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Development of Loop mediated isothermal amplification (LAMP) of SRY gene in human blood samples for sex determination

Panan Kanchanaphum[1]*, Titinan Sarataphan[1], Wirat Thirasan[1], and Gun Anatasomboon[2]

[1]Biochemistry Unit, Faculty of Science, Rangsit University, Patumthani 12000, Thailand
[2]Anatomy Unit, Faculty of Science, Rangsit University, Patumthani 12000, Thailand

*Corresponding author; E-mail: panan.k@rsu.ac.th

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Abstract

Sex determination of the source of forensic DNA evidence is an importance step in crime investigation. LAMP method is a novel molecular technique for determine the gender. *SRY* gene is male specific gene and used to indicate male genotype. In this study, we develop the new method to determine gender by using Loop mediated isothermal amplification (LAMP) technique from fresh blood sample and blood stained sample. DNA templates from fresh blood samples were extracted and added to a LAMP reaction. In addition, DNA extracted from blood stained sample in filter papers or cloths were used for LAMP reaction. Blood stained samples in filter and cloths were stored at room temperature for 0, 1, 7, 14, 30, and 45 days. Sex determination was achieved with LAMP technique for fresh blood samples and both blood stained samples on filter papers and cloths. LAMP products showed smear bands only in the male sample. LAMP products of samples from blood stained on filter papers kept at room temperature for up to 7 days were still detected while those of blood samples stained on cloth were detected after being kept at room temperature for up to 30 days. We have developed the LAMP method to amplify the *SRY* gene from fresh blood samples and blood stained samples on filter paper and cloth to determine the gender. Blood samples stained on cloths are more stable than those on filter papers. The authors of this study are confident that this finding will lead to a new practical application in the field of forensic and crime investigations.

Keywords: *sex determination, Loop-mediated isothermal amplification, LAMP, SRY gene, blood stained sample*

บทคัดย่อ

ทางนิติวิทยาศาสตร์ การแยกเพศจากตัวอย่างดีเอ็นเอในสถานที่เกิดเหตุเป็นขั้นตอนที่มีความสำคัญมาก ในงานวิจัยนี้ได้ใช้เทคนิค LAMP ซึ่งเป็นเทคนิคใหม่ในการแยกเพศ โดยใช้ยีนที่มีความจำเพาะต่อเพศชายคือยีน *SRY* ผู้วิจัยได้พัฒนาวิธีแยกเพศโดยเทคนิค LAMP จากตัวอย่างเลือดสดและคราบเลือด ในเลือดสดต้องมีการสกัด DNA ก่อนที่จะนำไปใส่ในปฏิกิริยา LAMP ส่วนตัวอย่างจากคราบเลือดบนกระดาษกรองและบนเศษผ้าก็จะต้องผ่านขั้นตอนทำการสกัด DNA แบบหยาบก่อนที่จะนำไปใส่ในปฏิกิริยา LAMP โดยที่ตัวอย่างคราบเลือดบนกระดาษกรองและบนเศษผ้า นั้นถูกตั้งทิ้งไว้ที่อุณหภูมิห้องเป็นเวลา 0, 7, 14, 30, และ 45 วัน หลังจากนั้นก็ทำการสกัด DNA แล้วนำไปใส่ในปฏิกิริยา LAMP จากการทดลองพบว่า สามารถแยกเพศจากตัวอย่างจากเลือกสดและคราบเลือดบนกระดาษกรองและบนเศษผ้าได้ โดยจำเพาะต่อตัวอย่างของเพศชายซึ่งให้ LAMP product เป็นแถบปื้นของ DNA สามารถแยกเพศจากตัวอย่างคราบเลือดบนกระดาษกรองที่ตั้งทิ้งไว้ที่อุณหภูมิห้องได้เป็นเวลาไม่นานเกิน 7 วัน แต่ในเชิงเปรียบเทียบปรากฏว่าสามารถแยกเพศจากตัวอย่างคราบเลือดบนเศษผ้าที่ตั้งทิ้งไว้ที่อุณหภูมิห้องได้เป็นเวลายาวนานถึง 30 วัน จากผลการทดลองที่ได้มานี้ทำให้ผู้วิจัยเกิดความเชื่อมั่นว่าสามารถพัฒนาวิธี LAMP ในการเพิ่มปริมาณยีน *SRY* จากตัวอย่างเลือดสดและคราบเลือดบนกระดาษกรองและเศษผ้าได้ ซึ่งตัวอย่างคราบเลือดบนเศษผ้าอยู่คงทนกว่าตัวอย่างคราบเลือดบนกระดาษกรอง คณะผู้วิจัยมีความเชื่อมั่นว่าผลการวิจัยชิ้นนี้จะมีประโยชน์อย่างยิ่งในการประยุกต์ใช้แยกเพศในงานทางด้านการสืบสวนทางด้านอาชญาวิทยาและนิติเวชวิทยาในอนาคตต่อไป

