Co-amplification of human specific and conserved region in cytochrome *b* gene confirms human bloodstains

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ABSTRACT

The objective of this study was to determine human bloodstains by duplex PCR analysis of cytochrome *b* gene (*Cyt b*). Two regions of *Cyt b* consisting of conserved and human specific regions were the targets of interest. The primers specific to these regions were designed and tested on DNA extracted from human and animal (canine, feline, pig, chicken and cow) blood samples. A PCR product of *Cyt b* conserved region (~309 bp) was found in both human and animal blood samples while a DNA fragment of human specific region (~412 bp) was seen only in human blood sample. Duplex PCR analysis was then carried out. Human blood could be discriminated from animal blood by the number of amplified fragments, two for human and one for animal blood samples. The duplex PCR analyses of mixed human and animal bloodstains were also investigated. Duplex PCR could determine human DNA even when the quantity of human blood was 100 fold less than that of animal blood. The effects of temperature and age of bloodstains on human blood determination were then examined. Human bloodstain samples were kept at different temperatures (25, 35, 45 and 55°C) for different periods of time (1, 7, 14 and 30 days). The results demonstrated that duplex PCR analysis is able to determine human bloodstains in all experimented conditions.

Keywords: human bloodstains; cytochrome *b* gene; duplex PCR; forensic science

INTRODUCTION

Forensic investigation of bloodstains mostly involves human blood identification, sex determination and age estimation. For human blood identification, techniques based on the interaction between human protein target and antibody e.g. precipitin test (Gomes *et al*., 2001) immunochromatographic test (Schweers *et al*., 2008) and enzyme-linked immunosorbent assay (ELISA) test (Hurley *et al*. 2009) are mostly used. However, the detection limit of these techniques is influenced by the protein content and the specificity of antibody (Tsutsumi *et al*., 1987; Bataille *et al*., 1999). Thus, other approaches such as near infrared spectroscopy (Edelman *et al*., 2012), raman spectroscopy (McLaughlin *et al*., 2014) and mass spectrometry (Donfack and Wiley, 2015) have been introduced to the study of human blood identification. However, the necessity for the expertise and specialized instruments limit the ability of some laboratories to analyze blood evidence using these techniques. Hence, simpler method, like polymerase chain reaction (PCR), is favorable.

For human blood identification, nuclear DNA either on non-coding sequence e.g. STR loci (Crouse and Schumm, 1995) or coding sequence e.g. *myoglobin* (Ono *et al*., 2001) and *FOXP2* (Soejima *et al*., 2012) has been used as a target for PCR analysis. Although, the success of these DNA targets has been reported, failure of analysis may occur due to DNA degradation. Mitochondrial DNA is considered as an alternative target from nuclear DNA because it is less easily destroyed due to its smaller size and higher copy number. It has been reported that mitochondrial DNA analysis could give results even when extracted from a highly decomposed corpse (Sullivan *et al*., 1992). Cytochrome *b* (*Cyt b*) gene is one of 37 genes located on mitochondrial DNA. The DNA sequence of *Cyt b* is species-specific. Therefore, primers specific to human *Cyt b* were used to identify human blood samples (Matsuda *et al*., 2005; Caine *et al*., 2006). However, as a single site amplification of DNA, results with no PCR product may cause misinterpretation. Co-amplification of more DNA target sites may confirm the negative PCR results.

Therefore, this study aims to confirm human bloodstains by duplex PCR analysis of *Cyt b*. Two pairs of primers were designed to bind the conserved region and human specific region. The designed primers were tested on human and animal blood samples. The duplex PCR analysis was then carried out to analyze human, animal and mixed blood samples. The effects of temperature and the age of bloodstains on duplex PCR analysis were also investigated.

MATERIALS AND METHODS

Sample preparation

Male and female blood samples (5 samples of each) were kindly provided by blood bank, Faculty of Medicine, Khon Kaen University with permission from the Khon Kaen University Ethics Committee in Human Research (Project no. HE 533019). The animal blood samples were supplied by Department of Veterinary Technology, Faculty of Technology, Udon Thani Rajabhat University. One milliliter of animal blood samples were taken from five species each: *Canis lupus* (dog), *Felis catus* (cat), *Sus scrofa* (pig), *Gallus gallus* (chicken) and *Bos Taurus* (cow). The blood samples were subsequently kept in collecting tubes containing anti-coagulants. For bloodstains samples, 200 μl of blood samples were dropped onto cotton swabs and allowed to dry at room temperature.

DNA extraction

DNA was extracted from fresh blood samples, 200 μl from each sample, using a genomic DNA isolation kit, Nucleospin® (Macherey-Nagel, Germany), according to the manufacturer's instructions. The quantity and quality of eluted DNA was then measured by NanoDrop (Bio-Active, Thailand). For bloodstain samples, the sample was cut into small pieces and then placed into microcentrifuge tube containing 1 ml deionised water for 30 min at room temperature and vortex for 10 seconds every 5 min. The sample was then centrifuged and 200 μl aliquots were taken for DNA extraction as described above.

