IDENTIFICATION OF HUMAN DNA BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) METHOD

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Oral Presentation

Abstract

Loop-mediated isothermal amplification (LAMP) is a highly specific and sensitive technique used for amplification of specific DNA region under isothermal conditions which utilizes four primers that are complementary to six distinct regions of the target sequence. In order to determine DNA of human origin, primers were designed based on the human cytochrome *b* (cyt *b*) gene obtained from the GeneBank database (accession number: FJ383711) to allow for human-specific amplification of DNA samples. Using the designed primers, amplification of the transformed pGEM HCYTB-plasmid (carrying the cyt *b* gene fragment) and extracted human DNA (buccal swab) was successful based on agarose gel electrophoresis analysis. Conversely, no product was visible when the following samples were used: shrimp, dog, pig, monkey (Nomascus Concolor), hedgehog (Erinaceinae), and bird (Red Whiskered Bulbul). Furthermore, results indicated that the minimum copy number of template required for successful amplification was 10 copies. Thus, identification of human DNA by LAMP is specific, sensitive, and proves to be a useful tool in undetermined forensic specimens.

Keywords: Loop-mediated Isothermal Amplification, Cytochrome *b* gene, Mitochondrial DNA, Species identification, Human identification

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Background

From a forensic standpoint, it is fundamental to identify the origin of forensic biological evidence, most importantly human origin. Many households have domestic pets, thus, discrimination between human and non-human samples becomes increasingly important especially when living species other than human are found in the crime scene.

The cytochrome *b* (cyt *b*) gene is one of the 37 genes of the circular mitochondrial DNA (mtDNA). This region has often been referred to as the phylogenetic region and has been widely used as DNA marker in species identification applications[1,2,3,4,5]. Matsuda *et al.* (2005) have shown that human origin can be determined by amplification of a human-specific mitochondrion sequence. The sequence is 157 contiguous base pairs long, their study involved designing primers in order to amplify, using conventional PCR, this region that resides in the cyt *b*.

Loop-mediated isothermal amplification (LAMP), first described by Notomi *et al* (2000)[6] allows for nucleic acid sequence amplification with high specificity, sensitivity and rapidity under isothermal conditions. This technique amplifies target nucleic acids from a few copies to 10^9 copies under an isothermal temperature of 60 - 65°C within 1h. Isothermal conditions are possible due to the strand-displacement activity of the large fragment Bst DNA polymerase. This technique has been used quite extensively in different fields [7,8].

Objective

The objective is to identify human DNA by applying the LAMP technique to amplify human specific cyt *b* nucleotide sequences.

Materials and methods

1.1 DNA sample preparation

Human and animal DNA [i.e., shrimp, dog, pig, monkey (Nomascus concolor), hedgehog (Erinaceinae) and bird (Red Whiskered Bulbul)] were extracted from either buccal swab of living animal or tissue (shrimp and pork) available in the market using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA) according to manufacturer's instruction.The pGEM-HCYTB plasmid, carrying the 190 base pair cyt *b* gene fragment, was used for sensitivity testing.

1.2 Primer design

LAMP primers (FIB, BIP, F3, and B3) were designed using the PrimerExplorer V3 software program (http://primerexplorer.jp/elamp3.0.0/index.html) based on the 190 base pair human cyt *b* gene sequence (accession number: FJ383711) obtained from the GeneBank database. Primer sequences are shown in **Table 1**.

Primers	5'-Sequence-3'	Length
CYTB-F3	AACTAGGAGGCGTCCTTG	18
CYTB-FIP	GCTTTGTTGTTTGGATATATGGAGG-CCCTATTACTATCCATCCTCATC	48
CYTB-B3	ATGATGGTAAAAGGGTAGCT	20
CYTB-BIP	ATTTCGCCCACTAAGCCAATC-GTTGTCCTCCGATTCAGG	39

Table 1. Primer sequences

1.3 LAMP reaction

LAMP reaction mixture contained 2 µM of CYTB-FIP and CYTB-BIP primers, 0.2 µM of CYTB-F3 and CYTB-B3 primers, 1.2 mM of dNTP mix (Promega, Madison, WI, USA), 0.4 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 4 mM $MgSO_4$, 1X thermopol buffer, 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA), DNA template and sterile water to make up a final volume of 25µl.

Amplification was carried out at 60^{o}C for 1 hour. Amplification products were separated and detected by 2%(w/v) ethidium bromide-stained agarose/TBE gel electrophoresis.

1.4 LAMP specificity and sensitivity testing

The specificity of the designed primers was tested by using DNA extracted from animals as template.

Sensitivity testing was performed using ten-fold serial dilution (10ng-10pg) of extracted DNA as template. Furthermore, LAMP sensitivity testing on copy number was also conducted. A ten-fold serial dilution (10^{10} - 1copy) of plasmid DNA was used as template.

Results and Discussion

When LAMP products are amplified, the resulting products come in many sizes. Therefore, when observing the bands on the agarose gel, it is normal that the banding covers a larger area.

Specificity testing demonstrated that only human DNA template was amplified successfully. A wide variety of species were tested with the LAMP primers and all but human DNA template had product when detected using 2% (w/v) gel electrophoresis as shown by the banding in Figure 2. LAMP technique has been demonstrated to be specific[6] in that it employs 4 primers which recognizes 6 distinct locations of the target sequence. Therefore, for amplification to occur, all primers must anneal to their complementary region. Furthermore, nucleotide sequences in GeneBank of the human cyt *b* target sequence region are identical for all Caucasians and negroid subjects. Thus, the designed primers for the LAMP technique can amplify the target sequence of all races.