คำสำคัญ: *การแยกเพศ, Loop-mediated isothermal amplification, LAMP, ยีน SRY, ตัวอย่างคราบเลือด*

1. Introduction

Sex determination from biological specimens gathered from crime scenes is very important in criminal investigations (von Wurmb-Schwark, Bosinski, & Ritz-Timme, 2007). Sex determination is the first step for person identification in forensic science. In general, the sexual determination of unidentified body can be indentified based on anatomical characteristics of the external genitalia or the gonads are ovaries or testes. Bones and teeth are used for sex determination in markedly decayed or skeletonized bodies (Murakami, Yamamoto, Yoshitome, Ono, Okamoto, Shigeta, et al., 2000). The molecular biological

methods for sex determination based on polymerase chain reaction (PCR) have been widely used, especially in forensic science (Kastelic, Budowle, & Drobnic, 2009). The sex-determining region Y or *SRY* gene on the Y chromosome is used for sex determination (Santos, Pandya, & Tyler-Smith, 1998; Thangaraj, Reddy, & Singh, 2002). In mammals, the inducing of male sex determination required the Y-chromosome gene *SRY*. *SRY* encodes a protein with a central high mobility group domain (HMG) of about 78 amino acid (Whitfield, Lovell-Badge, & Goodfellow, 1993). Methods for sex determination using PCR for amplification the *SRY* gene have also been reported (Santos et al., 1998; Thangaraj et al., 2002). These methods can indicate a male genotype by the presence of the amplified product of *SRY* gene.

Recently, loop-mediated isothermal amplification (LAMP) has become an interesting method to replace PCR due to its more rapid and sensitive reaction. Furthermore, LAMP has been developed and used for sex determination in bovine embryos, female calves, salmon and *Columbidae* bird (Chan, Liu, Yang, Kuo, Chang, & Wang, 2012; Hirayama, Katagiri, Kageyama, Minamihashi, Moriyasu, Sawai, et al., 2007; Zhang, Zhang, Liu, Zhang, An, Quan, et al., 2009). This method involved amplification of DNA targets under isothermal conditions in the temperature range 60°C-65°C for 60 minutes (Notomi, Okayama, Masubuchi, Yonekawa, Watanabe, Amino, et al., 2000). Two sets of primers, inner primer and outer primer sets used in LAMP were specific at six different areas located within the target sequence and primary DNA amplification was begun 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).

Therefore, the objective of this study was to determine the human gender from fresh blood and blood stained on filter paper and on cloth by using LAMP technique. The major advantages of our method are more rapid, more sensitive and inexpensive method for sexual determination. Another advantage is usage this technique at crime scenes.

2. Objective

Sex determination of the source of forensic DNA evidence is an importance step in crime investigation. LAMP method is a novel molecular technique for determining the gender. *SRY* gene is male specific gene and used to indicate male genotype. We sought to develop the new method to determine gender by using LAMP technique from fresh blood samples and blood stained samples.