Primer design

The DNA sequences of *Cyt b* from human (HQ843989.1) and animals including *Gallus gallus* (NC_001323.1), *Felis catus* (NC_001700.1), *Canis lupus* (AU598499.1), *Sus scrofa* (HM010474.1) and *Bos taurus* (HQ184045.1) were obtained from NCBI database. These sequences were then compared using CLUSTALW program (http://www.ebi.ac.uk/Tools/msa/clustalw/). Two regions of *Cyt b* conserved sequence and human specific sequence were determined. Two pairs of primers designated as ConservedCytb and HumanCytb were then designed from these regions. Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was also compared with the chromosome sequences of humans and all tested animals in order to confirm the specificity of designed primers. No sequences other than those of *Cyt b* regions were matched.

Simplex PCR amplification

This experiment was carried out to check whether the designed primers could amplify the targeted sequence. The DNA was extracted from human and animal blood samples as described earlier. The PCR reaction containing 2 ng DNA, forward and reverse primer at 0.1 μM each, 1x *Taq* master mix (Vivantis, Malaysia) was set up. Deionised water was then added to bring the reaction volume up to 20 μl. The conditions were initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C 30 sec, 50°C 30 sec, 72°C 1 min and a final extension at 72°C for 2 min. The amplicon was subsequently examined by gel electrophoresis in 1.2% agarose gel. After the electrophoresis, gel was stained in 0.5 μg/ml ethidium bromide solution in deionised water for 10 min with gentle agitation. The gel was then destained in deionised water for 10 min before being visualized under UV light and photographed.

Duplex PCR amplification

DNA extracted from human and animal blood samples were used for duplex PCR analysis of conserved and human-specific *Cyt b*. The reactions consisted of 2 ng DNA, 1xTaq Master Mix, HumanCytb forward (5′-CTTCCTTCTCTCCTTAATGACATTAAC-3′) and reverse primer (5′-TAGGGAGATAGTTGGTATTAGGAT-3′) at 0.1 μM each, ConservedCytb forward (5′-CCATCCACCATCTCAGCATGATGAAA-3′) and reverse primer (5′-CCCCTCAGAATGATATTTGTCCTC-3′) at 0.1 μM each were prepared. Deionized water was then added to make the total volume up to 20 μl. The duplex PCR condition was the same as simplex PCR as described earlier. To determine the presence of PCR products, gel electrophoresis followed by gel staining were then carried out. Apart from human and animal blood samples, DNA samples extracted from male and female bloodstains were also analyzed by duplex PCR.

Duplex PCR analysis of mixed bloodstains

Human and dog mixed blood was prepared in various ratios (1:1, 1:2, 1:5, 1:10, 1:20, 1:50 and 1:100 of human:dog). The mixed bloodstains were prepared on cotton swabs as described earlier. The DNA was then extracted and the extracted DNA was used as a template for duplex PCR analysis.

Effects of temperature and bloodstain age on duplex PCR analysis

Male and female (5 each) bloodstains prepared by adding 200 μl onto cotton swab were kept in the incubator chamber at different temperatures including 25, 35, 45 and 55°C under 70% relative

humidity. At each temperature, the bloodstains were left for various periods of time e.g. 1, 7, 14 and 30 days. Experiment of each condition was performed in replicate. DNA was then extracted from human bloodstain samples and then analyzed by duplex PCR as described before.

RESULTS

The designed primers

The *Cyt b* sequences of human, chicken, cat, dog, pig and cow were compared using CLUSTALW program (Figure 1). The first primer pair (ConservedCytb) and the second primer pair (HumanCytb) were designed from the *Cyt b* conserved and human specific region, respectively. For human-specific *Cyt b* primers (HumanCytb), the forward and reverse primers bind to nucleotide 702–728 and 1090–1113, respectively. The human *Cyt b* sequences at the nucleotide 14816–14841 and 15149–15163 were used for forward and reverse primers of *Cyt b* conserved region (ConservedCytb). The expected sizes of amplicons amplified from the conserved and human-specific regions of *Cyt b* were 309 and 412 bp, consecutively.

Simplex PCR analysis of primer specificity

This experiment aimed to examine whether or not the designed primers are specific to the target regions. DNA extracted from human and animal blood fresh samples were used as template DNA. These DNA samples were separately amplified by PCR with either ConservedCytb or HumanCytb. The results of PCR products amplified by ConservedCytb and HumanCytb are shown in Figure 2a and 2b. For the PCR analysis on *Cyt b* conserved region, a DNA band sized 309 bp was observed in all samples. An amplicon sized 412 bp was only amplified from the DNA extracted from human blood. No other fragments than those of expected fragments are seen. These results suggest that PCR amplification by designed primers could differentiate human blood from animal blood samples.

