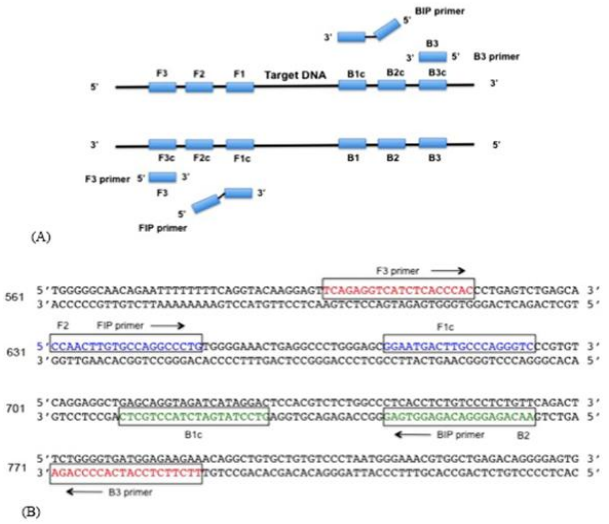

Figure 1. (A) Schematic diagram of the two inner (FIP and BIP) and the two outer (F3 and B3) primers for LAMP. This diagram partly reproduces material from Eiken Chemical Co.Ltd. (B) Nucleotide sequence of the*ApoL1* gene (GenBank accession No. GCA_000001405.15) that was used for the inner and outer primers.

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 ultraviolet light. To visually inspect LAMP products, 100 mM $CuSO_4$ was used to form a white ring-shaped deposition of precipitate.

3. Results

In this study, the specificity of LAMP reaction was assessed with DNA samples representing 8 species as shown in Figure 2. Figure 2A indicates that the LAMP product of *ApoL* 1 gene was present in only the human DNA, in lane 1. No LAMP product was detected in the other lanes. In contrast, the 203 base pair PCR product of actin gene is seen in every lane of Figure 2B. These PCR products confirm that there was genomic DNA in all the samples tested. This test suggests that the LAMP primer set was appropriate for the specific detection of human DNA.

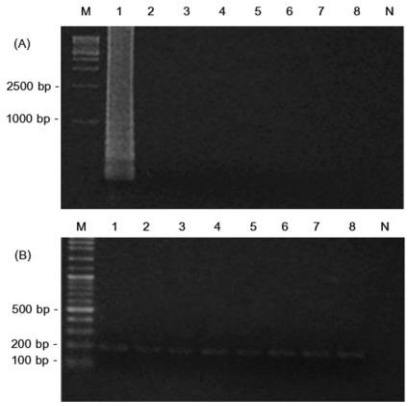

Figure 2. Specificity test. DNA samples included in the analyzed in (A), showing that the LAMP primer set was specific to human DNA: the LAMP product is only detected in lane 1. The DNA samples represent *Homo sapiens sapiens* (human) in lane 1, *Pan* sp. (Chimpanzee) in lane 2, *Mus musculus* ssp. (house mouse) in lane 3, *Canis lupus familiaris* (domestic dog) in lane 4, *Feliscatus* (domestic cat) in lane 5, *Susscrofa* (domestic pig) in lane 6, and *Bos* sp. (cow) in lane 7, *Gallus gallus* (domestic chicken) in lane 8, and the negative control in lane 9. (B) shows the presence of 203 bp PCR product from theβ-actin gene in all 8 DNA samples.

Sensitivity and reproducibility test of the human specific *ApoL* 1 gene by the LAMP primer set was done using approximately 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg of human DNA in three replicates (Figure 3). The results show that the limit of DNA detection by LAMP was 10 pg.

When $CuSO_4$ was added to the LAMP products, a white precipitate of Cu $(OH)_2$ formed a ring-shaped deposit only with negative LAMP reactions, as shown in Figure 4B. In contrast, the human DNA sample solution remained clear, as in Figure 4A. These results indicate that the LAMP technique can be used for species identification, and the addition of $CuSO_4$after LAMP reaction is an easy way to directly observe the results of LAMP reaction by naked eye. Moreover, the white ring of Cu $(OH)_2$ precipitate was easier to observe than the white precipitate of $Mg_2P_2O_7$.