3. Materials and methods

3.1 Materials

Bst DNA polymerase and chemicals and reagents were purchased from New England Biolabs and Sigma chemical companies, respectively. Nucleospin-blood extraction kit was obtained from Macherey-Nagel, Germany. The 1 Kb DNA ladder was bought from New England Biolabs.

3.2 Template preparation

Genomic DNA was extracted from fresh blood samples by Nucleospin-blood extraction kit. To prepare DNA template from stained blood samples, one drop of blood samples was spread to filter papers and let them dried and kept at room temperature until used. The blood stained samples on filter papers or cloths were cut into small pieces and added 100 µl of Red blood cell lysis buffer (RBC buffer) that contained 10% W/V sucrose, 10% Trition X-100, and 5mM $MgCl_2$. The 2 µl of proteinase K was also added in this reaction and incubated at 56 °C for 2 h then inactivated enzyme at 95°C for 5 min. The supernatant was taken in 1.5 ml microcentrifuge tube. After adding phenol: chloroform:isoamyl alcohol (25:24:1) (Sigma) at ratio 1:1, the reactants were centrifuged at 10,000 x g for 10 min. Then the upper solution was taken and added with isopropanol (Sigma) at equal volume. The solution was centrifuged at 10,000 x g for 10 min and the supernatant was discarded. The DNA pellet was washed with 70% ethanol and centrifuged at 10,000 x g for 10 min. After that the pellet was air dried and suspended with 20 µl of sterile distilled water.

For time course detection in both blood stained samples on filter papers and on cloths, the blood stained samples were stored for 0, 1, 7, 14, and 30 days in room temperature. Then the blood stained samples were cut into small pieces and one small piece of these cut blood stained samples was further processed for DNA extraction. Subsequently, these DNA solutions were used as templates for LAMP amplification.

3.3 LAMP primer design

The design of the LAMP primers was based on a human *SRY* gene (GenBank accession No. JQ811934). 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	AACAGTAAAGGCAACGTCCA
B3	TCTCTGTGCATGGCCTGTA
FIP	CCATCTTGCGCCTCTGATCGCTTTTAGAGTGAAGCGACCCATGAA
BIP	AGAGATCAGCAAGCAGCTGGGTTTTAGAATGGCCATTTTTCGGCT

3.4 LAMP reactions and analysis

All reactions were carried out in 25 µl of 1 x *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, and 8 U *Bst* DNA polymerase (New England Biolabs), and 5 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. The LAMP products were analyzed by loading 10 µl of reaction products on 1.5% agarose gel. After gel electrophoresis, gel was stained with ethidium bromide and visualized under an ultraviolet light.

4. Results

4.1 LAMP reactions and analysis

After detection of LAMP of *SRY* reaction by gel electrophoresis, the smeared bands of amplified products indicated the positive LAMP-*SRY* reaction. Our result revealed the positive LAMP-*SRY* reaction was highly specific only with fresh male blood samples, as shown in Lane 1 and 2 of Figure 1 while no amplification was found in female blood sample, as shown in Lane 3 of Figure 1. When LAMP of *SRY* reactions were performed in blood samples stained on filter papers (lane 1, 2, and 3 of Figure 2) and on cloths (Lane 1, 2, and 3 of Figure 3), which were kept at room temperature for a day, the LAMP results of the stained blood samples show as similar as those of fresh human blood samples as shown in Figure 2. These results indicated that the LAMP techniques can be used for sex determination not only in fresh human blood but also human blood stained on papers and cloths.

For the determination of detection limit for storage time of blood samples stained on filter papers and cloths, the LAMP reactions were performed using DNA extracted from blood samples stained on filter papers and cloths, which were kept at room temperature for 0, 7, 14, 30, and 45 days, respectively. The results of stability time course for detection by LAMP of *SRY* gene in the stained blood samples showed that LAMP products were still detected in male blood samples stained on filter paper, which were kept at room temperature for up to 7 days, as shown in Figure 4. While the smeared bands of LAMP products were still found in male blood samples stained on cloths, which were kept at room temperature for up to 30 days, as shown in Figure 5.