Duplex PCR analysis of blood samples

The amplification of conserved and human specific *Cyt b* in a single reaction was investigated on human and animal blood samples. Two amplicons (~309 bp and ~412 bp) were found in human blood samples while only one amplicon (~309 bp) appeared in animal blood samples (Figure 3). These results confirmed that duplex PCR could identify human blood samples. However, a non-specific band sized approximately 1,100 bp was seen in duplex analysis of human blood samples. The human bloodstains samples were then analyzed. DNA extracted from 5 male and 5 female bloodstains were used as templates. Two amplicons (~309 and ~412 bp) were produced in both male and female samples as expected, though a non-specific band (~1,100 bp) was produced (Figure 4). These results suggest that human bloodstains could be determined by duplex PCR analysis of the conserved and human specific *Cyt b*.

Duplex PCR analysis of mixed bloodstains

The capability of duplex PCR analysis for the determination of human blood in mixed bloodstains sample was examined. DNA extracted from various ratio of mixed human and dog bloodstains was investigated. Clearly, duplex PCR could determine human DNA in the mixed bloodstains sample because two DNA bands of conserved (309 bp) and human specific (412 bp) *Cyt b* were shown (Figure 5). However, the band intensities gradually decreased with lower amounts of human blood. Interestingly, even small amounts of human blood (1:100) in mixed bloodstains could be examined by duplex PCR analysis of *Cyt b*. These results suggest that duplex PCR analysis of *Cyt b* is capable of determining human blood in mixed bloodstains samples. The non-specific band (~1,100 bp) was not visible in this examination.

The effects of temperature and bloodstain age on duplex PCR analysis

The proficiencies of duplex PCR analysis on different conditions of human bloodstains were investigated. Male and female bloodstains kept at different temperatures for different periods of time were examined. Two DNA bands sized 309 and 412 bp were found in all tested conditions (Figure 6). Non-specific DNA bands (~1.1 and ~2 kb) were found in samples that had been kept at 25°C. Samples kept at 45°C and 55°C also had a non-specific DNA band sized ~1.1 kb. However, no non-specific DNA band was revealed from the sample kept at 35°C. Although, non-specific band was found in some kept conditions, two expected amplicons were clearly presented in all kept conditions. These results indicate that duplex PCR analysis of *Cyt b* was capable of determining bloodstain samples as human blood even with age and the temperature conditions of bloodstains up to 30 days at 55°C, respectively.

Figure 1 Sequence comparison of cow, pig, cat, human, chicken and dog cytochrome *b* genes. The symbol (*) represents identical nucleotides. Open boxes indicate the forward and complementary sequences of reverse primer ConservedCytb. Underlines indicate the forward and complementary sequences of reverse primer HumanCytb.

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Homo sapiens        TCCCGTGAGGCCAAATATCATTCTGAGGGGCCACAGTAATTACAAACTTA
Canis lupus         TACCATGAGGACAAATATCATTTTGAGGGGCAACTGTAATCACTAATCTT
Felis catus         TACCATGAGGCCAAATGTCCTTCTGAGGAGCAACCGTAATCACTAACCTC
Bos taurus          TACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATTACCAACCTC
Sus scrofa          TGCCCTGAGGACAAATATCATTCTGAGGAGCTACGGTCATCACAAATCTA
Gallus gallus       TCCCATGGGGCCAAATATCATTCTGAGGGGCCACCGTTATCACAAACCTA
                    * ** ** ** ***** ** ** ***** ** ** ** ** ** **  *

Homo sapiens        CTATCCGCCATCCCATACATTGGGACAGACCTAGTTCAATGAATCTGAGG
Canis lupus         CTCTCTGCCATCCCTTATATCGGAACTGACTTAGTAGAATGAATCTGAGG
Felis catus         CTGTCAGCAATTCCATACATCGGGACTGAACTAGTAGAATGAATCTGAGG
Bos taurus          TTATCAGCAATCCCATACATCGGCACAAATTTAGTCGAATGAATCTGAGG
Sus scrofa          CTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGAGG
Gallus gallus       TTCTCAGCAATTCCCTACATTGGACACACCCTAGTAGAGTGAGCCTGAGG
                     * ** ** ** ** ** ** **        * **  * ***  ******

Homo sapiens        AGGCTACTCAGTAGACAGTCCCACCCTCACACGATTCTTTACCTTTCACT
Canis lupus         CGGCTTCTCAGTGGACAAAGCAACCCTAACACGATTCTTTGCATTCCATT
Felis catus         GGGGTTCTCAGTAGACAAAGCCACCCTAACACGATTCTTTGGCTTCCACT
Bos taurus          CGGATTCTCAGTAGACAAAGCAACCCTTACCCGATTCTTCGCTTTCCATT
Sus scrofa          GGGCTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCCTTTCACT
Gallus gallus       GGGATTTTCAGTCGACAACCCAACCCTTACCCGATTCTTCGCTTTACACT
                     ** *  ** ** ****   * ***** ** ********    ** ** *