Sensitivity test indicated that the minimum copy number of template required was 10 copies as shown by the visible banding on the agarose gel in Figure 3a. This is in agreement with Notomi *et al* (2000) that a few copies of DNA can be amplified. Since cyt *b* comprises part of the mtDNA, copy number of mtDNA per skeletal muscle cell was approximately 3650 ± 620 [9]. This indicates that even with highly degraded DNA samples, amplification of the cyt *b* region may be possible. Furthermore, 10 pg of extracted genomic DNA produced visible bands on the agarose gel as demonstrated in Figure 3b.

Currently, there are a variety of methods in determining human origin. In the present study, we report the development of a specific and sensitive LAMP test that can be used to amplify human cyt *b* sequence. The target sequence is a 190 contiguous base pair nucleotide sequence that is located in the cyt *b*. Matsuda's *et. al.* (2005) 157 contiguous base pair is located within the aforementioned 190 contiguous base pair of the present study. They were able to demonstrate that these sequences were human specific by testing it against a variety of other species (chimpanzee, gorilla, Japanese monkey, crab-eating monkey, cow, pig, dog, goat, rat, chicken, and tuna). The utilization of LAMP permitted higher sensitivity and specificity under an isothermal condition (60°C).

Since analysis is based on the mtDNA genome, this extends the type of sample that is compatible with this technique. Determination of human origin DNA can also be accomplished by analysing hair follicles without its root.

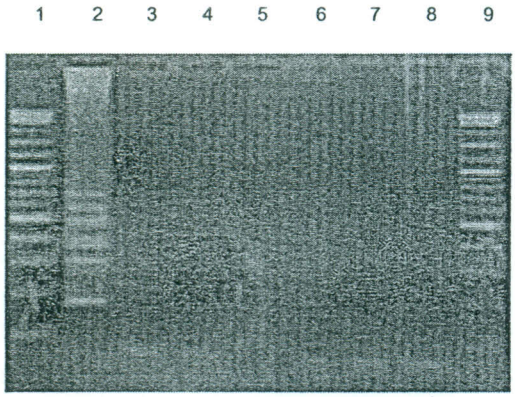

Figure 2. Amplification products of specificity testing were electrophoresed on 2% (w/v) agarose gel and stained with ethidium bromide. Lane 1, 2 log ladder; Lane 2, human; Lane 3, bird; Lane 4, dog; Lane 5, pig; Lane 6, shrimp; Lane 7, hedgehog; Lane 8, monkey

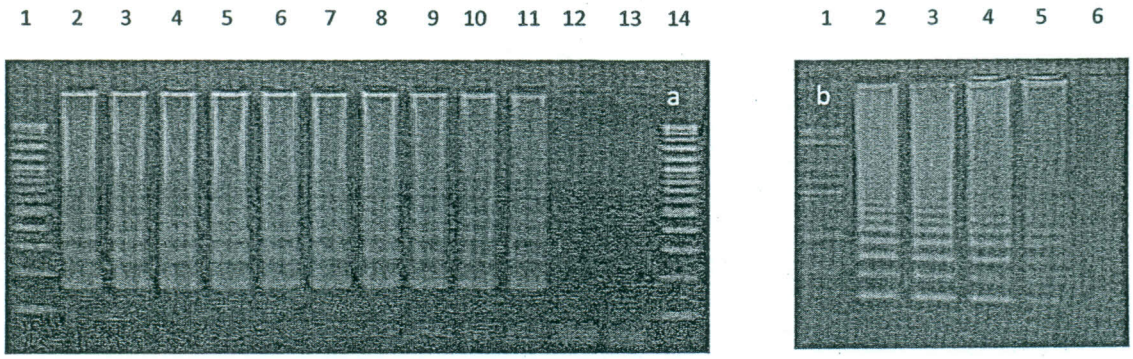

Figure 3. Amplification products of sensitivity testing, 10-fold serial dilution of human DNA.

a) Serial-dilution of pGEM HCYTB-plasmid (copy number). Lane 1, 2 log ladder; Lane 2, 10^{10} copy; Lane 3, 10^{9} copy; Lane 4, 10^{8} copy; Lane 5, 10^{7} copy; Lane 6, 10^{6} copy; Lane 7, 10^{5} copy; Lane 8, 10^{4} copy; Lane 9, 10^{3} copy; Lane 10, 10^{2} copy; Lane 11, 10^{1} copy; Lane 12, 1 copy; Lane 13, negative control; Lane 14, 2 log ladder

b) Serial-dilution of extracted genomic DNA. Lane 1, 2 log ladder; Lane 2, 10 ng; Lane 3, 1 ng; Lane 4, 0.1 ng; Lane 5, 10 pg; Lane 6, negative control (no DNA template).

Conclusion

In this study, we have developed LAMP primers that can amplify the human specific cyt *b* region using the LAMP technique. We are able to state that this set of LAMP primers will not amplify shrimp, dog, pig, monkey (Nomascus Concolor), hedgehog (Erinaceinae) and bird (Red Whiskered Bulbul).

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