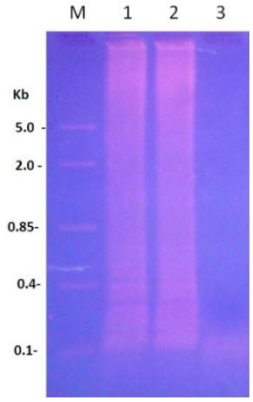

Figure 1 Agarose gel electrophoresis of LAMP products from fresh blood samples. Lane M is FastRuler Middle Range DNA ladder. Lane 1 and 2, fresh blood samples from male No.1 and No.2. Lane 3, fresh blood sample from female.

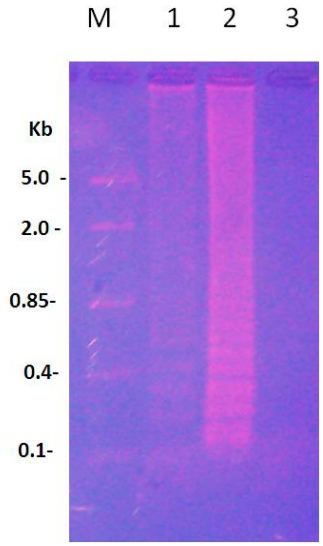

Figure 2 Agarose gel electrophoresis of LAMP products from blood stained samples on filter paper. Lane M is FastRuler Middle Range DNA ladder. Lane 1 and 2, blood stained samples from male No.1 and No.2. Lane 3, blood stained sample from female.

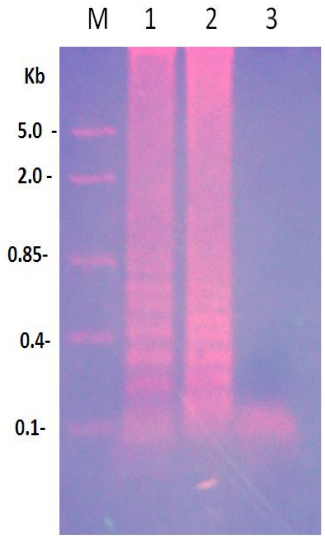

Figure 3 Agarose gel electrophoresis of LAMP products from blood stained samples on cloth. Lane M is FastRuler Middle Range DNA ladder. Lane 1 and 2, blood stained samples from male No.1 and No.2. Lane 3, blood stained sample from female.

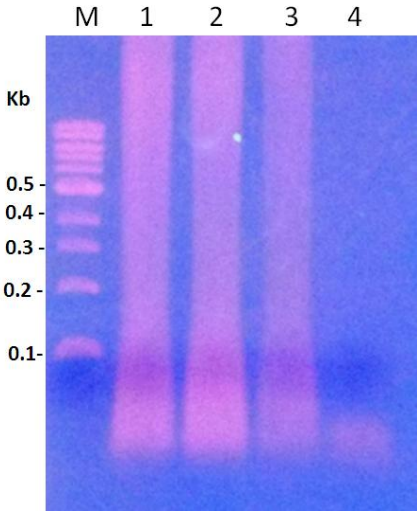

Figure 4 Agarose gel electrophoresis of LAMP products from blood stained samples on filter paper of various time. Lane M is 100 bp DNA ladder. Lane 1-4 are dry blood samples on filter paper that stored in room temperature for 0, 1, 7, and 14 days, respectively.