Homo sapiens        TCATCTTGCCCTTCATTATTGCAGCCCTAGCAGCACTCCACCTCCTATTC
Canis lupus         TCATCCTCCCTTTCATCATCGCAGCTCTAGCAATAGTACACCTCCTATTT
Felis catus         TCATTCTTCCATTCATTATCTCAGCCTTAGCAGGAGTACACCTCTTATTC
Bos taurus          TTATTCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTC
Sus scrofa          TTATCCTGCCATTCATCATTACCGCCCTCGCAGCCGTACATCTCCTATTC
Gallus gallus       TCCTCCTCCCCTTTGCAATCGCAGGTATTACTATCATCCACCTCACCTTC
                    *  *  * ** **    **    *   *  *     * ** **    **

Homo sapiens        TTGCACGAAACGGGATCAAACAACCCCCTAGGAATCACCTCCCATTCCGA
Canis lupus         CTACACGAAACCGGATCCAACAACCCTTCAGGAATCACATCAGACTCAGA
Felis catus         CTTCATGAAACAGGATCTAACAACCCCTCAGGAATTACATCCGATTCAGA
Bos Taurus          CTCCACGAAACAGGCTCCAACAATCCAACAGGAATTTCCTCAGACGTAGA
Sus scrofa          CTGCACGAAACCGGATCCAACAACCCTACCGGAATCTCATCAGACATAGA
Gallus gallus       CTACACGAATCAGGCTCAAACAACCCCCTAGGCATCTCATCCGACTCTGA
                     * ** *** * ** ** ***** **    ** **  * **  *    **

Homo sapiens        TAAAATCACCTTCCACCCTTACTACACAATCAAAGACGCCCTCGGCTTAC
Canis lupus         CAAAATTCCATTTCACCCTTACTACACAATCAAGGATATCCTAGGAGCCT
Felis catus         CAAAATCCCATTCCACCCATACTATACAATCAAAGACATCCTAGGTCTTC
Bos taurus          CAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCC
Sus scrofa          CAAAATTCCATTTCACCCATACTACACTATTAAAGACATTCTAGGGGCCT
Gallus gallus       CAAAATTCCATTTCACCCATACTACTCCTTCAAAGACATTCTGGGCTTAA
                     *****  * ** ***** *****  *  * ** **     * **

Homo sapiens        TTCTCTTCCTTCTCTCCTTAATGACATTAACACTATTCTCACCAGACCTC
Canis lupus         TACTCCTACTCCTAATCCTAATATCACTAGTTTTATTTTCACCTGACCTA
Felis catus         TAGTACTAGTTTTAACACTCATACTACTCGTCCTATTTTCACCAGACCTG
Bos taurus          TCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTC
Sus scrofa          TATTTATAATACTAATCCTACTAATCCTTGTACTATTCTCACCAGACCTA
Gallus gallus       CTCTCATACTCACCCCATTCCTAACACTAGCCCTATTCTCCCCCAACCTC
                       *  *  *        *  *     *     ****  * **  ****

Homo sapiens        CTAGGCGACCCAGACAATTATACCCTAGCCAACCCCTTAAACACCCCTCC
Canis lupus         TTAGGAGACCCAGATAACTACACCCCTGCAAACCCCCTAAACACCCCTCC
Felis catus         CTAGGAGACCCAGACAACTACATCCCAGCCAACCCTTTAAATACCCCTCC
Bos taurus          CTCGGAGACCCAGATAACTACACCCCAGCCAATCCACTCAACACACCCCC
Sus scrofa          CTAGGAGACCCAGACAACTACACCCCAGCAAACCCACTAAACACCCCACC
Gallus gallus       CTAGGAGACCCAGAAAACTTCACCCCAGCAAACCCACTAGTAACCCCCCC
                     * ** ******** ** *  * **  ** ** **  *    ** ** **
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Figure 1 (continued).

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Homo sapiens        CCACATCAAGCCCGAATGATATTTCCTATTCGCCTACACAATTCTCCGAT
Canis lupus         ACATATTAAACCTGAGTGATATTTTCTATTCGCCTATGCTATCCTACGAT
Felis catus         CCATATTAAACCTGAATGATACTTCCTATTCGCATACGCAATTCTCCGAT
Bos taurus          TCACATCAAACCCGAGTGATACTTCTTATTTGCATACGCAATCTTACGAT
Sus scrofa          CCATATTAAACCAGAATGATATTTCTTATTCGCCTACGCTATCCTACGTT
Gallus gallus       ACATATCAAACCAGAATGATATTTTCTATTCGCCTATGCCATCCTACGCT
                     ** ** ** ** ** ***** **  **** ** **  * **  * ** *

