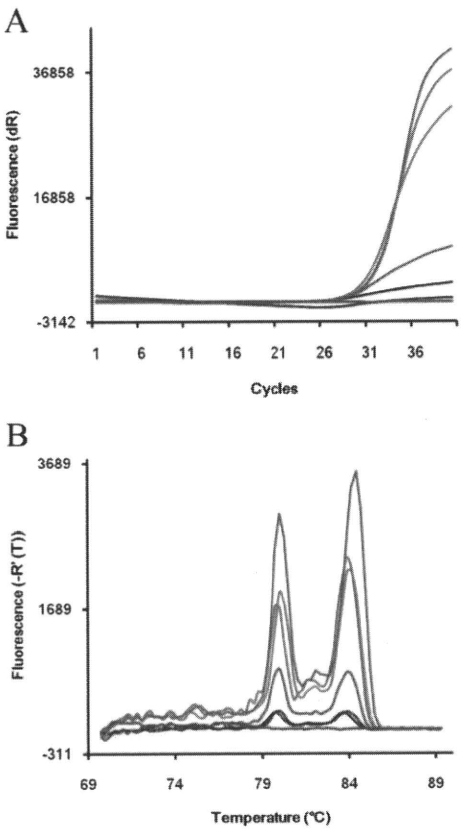

Fig. 3. Effect of dilution of blood on the fluorescent signal of real-time PCR analysis. The results of severalfold diluted blood sample from an *HP*/*HP*^{del} individual are shown by navy (no dilution), red (1:4), green (1:16), gray (1:64), chartreuse (1:256), blue (1:1024), and purple (1:4096). A negative control is shown by orange. (A) Real-time amplification plot. (B) Dissociation curves provide a graphic representation of the PCR product after amplification.

50 mmol/L NaOH, the T_m value seemed to have dropped slightly (approx. 0.5°C for both products) compared to that observed when water is used as the diluent. Although undesired fluorescent signals were detected in some negative control samples (with a frequency of less than 10%), they were not reproducible, and the T_m value (76.8°C) of the undesired products was lower than that of the targets.

For safe transfusion medicine for as many people as possible, the screening test of *HP*^{del} of every potential transfusion recipient before transfusion is desirable. However, as mentioned above, the prevalence of absolute Hp deficiency is relatively low, and it is a difficult issue to include the screening test considering the cost-effectiveness, while they would only need to be tested once in their lifetime.

The advantages of the TaqMan-based method over the SYBR Green I–based are superior specificity and the possibility of determination of common Hp genotypes (*HP*^{1} and *HP*^{2}) when one probe and primers set is added.[21] However, when confirmation of the length of PCR products by electrophoresis was included, the reliability of the SYBR Green I–based method with melting curve analysis was comparable with that of the TaqMan-based method. In addition, the results of the present SYBR Green I–based method with melting curve analysis were completely concordant with that of the TaqMan-based method. When melting curve analysis is included, less than 1.5 hours after taking blood from the subject is required for allele determination like the TaqMan-based method. Thus, the present SYBR Green I–based method for diagnosis of the *HP*^{del} allele seems to be comparable to the TaqMan-based method in specificity and suitability for high-throughput analysis. It seems to be better adapted to clinical diagnosis of patients before blood transfusion or infusion of human blood components in clinical laboratories as an alternative method for allelic discrimination of *HP*^{del} to prevent anaphylactoid shock caused by anti-Hp.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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Rapid Detection of Haptoglobin Gene Deletion in Alkaline-Denatured Blood by Loop-Mediated Isothermal Amplification Reaction