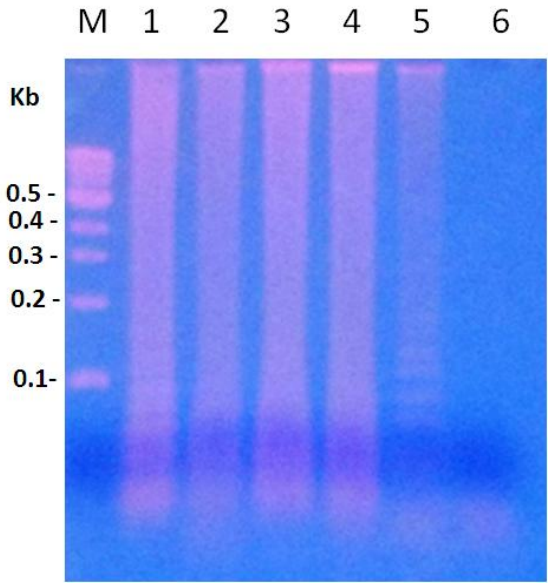

Figure 5 Agarose gel electrophoresis of LAMP products from blood stained samples on cloth of various time. Lane M is 100 bp DNA ladder. Lane 1-6 are dry blood samples on filter paper that stored in room temperature for 0,1,7,14, 30, and 45 days, respectively.

5. Discussion

So far, the molecular biology techniques mainly use for sex determination in human have been attributed to PCR of several genes in Y-chromosome (Hanley, Hagan, Clement-Jones, Ball, Strachan, Salas-Cortes, et al., 2000; Finch, Hope, & van Daal, 1996) Particularly, *SRY* gene (Luptakova, Babelova, Omelka, Kolena, Vondrakova, & Bauerova, 2010; Sato, Shinka, Chen, Yan, Sakamoto, Ewis, et al., 2009). Recently, a novel LAMP technique has been developed for sex determination, which was initiated by Notomi et al. (Notomi et al., 2000). LAMP does not require any reagents or sophisticated equipments and is an easy, fast, specific and sensitive technique involving 4 primers based on 6 specific sequence on the target gene to generate the smear and/or ladder band. In addition the amplification results can be evaluated by electrophoresis or fluorescent dye stain. Nowadays, it has also been used for detection of infectious diseases, food-borne bacteria, virus and parasites (Ikadai, Tanaka, Shibahara, Matsuu, Uechi, Itoh, et al., 2004; Maruyama, Kenzaka, Yamaguchi, Tani, & Nasu, 2003; Song, Li, Hou, Li, & Chen, 2012; Yoshikawa, Ihira, Akimoto, Usui, Miyake, Suga, et al., 2004). However, there has been no such a report on the application of LAMP method for sex determination. In this study, we successfully developed the novel LAMP of *SRY* gene technique for sex determination in fresh human blood and blood stained on materials. The positive LAMP-*SRY* reaction was highly specific with only fresh male blood samples and male blood samples stained on materials, but not in female blood samples.

Blood stained sample is the common evidence that found in crime scene. In real crime scene, blood stained sample is usually found and difficult to determine sex. Nevertheless, LAMP technique in this study can overcome this hindrance. For this LAMP technique, anticoagulants were not necessary for DNA extracted from blood samples stained on materials. RBC buffer is used to dissolve blood stains from filter paper or cloth after that proteinase K lyses membrane of white blood cell. Then, the extracted DNA is purified and precipitated before using in LAMP reaction.

LAMP of the *SRY* gene products of male blood stained on filter paper for up to 7 days storage and on cloths for up to 30 days storage were still found. These results indicated that the DNA in blood left on cloth are more stable than DNA in the blood left on filter paper. On the other hand, sex determination by LAMP of *SRY* gene in the dried blood left on cloths is easier to detect in the crime scene than blood left on paper.

The authors are convinced that the detection of *SRY* gene by LAMP technique for sex determinations of the source of forensic DNA samples can be applied for many forensic investigations, especially sexual assault cases. The LAMP of SRY gene detected in the dried blood which is collected evidences in crime scenes, can be applied for sex determination.

6. Conclusion

Sex determination using detection of *SRY* gene by LAMP method in both fresh human blood and blood stained on cloth and papers show the smear and/or ladder band only in male blood samples, but not detectable in female samples. DNA of blood stained on filter paper is more stable than those of blood stained on filter papers when used for sources of the extraction of template for LAMP technique. On the other hand, sex determination by LAMP of *SRY* gene in the dried blood on cloths is easier to detect in the crime scene than blood left on papers.

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