Homo sapiens        CCGTCCCTAACAAACTAGGAGGCGTCCTTGCCCTATTACTATCCATCCTC
Canis lupus         CCATTCCTAATAAATTAGGAGGTGTACTCGCCCTAGTATTCTCCATCCTA
Felis catus         CCATCCCTAACAAACTAGGGGGAGTCCTAGCCCTAGTACTCTCCATCCTA
Bos taurus          CAATCCCCAACAAACTAGGAGGAGTACTAGCCCTAGCCTTCTCTATCCTA
Sus scrofa          CAATTCCTAATAAACTAGGTGGAGTGCTAGCTCTAATAGCCTCCATCCTA
Gallus gallus       CCATCCCCAACAAACTTGGAGGTGTACTAGCCCTAGCAGCCTCAGTCCTC
                    *  * ** ** *** * ** ** ** ** ** ***      **  ****

Homo sapiens        ATCCTAGCAATAATCCCCATCCTCCATATATCCAAACAACAAAGCATAAT
Canis lupus         ATCTTGGCATTCATTCCACTCCTCCACACATCTAAGCAACGCAGCATAAT
Felis catus         GTACTAGCAATCATTCCAATCCTCCACACCTCCAAACAACGAGGAATAAT
Bos taurus          ATTCTTGCTCTAATCCCCCTACTACACACCTCCAAACAACGAAGCATAAT
Sus scrofa          ATCCTAATTTTAATGCCCATACTACACACATCCAAACAACGAAGCATAAT
Gallus gallus       ATCCTCTTCCTAATCCCCTTCCTCCACAAATCTAAACAACGAACAATAAC
                     *  *     * ** **  * ** ** *  ** ** ****     ****

Homo sapiens        ATTTCGCCCACTAAGCCAATCACTTTATTGACTCCTAGCCGCAGACCTCC
Canis lupus         ATTCCGGCCCCTTAGCCAATGCCTATTCTGACTTTTAGTCGCCGATCTTC
Felis catus         GTTTCGACCACTAAGCCAATGTCTATTCTGACTCCTAGTAGCGGATCTCC
Bos taurus          ATTCCGACCACTCAGCCAATGCCTATTCTGAGCCCTAGTAGCAGACCTAC
Sus scrofa          ATTTCGACCACTAAGTCAATGCCTATTCTGAATACTAGTAGCAGACCTCA
Gallus gallus       CTTCCGACCACTCTCCCAAACCCTATTCTGACTTCTAGTAGCCAACCTTC
                     ** ** ** **    ***   ** *  ***    ***  **  * **

Homo sapiens        TCATTCTAACCTGAATCGGAGGACAACCAGTAAGCTACCCTTTTACCATC
Canis lupus         TCACTTTAACATGAATTGGAGGACAACCAGTTGAACACCCTTTCATCATT
Felis catus         TAACCCTAACATGAATCGGTGGCCAACCTGTAGAACATCCGTTCATCACC
Bos taurus          TGACACTCACATGAATTGGAGGACAACCAGTCGAACACCCATATATCACC
Sus scrofa          TTACACTAACATGAATTGGAGGACAACCCGTAGAACACCCATTCATCATC
Gallus gallus       TTATCCTAACCTGAATCGGAAGCCAACCAGTAGAACACCCCTTCATCATC
                    * *   * ** ***** **  * ***** **     * ** *  * **

Homo sapiens        ATTGGACAAGTAGCATCCGTACTATACTTCACAACAATCCTAATCCTAAT
Canis lupus         ATCGGACAAGTCGCTTCAATCTTATATTTCACCATCTTATTGATCCTAAT
Felis catus         ATCGGGCAACTAGCCTCCATCCTATATTTCTCAACCCTCCTAATCCTAAT
Bos taurus          ATCGGACAACTAGCATCTGTCCTATACTTTCTCCTCATCCTAGTGCTAAT
Sus scrofa          ATCGGCCAACTAGCCTCCATCTTATATTTCCTAATCATTCTAGTATTGAT
Gallus gallus       ATTGGCCAAATAGCATCCCTCTCTTACTTCACCATCCTACTTATCCTCTT
                    ** ** *** * ** **  *    ** **        *  *  *  *  *

Homo sapiens        ACCAACTATCTCCCTAATTGAAAACAAAATACTCAAATGGGCCT
Canis lupus         ACCAACAGTTAGCGTTATCGAAAACAACCTTCTAAAATGAAGA-
Felis catus         ACCCATCTCAGGCATTATTGAAAACCGTCTACTCAAATGAAGA-
Bos taurus          ACCAACAGCCGGCACAGTCGAAAACAAATTACTAAAATGAAGA-
Sus scrofa          ACCAATCACTAGCATCATCGAAAACAACCTATTAAAGTGAAGA-
Gallus gallus       CCCCACAATCGGAACACTAGAAAACAAAATACTCAACTACTAA-
                     ** *            * ******    *  * ** *
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Figure 1 (continued).