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Anhaptoglobinemic patients run the risk of severe anaphylactic transfusion reaction because they produce serum haptoglobin antibodies. Being homozygous for the haptoglobin gene deletion allele (HP^{del}) is the only known cause of congenital anhaptoglobinemia, and detection of HP^{del} before transfusion is important to prevent anaphylactic shock. In this study, we developed a loop-mediated isothermal amplification (LAMP)-based screening for HP^{del}. Optimal primer sets and temperature for LAMP were selected for HP^{del} and the 5′ region of the *HP* using genomic DNA as a template. Then, the effects of diluent and boiling on LAMP amplification were examined using whole blood as a template. Blood samples diluted 1:100 with 50 mmol/L NaOH without boiling gave optimal results as well as those diluted 1:2 with water followed by boiling. The results from 100 blood samples were fully concordant with those obtained by real-time PCR methods. Detection of the HP^{del} allele by LAMP using alkaline-denatured blood samples is rapid, simple, accurate, and cost effective, and is readily applicable in various clinical settings because this method requires only basic instruments. In addition, the simple preparation of blood samples using NaOH saves time and effort for various genetic tests. *(J Mol Diagn 2011, 13:334–339; DOI: 10.1016/j.jmoldx.2011.01.005)*

The absence of a serum protein such as IgA or haptoglobin (Hp) is one of the factors that can lead to anaphylactic transfusion reactions due to production of serum antibodies against the absent protein after a transfusion.[1] At present, a homozygous deletion of the haptoglobin gene (HP^{del}) is the only known cause of anhaptoglobinemia.

Human Hp has a genetic polymorphism of two codominant alleles, HP^1 and HP^2, that give rise to the three common phenotypes, Hp1, Hp2-1, and Hp2.[2,3] The HP^2 allele appears to have occurred by a 1.7-kb intragenic duplication of exons 3 and 4 of the HP^1 allele (Figure 1A). Anomalous inheritance of the Hp phenotypes was encountered during determinations of parentage, and HP^{del} was identified by genetic analyses of one such family in Japan.[4] The HP^{del} allele lacks an approximately 28-kb segment of chromosome 16 extending from the promoter region of the *HP* gene to intron 4 of the haptoglobin-related gene (*HPR*) (Figure 1A).[4,5]

The HP^{del} allele has been found only in East and Southeast Asian populations (Chinese, Korean, Japanese, Mongols, Thais, and Indonesians), not in African, West and South Asian, and European populations so far.[5–9] Detection of homozygosity for HP^{del} before blood transfusion or blood component infusion is important to prevent severe side effects of transfusion because washed red blood cells and platelet concentrate do not cause transfusion-related anaphylactic reactions.[10] Recently, we established two real-time PCR methods for detection of HP^{del} by use of a 5′-nuclease assay using dual-labeled (TaqMan; Applied Biosystems, Foster City, CA) probes and SYBR Green I (Invitrogen, Carlsbad, CA).[11,12] These methods are rapid, robust, and suitable for high-throughput analysis but require a real-time PCR apparatus.

A technique called loop-mediated isothermal amplification (LAMP) of DNA has recently been developed.[13] LAMP employs a DNA polymerase with strand-displacement activity; the reaction proceeds when the forward inner primer anneals to the target DNA and the first strand is synthesized, and then the outer forward primer hybridizes and displaces the first strand, forming a loop structure at one end. The single-stranded DNA serves as a template for backward inner primer–initiated DNA synthesis and subsequent outer backward–primed strand-displacement DNA synthesis. The stem loop formed acts as a template, and the final products are stem-loop DNAs with several inverted repeats of the target DNA. Loop primers (forward and backward), which are additional primers designed to anneal at the loop structure (be-

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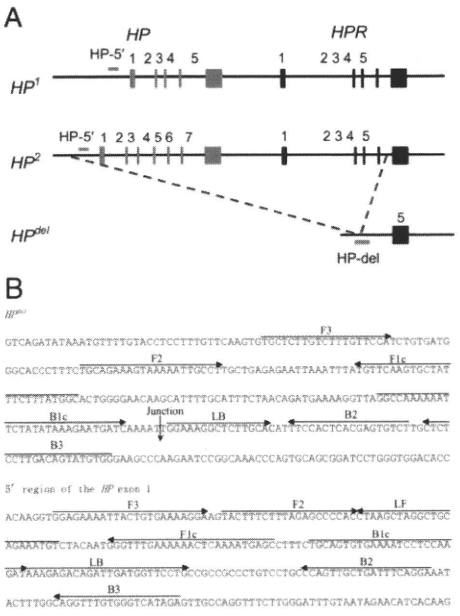