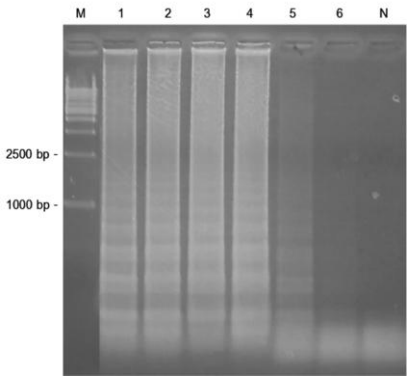

Figure 3. Sensitivity test. The sensitivity of the LAMP primer set was tested with the human genomic DNA amounts 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg, shown in this order in lanes 1-6. Lane N is the negative control.

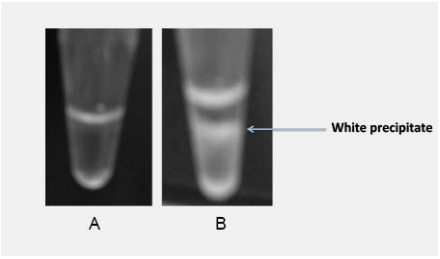

Figure 4. White precipitate of $Cu(OH)_2$added to the LAMP products. (A) The LAMP products of *Homo sapiens sapiens* (human) *APOL1* gene. (B) Negative LAMP reaction.

4. Discussion

Nowadays, the main molecular biology technique used to determine human DNA presence in a forensic study is PCR, such as conventional PCR (Matsuda *et al.*, 2005) or 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 DNA presence, initiatially by Notomi and colleagues (Notomi *et al*., 2000).In our current study, the sensitivity of LAMP primer set was 10 pg of human genomic DNA. The DNA amount in a human diploid cell is approximately 3.59 pg (Butler, 2009), so that 10 pg of genomic DNA is the equivalent of about 3 human diploid cells. We can state the limit of detection as about 3 human cells on using this primer set.

Farrall M. (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 that are absent in Asian and European populations. Although there are nonsynonymous (p.S342G and p.I384M) or in-frame deletion (p.NYK388K) alleles in Africans population(Farrall, 2014), these should not affect the LAMP primer set of current study. The in-frame deletion of this gene is only 6 base pairs and does not involve in the binding site of this LAMP primer set. However, a further study could use samples more widely representing human DNA.

LAMP does not require sophisticated instruments, such as a thermal cycle machine. It is fast and specific, involving 4 primers based on 6 specific sequences of the target gene to generate the ladder bands. The reaction time of LAMP was short at about 1 hr, at a single constant temperature (60-65°C) for DNA amplification. As another important advantage of LAMP, the amplified product can be observed directly with the naked eye from the white precipitate of magnesium pyrophosphate ($Mg_2P_2O_7$) in positive LAMP product cases (Notomi *et al*., 2000) and from fluorescence in the presence of either ethidium bromide or PicoGreen as response to UV illumination .

Our results are in alignment with (Watthana-panpituck *et al*., 2014)who used LAMP combined with a colorimetric gold nanoparticle hybridization probe to identify human DNA in forensic evidence, by targeting the human cytochrome b gene.

When adding $CuSO_4$to negative samples after LAMP reaction, a white ring of $Cu(OH)_2$ precipitate was formed (Zoheir *et al.*, 2010). The reason for this phenomenon is the excess dNTPs in the reaction that are almost uncharged, so they bind with $CuSO_4$ to form the white Cu $(OH)_2$

precipitate. At this time, the observation of white ring of precipitate is used in many applications. This method has been used for the rapid LAMP-based sexing of bovine embryos (Zoheir *et al*., 2010) and of Columbidae birds (Chan *et al*., 2012).

5. Conclusions

The specificity and reproducibility of human DNA identification by using *ApoL* 1 gene was tested using the LAMP technique. This method can be conducted quickly and without complex instrumentation. The addition of $CuSO_4$ makes it easy to visually detect the results, making the approach suitable for field use where gel electrophoresis apparatus is normally impractical.

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