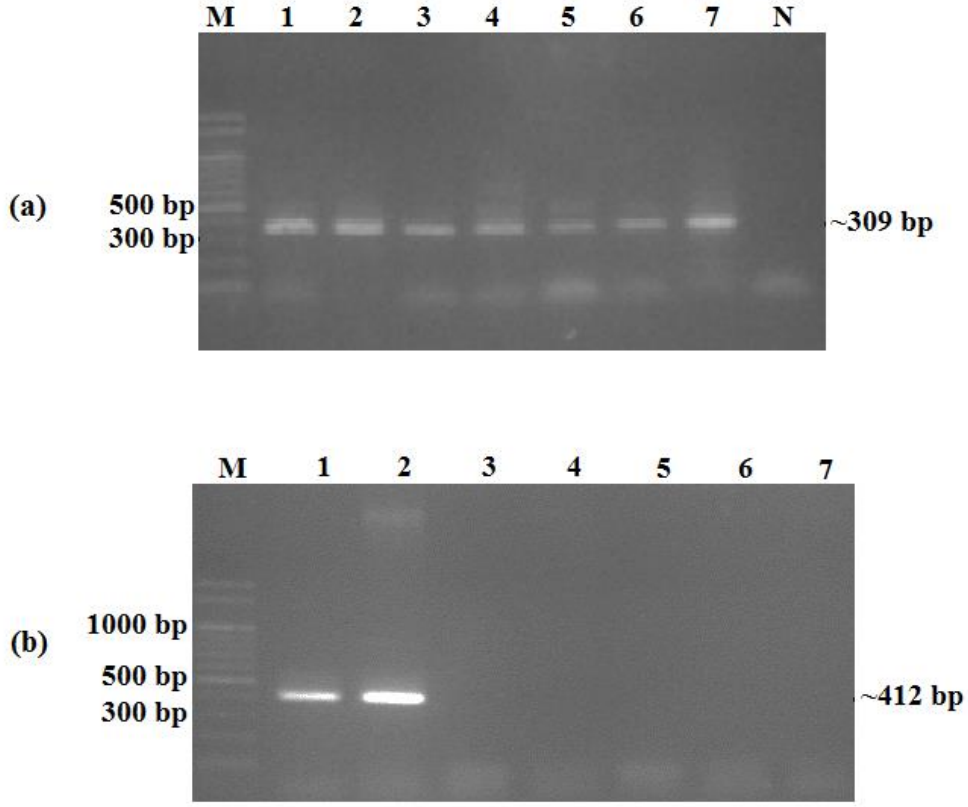

Figure 2 The PCR products of cytochrome *b* (*Cyt b*) conserved region (a) and human-specific *Cyt b* (b) amplified from DNA extracted from blood samples by using ConservedCyt primers. Lane 1: male, Lane 2: female, Lane 3: pig, Lane 4: dog, Lane 5: cat, Lane 6: cow, Lane 7: chicken, Lane 8: negative control (no DNA). Lane M represents a 100-bp DNA ladder.

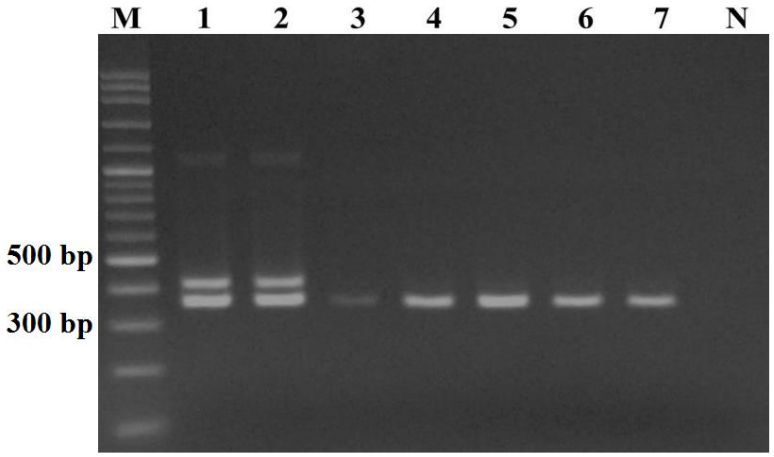

Figure 3 Duplex PCR analysis of human and animal blood samples compared with 100 bp DNA ladder (M) and negative control (N). Male and female blood samples are in lane 1 and 2. Animal blood samples: pig (Lane 3), dog (Lane 4), cat (Lane 5), cow (Lane 6) and chicken (Lane 7) were examined.

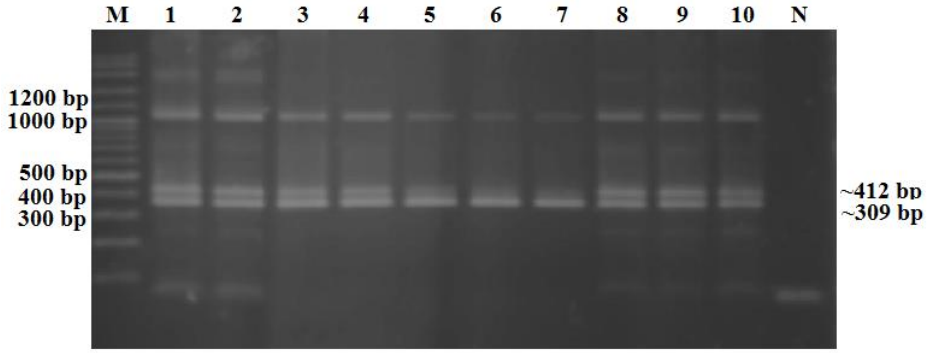

Figure 4 Duplex PCR amplification of male (Lanes 1–5) and female (Lanes 6–10) bloodstain samples. Lane N is negative control. A 100 bp DNA ladder was used as DNA marker (Lane M).