Figure 1. Gene structure of *HP*^{1}, *HP*^{2}, and *HP*^{del}, and positions of the target regions (**A**) and locations of two primer sets used in the LAMP reaction (**B**). The position and direction of each primer are shown by **arrows**. A **downward arrow** indicates the junction of gene deletion of the *HP*^{del}.

tween F1c and F2, B1c and B2) in LAMP amplicons, can accelerate and enhance the sensitivity of the LAMP reaction. In DNA polymerization by DNA polymerase, pyrophosphate ions are released from dNTP as a by-product, which react with magnesium ions in the LAMP reaction buffer, yielding an insoluble white precipitate.[13,14] This method amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions, and the advantages are i) easy identification of positive reaction by visual inspection of turbidity, and ii) requirement of only a heating block or water bath.[13] In this study, we developed a LAMP reaction for detection of *HP*^{del} with the aim of establishing a feasible detection method for *HP*^{del} in clinical diagnostic laboratories.

Materials and Methods

The study protocol was approved by the ethics committee of Kurume University School of Medicine.

LAMP Amplification

LAMP primers, which specifically amplify two regions, the region encompassing the *HP*^{del} breakpoint (HP-del) and the 5′ region of *HP* exon 1 (HP-5′), were designed with the aid of PrimerExplorer V4 (*http://primerexplorer.jp/elamp4.0.0*, last accessed December 2, 2010) and synthesized by Operon Biotechnologies (Tokyo, Japan). The locations and sequences of primers that brought the optimal results are presented in Figure 1 and Table 1. The LAMP reaction was performed in a volume of 25 μL containing 12.5 μL of 2× Reaction Mix, 1 μL of BstDNA Polymerase (Eiken, Tokyo, Japan), 40 pmol each of the forward inner and the backward inner primers, 20 pmol each of the backward loop primer and/or the forward loop primer, 5 pmol each of the outer forward and the outer backward primers, and 2 μL of the template (diluted and/or heated venous whole blood or genomic DNA) in a Loopamp Reaction tube (Eiken). Wells without a template were included as negative controls. Reactions for HP-del and HP-5′ were conducted in discrete tubes. The reaction mixture was incubated at 60°C to 65°C for 60 minutes, and turbidity was measured every 6 seconds using a real-time turbidity meter (Loopamp EXIA; Eiken).[15] The reaction was followed by incubation at 80°C for 5 minutes for inactivation of the enzyme. At least three assays using different samples were performed for every group for assay validation. The turbidities were also assessed by visual inspection. Fluorescent Detection Reagent (Eiken) was added to the reaction mix before the reaction to inspect the amplification visually by the color.

Preparation of Templates

To determine the optimal primer sets and temperature for detection of *HP*^{del}, LAMP reactions were performed using

Table 1. Sequences of Primers Used in the LAMP Assay

	Primer sequence
Set for *HP*^{del} (HP-del)	
F3	5′-TGCTCTTGTCTTTGTTCCA-3′
B3	5′-CCACATACTGTCAAGGAGAG-3′
FIP (F1c + F2)	5′-GCCATAAAGAAATAGCACTTGAACATGCAGAAAGTAAAAATTGCCT-3′
BIP (B1c + B2)	5′-GGCCAAAAAATTCTATATAAAGAATGATCAGACACTCGTGAGTGGAA-3′
LB	5′-GGAAAGGCTCTTGCA-3′
Set for 5′ region of *HP* (HP-5′)	
F3	5′-GGAGAAAATTACTGTGAAAAGGA-3′
B3	5′-TCTATGACCCACAAACCTG-3′
FIP (F1c + F2)	5′-GCTCATTTTGAGTTTTTTCAAACCCTACTTTCTTTAGAGCCCCAC-3′
BIP (B1c + B2)	5′-TGCAGTGTGAAAATCCTCCAAGATTCCTGAAATCAGCAACTGG-3′
LF	5′-ACATTTCTGCAGCCTAGCTTAG-3′
LB	5′-AAAGAGACAGATTGATGGTTCCTG-3′

10 ng of genomic DNAs from known genotypes lacking HP^{del} (*HP/HP*), heterozygous for HP^{del} (*HP/HP*^{del}), and homozygous for HP^{del} (*HP*^{del}/*HP*^{del}) as templates. For determination of the detection limit of the method, a dilution series of *HP/HP*^{del} DNA, 100, 10, 1, 0.1, 0.01, and 0.001 ng/reaction, was used. To determine analytical sensitivity, a dilution series of *HP/HP*^{del} DNA, 0.2, 0.1, 0.05, 0.025, and 0.0125 for Hp-del and 0.02, 0.01, 0.005, 0.0025, and 0.00125 for Hp-5′ (in nanograms/reaction), was also examined. The detection limit was estimated by using probit analysis (SAS Statistical Software Package, SAS, Cary, NC). In addition, 1:10, 1:30, 1:100, 1:300, 1:1000, and 1:3000 dilutions of whole-blood samples of *HP/HP*^{del} individuals with 50 mmol/L NaOH were prepared for evaluation of NaOH dilution. For investigation of the effect of diluents (water or NaOH) and heat treatment (98°C for 5 minutes), we prepared blood samples diluted 1:2 with water and heated, 1:10 with water and heated, 1:100 with NaOH and heated, and 1:100 with NaOH. Denaturation (boiling) of diluted blood samples was performed in a block incubator and subsequent centrifugation at 12,000 rpm for 3 minutes. We also determined the optimal proportion of NaOH in the mixture by using 1, 2, 4, and 8 μL of 1:100 diluted samples with 50 mmol/L NaOH in a 25-μL mixture.

Confirmation of Specific Amplification of LAMP Products

After amplification, 2 μL of reaction mixtures were electrophoresed on 1.5% agarose gel and stained by ethidium bromide. Some fragments were gel purified and inserted into a pUC118 plasmid using a Mighty Cloning Kit (TaKaRa, Shiga, Japan). Sequence analyses of three clones for each of band were performed using BigDye Terminators v1.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Life Technologies Japan, Tokyo, Japan).

Results

To determine the optimal primer sets and temperature for detection of HP^{del}, LAMP reactions were performed using genomic DNA of *HP/HP*, *HP/HP*^{del}, and *HP*^{del}/*HP*^{del} as templates. The optimal results were obtained by incubation at 61.5°C for both amplifications of HP-del and HP-5′. An amplification curve of HP-del was obtained with *HP/HP*^{del} and *HP*^{del}/*HP*^{del}, whereas that of HP-5′ was obtained with *HP/HP* and *HP/HP*^{del} (Figure 2A). No increase in turbidity was detected in the negative controls of HP-del and HP-5′. As expected, the HP-5′ results obtained using genomic DNA of HP^1/HP^1, HP^1/HP^2, and HP^2/HP^2 were indistinguishable from each other (n = 3, data not shown), and no amplifications were observed using HP-del in these genomic DNAs. In addition, only HP-5′ tubes were positive when using DNA of three Ghanaians with acquired anhaptoglobinemia (without any causal mutations in the promoter and coding regions) as templates.[6] The threshold time required for the turbidity of the solution to develop was about 25 minutes for HP-del and about 23 minutes for HP-5′. After amplification, all of the positive reactions produced white precipitates and a characteristic ladder of multiple bands on an agarose gel, whereas no bands were detected in negative controls. In addition, sequence analyses revealed the target *HP* sequences in the plasmids inserted the LAMP products (data not shown). These results confirmed the specific amplifications of the HP^{del} and 5′ region of *HP* exon 1.