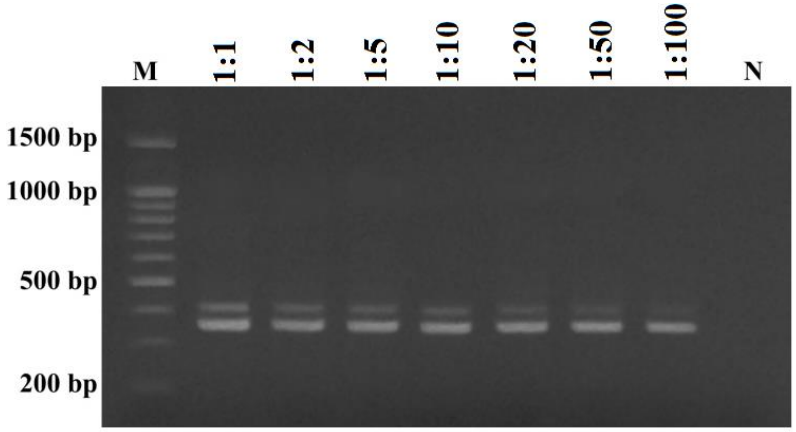

Figure 5 Duplex PCR analysis of human and dog mixed bloodstains samples at various ratio. A 100-bp ladder and negative control are represented by M and N, respectively.

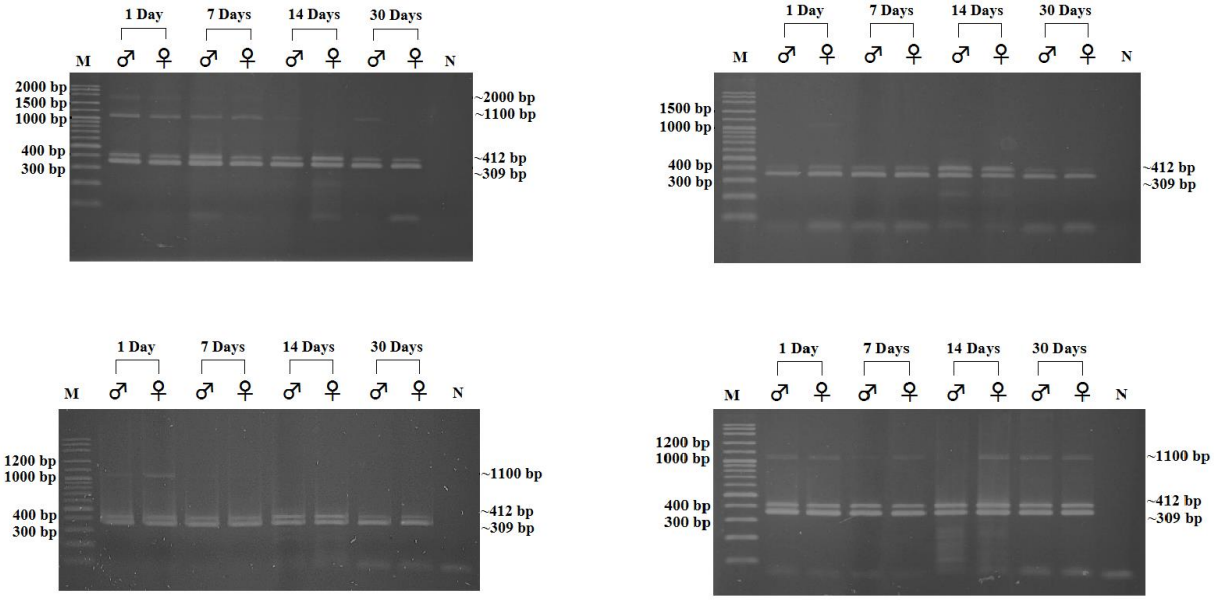

Figure 6 Duplex PCR analysis of human bloodstains (male; ♂ and female; ♀) kept at different temperatures for 1, 7, 14 and 30 days.

DISCUSSION

Determination of human blood origin is a crucial step for crime investigation especially for DNA analysis of blood evidence. This work has studied human blood determination by using duplex PCR analysis of the *Cyt b* gene. Two regions of *Cyt b*, consisting of conserved and human-specific regions, have been focused upon. These two regions were of interest because not only could they differentiate human blood from those of animals, but also from non-biological evidence e.g. rust and mud stains. So, this method helps investigators in terms of reducing time spent and cost on DNA analysis of irrelevant evidence.