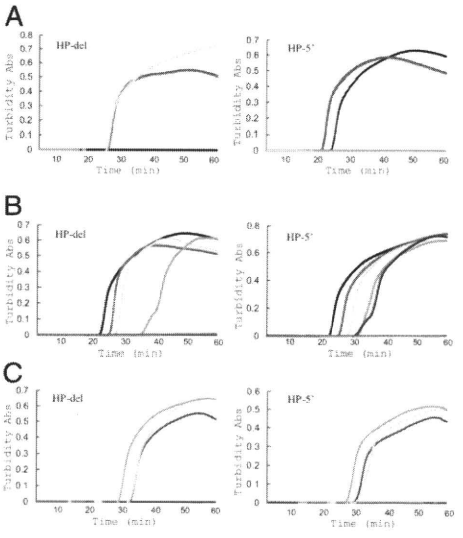

Figure 2. Results of the LAMP reaction for HP^{del} (**left**) and the 5′ region of *HP* exon 1 (**right**). **A:** Validation of specificity using DNA samples of known genotypes. Real-time amplification curves with turbidity detection of triplicate experiments with *HP/HP*, *HP/HP*^{del}, and *HP*^{del}/*HP*^{del}. The results are shown by navy (*HP/HP*), pink (*HP/HP*^{del}), and yellow (*HP*^{del}/*HP*^{del}). Negative control is shown by aqua. **B:** Validation of sensitivity using DNA samples of known genotypes. Amplification curves of triplicate experiments with various amounts of initial template DNA of *HP/HP*^{del}: 100, 10, 1, 0.1, 0.01, and 0.001 ng/reaction. The results are shown by navy (100 ng), pink (10 ng), yellow (1 ng), aqua (0.1 ng), purple (0.01 ng), and red (0.001 ng). A negative control is shown by green. **C:** Comparison of various preparations of blood samples. The results obtained from four different sample preparations are shown by red (1:2 dilution with water followed by heat), pink (1:10 dilution with water followed by heat), yellow (1:100 dilution with NaOH followed by heat), and aqua (1:100 dilution with NaOH). Negative control is shown by purple.

We then determined the sensitivity of the method by a dilution series of *HP/HP*^{del} DNA and found that the detection limit of HP-del was about 0.1 ng and that of HP-5′ was about 0.01 ng (Table 2 and Figure 2B). The threshold time (Tt) decreased roughly in proportion to the quantity of the initial template DNA for HP-del but not always for HP-5′. To determine analytical sensitivity, a total of 20 replicates were tested at each concentration (Table 3). Probit analysis showed that the analytical sensitivity for HP-del was estimated at 0.118 ng/reaction with a 95% confidence interval (CI) between 0.096 and 0.161 ng/reaction, and that for HP-5′, it was 0.021 ng/reaction with a 95% CI between 0.018 and 0.027 ng/reaction. No increase in turbidity was observed in the tubes of HP-del and Hp-5′ when using 100 ng/μL of DNA of *HP/HP* and

Table 2. Threshold Time Required for Turbidity of Solution to Exceed a Given Value with Various Templates

Sample preparation	Mean (SD) Tt value (min)	
	HP-del	HP-5′
Detection limit of purified DNA		
100 ng	24.9 (4.0)	23.7 (2.4)
10 ng	26.8 (4.2)	26.3 (2.8)
1 ng	30.9 (5.5)	30.0 (4.0)
0.1 ng	39.5 (5.3)	33.0 (5.4)
0.01 ng	—	32.7 (5.4)
0.001 ng	—	—
Serial dilution by NaOH		
1:10	*	*
1:30	28.6 (1.5)	29.9 (2.4)
1:100	34.0 (3.7)	32.6 (3.9)
1:300	41.5 (10.1)	37.4 (2.5)
1:1000	45.8 (9.2)	37.9 (7.9)
1:3000	*	*
Water- or NaOH-diluted with/without heat		
⁄2 with water and heat	31.8 (3.3)	33.3 (4.5)
\10 with water and heat	—	*
×100 with NaOH and heat	35.7 (4.0)	36.7 (5.4)
×100 with NaOH	31.4 (2.9)	32.2 (5.1)
Serial proportion of NaOH		
1	33.5 (1.0)	35.4 (6.4)
2	35.4 (2.3)	36.0 (3.9)
4	42.4 (2.8)	42.8 (8.4)
8	—	—

Note: — represents the group in which turbidity was not observed, and **asterisks** represent the group in which turbidity was not consistently observed.
Tt, threshold time.

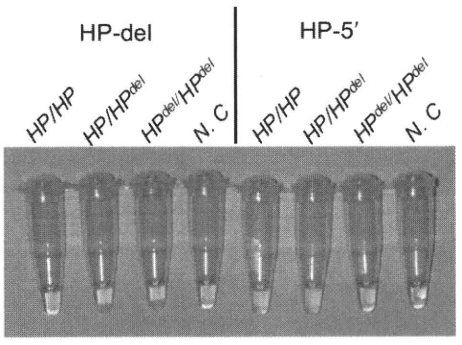

Figure 3. Evaluation of results by inspection of colors of reaction mixtures. One microliter of Fluorescent Detection Reagent (Eiken) was included in a 25-μL reaction mixture before the amplification reaction. Green and orange indicate positive and negative reactions, respectively. A 50 mmol/L NaOH-diluted blood sample of an *HP/HP* individual was green in the tube for HP-5′ and orange in the tube for the HP-del, that of *HP/HP^{del}* was green in both tubes, whereas that of *HP^{del}/HP^{del}* was green in the tube for HP-del and orange in the tube for the HP-5′. Negative controls (N.C) were orange in both tubes.

HP^{del}/HP^{del}, respectively. Thus, the analytical specificity of the present method seems to be 100%.

Recent studies have demonstrated that heat-treated blood samples are applicable for LAMP (LAMP-HB) as a template.[16,17] This time- and cost-saving method is now in common use for LAMP analysis. On the other hand, a blood sample diluted with 50 mmol/L NaOH (and heated) is used as a template for our real-time PCR for diagnosis of HP^{del} and isothermal single nucleotide polymorphism genotyping.[11,12,18] We first prepared serial dilutions to identify the suitable dilution range, and turbidity was consistently observed in the tubes with dilutions from 1:30 to 1:1000 for both amplifications. The Tt increased roughly in proportion to the dilution ratio for HP-del, but not for HP-5′ (Table 2). A failure of amplification or an irregular curve was sometimes observed in the tubes containing 1:10 diluted bloods. This may due to incomplete lysis of cells by NaOH and subsequent formation of heat-denatured blood component(s) such as hemoglobin during the reaction. Use of such low dilutions produces a risk of false-positive results. In addition, the color of the mixture itself is brownish, making it difficult to evaluate the result visually. We also did not always observe positive results using 1:3000 dilutions of bloods. The reaction mixture with a 1:1000 dilution is estimated to contain about 30 pg of DNA. This sensitivity is consistent with that obtained using purified DNA and is comparable to that of real-time PCR (data not shown). These results suggested that blood sample dilutions between 1:30 and 1:1000 provide a good template for this LAMP reaction. Thus, we used 1:100 dilution of blood samples for further studies. Further, no change was observed in the optimal temperature for both amplifications when using NaOH-diluted templates instead of purified DNA (data not shown). We also compared the results using frozen bloods with those of freshly drawn blood samples and observed similar amplifications in both types of samples (data not shown).

We next investigated the effect of diluents (water or NaOH) and heat treatment. As shown in Figure 2C and Table 2, the shortest Tt was obtained using 1:100

Table 3. Limit of Detection Determination of LAMP Reactions for HP-del and HP-5′

HP-del				HP-5′			
Amount of DNA, ng	Number tested	Number positive	Positive rate, %	Amount of DNA, ng	Number tested	Number positive	Positive rate, %
0.2	20	20	100.0	0.05	20	20	100.0
0.1	20	16	80.0	0.02	20	17	85.0
0.05	20	13	65.0	0.01	20	12	60.0
0.025	20	5	25.0	0.005	20	3	15.0
0.0125	20	3	15.0	0.0025	20	0	0.0
0	20	0	0.0	0	20	0	0.0

NaOH-diluted blood without heat treatment (LAMP-NaOH) and LAMP-HB (1:2). On the other hand, heat following NaOH dilution decreased the efficiency of the LAMP reaction.

We then tested the concentration of NaOH in the mixture, and the inhibitory effect of the NaOH concentration on enzyme activity was observed (Table 2). Less than 8 mmol/L NaOH seems desirable as a final concentration.