Two pairs of primers specific to those two regions of *Cyt b* were designed by comparing the *Cyt b* sequences of human and animals. Five domestic animals including pets (dog and cat) and livestock (cow, chicken and pig) were chosen because they are usually found in the household. Therefore, bloods from these animals may be found and contaminated the crime scenes. The designed primers showed the expected sizes of PCR products at ~309 bp and ~412 bp for target site amplification of the conserved and human-specific *Cyt b* regions, respectively. To confirm the success of the designed primers, simplex PCR analyses of either conserved or human-specific *Cyt b* regions on human and animal blood samples were conducted. A PCR product sized ~309 bp was observed in both human and animal blood samples. On the contrary, a ~412 bp PCR product was found only in human blood samples. These results confirmed that the target regions of *Cyt b* could be amplified by the designed primers.

The duplex PCR analysis was then conducted. The results showed two DNA bands (~309 and ~412 bp) in human blood samples and one DNA band (~309 bp) in animal blood samples, indicating that analysis of conserved and human-specific *Cyt b* regions by duplex PCR could differentiate between human and animal blood samples. Similar study on differentiation of human and animal blood sample based on sequence analysis of *Cyt b* has successfully been reported but a SNP was targeted (Nakaki *et al*., 2007). Unexpectedly, this study found non-specific PCR fragment (~1,100 bp) in human samples. This could not be explained by the primer-dimer or by non-specific binding of primer to the DNA template, as such DNA fragment was not seen in the simplex PCR results or the duplex analysis of animal blood samples. Therefore, it was possibly caused by the PCR products of conserved and human-specific *Cyt b* that may sometimes bind to each other at the complementary region during annealing step. This fragment was then amplified along with the target sites and become a non-specific target. In addition, non-specific band may be resulted from amplification of binding forward ConservedCytb primer and reverse HumanCytb primer because this non-specific band was only observed in human samples. To overcome this problem, the appropriate duplex PCR conditions e.g. increased annealing temperatures, decreased quantity of DNA template and increased concentration of $MgCl_2$ should be investigated (Henegariu *et al*, 1997). Additionally, reducing extension time may be another method that could solve the non-specific band problem.

Human bloodstains samples were then analyzed by duplex PCR analysis. Two targeted DNA fragments and non-specific fragment were revealed in all male and female blood samples. To determine the capability of duplex PCR analysis for determination of human blood in mixed blood samples, the various ratios of human and dog mixed blood samples were tested. The duplex PCR analysis of *Cyt b* could determine human DNA in a mixed blood sample, even when the quantity of human was 100 fold less than that of animal blood. Therefore, the sensitivity of this technique on human blood determination is quite high. However, to examine the accurate sensitivity of the assay, quantification of human and animal mtDNA in a mixed sample needs to be conducted (Tobe and Linacre, 2008). In addition, quantitative real-time PCR (qPCR) assay may also be applied to quantify mixed species DNA template (Kanthaswamy *et al*., 2012). However, a non-specific DNA fragment was not visible in duplex analysis of mixed bloodstains samples. This is probably due to the lower quantity of human DNA in mixed blood. Hence, the condition of duplex PCR was more suitable as described above.

This study showed that temperature and age of bloodstains had no effects on human blood determination by duplex analysis of *Cyt b* because two target DNA fragments were found in all conditions. Previous studies had shown that mitochondrial DNA can be preserved in long-term buried bodily remains because analysis of evidence can be revealed by mitochondrial DNA analysis (Just *et al*., 2011; Barta *et al*., 2014). Our observed result confirmed that the duplex PCR analysis of *Cyt b* can determine human blood in various conditions of blood evidence. New methods, for example, genome profiling (Suwa *et al*., 2012), mRNA markers (Richard *et al*., 2012), mRNA profiling (Jakubowka *et al*., 2013) and microRNA

markers (Wang *et al*., 2013) have recently been studied for identification of human samples. However, the multiple steps required for these methods, along with the need for expertise and special equipment is a limiting factor for some laboratories. Therefore, duplex PCR analysis of *Cyt b* for human blood determination is still useful for forensic investigation.

CONCLUSION

This work focused on human blood determination by analyzing two regions of *Cyt b* gene which were conserved (~309 bp) and human specific (~412 bp) regions by duplex PCR. Primers were designed and separately tested for their specificity on human and animal blood samples by simplex PCR. The duplex PCR analysis was then carried to confirm the amplified results on human and animal blood samples as well as human bloodstain samples. The expected size of PCR fragments were visible indicating that the duplex PCR analysis for human blood determination had worked successfully. The capability of duplex PCR analysis for human blood determination in human and animal mixed blood samples demonstrated that this technique can determine human DNA in mixed blood even where the quantity of human blood is less than 100 fold. Moreover, the temperature and age of bloodstains had no effect on the duplex PCR analysis. These results demonstrate clear evidence that duplex PCR analysis of *Cyt b* can be applied for human blood determination.

ACKNOWLEDGEMENTS

This research was supported by grants funded by Khon Kaen University under the new researcher project.

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