We then examined blood samples from 100 patients who were scheduled for blood transfusion at Kurume University Hospital. Before the LAMP amplification, detection assays for the HP^{del} were performed by the TaqMan-based and SYBR Green I–based real-time PCR methods, and the quality of the samples were checked. Seven of 100 samples were HP/HP^{del}, and 93 samples were *HP/HP*. In the TaqMan assay, the mean (SD) threshold cycle (Ct) values were 31.4 (0.8) for HP-5′ and 35.5 (0.8) for HP-del, and the 90th percentile for the Ct for HP-5′ was 32.4. The mean (SD) Tt value was 29.8 (2.9) minutes, and the 90th percentile for the Tt was 32.4 minutes for HP-5′ and 31.9 (3.0) minutes for HP-del in the LAMP amplification. The zygosity of the HP^{del} of every sample was fully consistent with real-time PCR results.[11,12] Amplifications were inspected visually by not only the turbidity, but also the color with the addition of Fluorescent Detection Reagent (Eiken) to the reaction mix before the reaction without the risk of contaminating products by opening the tubes as required when using SYBR Green I.[13] We also used a frozen sample of HP^{del}/HP^{del} blood and got a positive result only in the HP-del tube (Figure 3).

Discussion

We established rapid detection method of the HP^{del} by LAMP reaction using NaOH-diluted whole-blood samples. Although the results obtained by using both 1:100 NaOH-diluted blood without heat treatment (LAMP-NaOH) and LAMP-HB (1:2) were comparable, LAMP-NaOH was faster than LAMP-HB. Unlike PCR, which needs a heat-denaturing step, DNA amplification in the LAMP reaction is preceded by strand separation under isothermal conditions using betaine, which destabilizes the DNA helix. However, Njiru et al reported that preheating the template increased the efficiency of the assay because preheating produced a faster and/or a greater amount of strand separation, which translated into a far more rapid assay.[14] Alkaline treatment also seems to promote single-stranded DNA formation and thus to facilitate the LAMP reaction. Accordingly, this treatment might be used in a wide variety of experiments as well as heat denaturation.

The two methods based on real-time PCR have two advantages over the LAMP method in the present study: we need only one tube per sample, and they are applicable to high-throughput analyses. Although two reaction tubes are needed for each sample, the LAMP method is cost effective and suitable for running only a few tests. Only about 1 hour after taking blood from the subject is required for allele determination, and this method seems to be better adapted to clinical diagnosis of patients before transfusion in clinical laboratories to prevent anaphylactic shock caused by anti-Hp antibody. In addition, the simple sample preparation using 50 mmol/L NaOH, LAMP-NaOH, may be suitable for various LAMP-based genotyping such as pharmacogenetics and even for diagnosis of infectious diseases.

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神田芳郎、副島美貴子、川野洋之、江頭弘一、佐川公矯

輸血副作用原因遺伝子ハプトグロビン欠失アリルの輸血前診断法の検討
（第 58 回日本輸血・細胞治療学会座長推薦論文）
日本輸血細胞治療学会誌, 57(1):34-38, 2011.

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—【原　著】— Original —

輸血副作用原因遺伝子ハプトグロビン欠失アリルの輸血前診断法の検討

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重篤な非溶血性輸血後副作用であるアナフィラキシーショックのリスク因子であるハプトグロビン欠損症の原因遺伝子はハプトグロビン遺伝子欠失（HP^{del}）であり，その頻度は約4,000人に1人であると予想される．我々は最近安全な輸血医療の遂行を目的とし迅速・簡便なリアルタイムPCR法に基づく2種のHP^{del}診断法を開発し報告した．今回，臨床現場への導入を目的として久留米大学病院で輸血予定患者の血液を鋳型とし，TaqMan probeを用いる方法とSYBR green Iを用い融解曲線をおこなう方法の2法を実施し結果を比較した．約1時間半で得られた結果は全サンプルで一致し，2009年1月から2010年3月末に解析した2,954名のうち91名がHP/HP^{del}，1名がHP^{del}/HP^{del}であった．TaqMan法は増幅シグナルそのものが結果を反映することから反応中に診断結果を予想でき，HP^{del}/HP^{del}がソフトウエアで自動検出できるため多検体処理能力に優れた方法であり，SYBR法は初期費用が低く抑えられ，より幅広い臨床現場での導入が容易であると考えられた．

キーワード：無ハプトグロビン血症，アナフィラキシーショック，輸血前診断

第58回日本輸血・細胞治療学会総会座長推薦論文

緒　言

免疫学的機序による非溶血性輸血副作用には，発熱，蕁麻疹等の軽微なものが多いが，輸血関連急性肺障害（TRALI），アナフィラキシーショックなど重篤な副作用も希に認められる[1)]．アナフィラキシーショックの原因は大部分不明であるが，血漿蛋白欠損例でアナフィラキシーショックを惹起する事例が報告されている．西洋人の主な血漿蛋白欠損はIgA欠損であるが，日本人ではハプトグロビン（HP）欠損症の方が高頻度であり[2)]，2005年から各種血液製剤添付資料「慎重投与の項」に記載されている．

HPは急性期反応物質として知られる糖蛋白であり，血中で遊離型ヘモグロビン（Hb）と結合しHP-Hb複合体を形成し，溶血時に体内からの鉄の喪失を防ぐ，あるいはHbの酸化作用による腎臓障害を防ぐ，という生理学的役割を担うと考えられている[3)]．HP遺伝子（*HP*）は優劣のないHP^1とHP^2の対立遺伝子の組み合わせにより，HP1-1，HP2-1，HP2-2の代表的な3つの表現型が決定される．HP^1は，5つのエクソン，HP^2にはHP^1のエクソン3，4の重複（1.7-kb）により生じたエクソン5，6が存在し7つのエクソンで構成されている[3)]．

我々はHP欠損症患者の解析から*HP*欠失対立遺伝子（HP^{del}）を同定しHP^{del}のホモ接合体がHP欠損症（先天性無HP血症）となることを報告した[4)]．その後，欠失領域をクローニングし，欠失は*HP*の上流約5.2kbから*HP*下流に位置するHP関連遺伝子のイントロン4まで及ぶことを示し，エクソン1とのデュプレックスPCR法による遺伝子診断法を確立した[5)]．HP^{del}は日本人集団には約1.5%の頻度で存在し，したがってHP欠損症患者（HP^{del}/HP^{del}）は約4,000人に1人と推定される．HP欠損症患者は普段は無症状であるが，輸血やアルブミン輸液等に伴うアナフィラキシーショック発生症例が報告され輸血副作用発生のリスク因子と考えられる[6)]．

HP欠損はアフリカ人に多いものの，溶血などの二次的な無HP血症も多いと考えられており，その遺伝的原因は未だ解明されておらず，現時点でHP^{del}は同定されている唯一のHP欠損症の原因変異である．日本赤十字社でこれまでに調べられた抗HP抗体を有する欠損症患者は例外無くHP^{del}のホモ接合体でありHP^{del}の輸血前診断はHP欠損症による輸血後副作用の回避を可能にする．そこで，我々は臨床現場でも利用可能な迅速診断法として，DNA抽出をおこなわず希釈血液を直接PCRの鋳型とする，2種のリアルタイムPCR法（TaqMan

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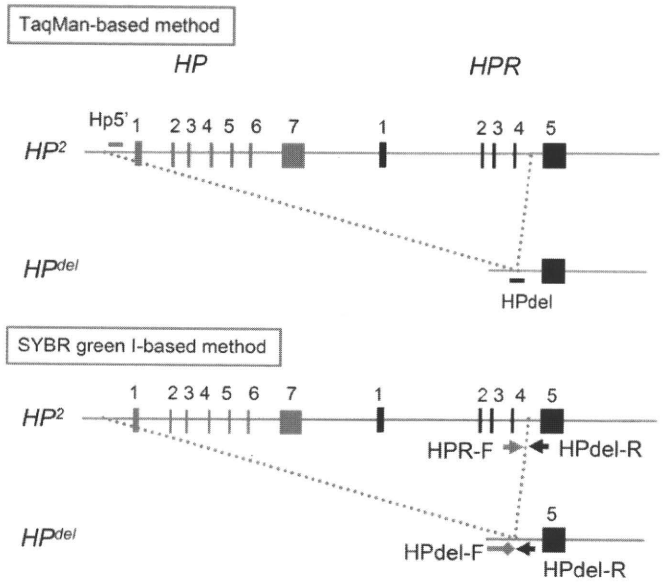

Fig. 1　Relative positions of primers and probes used in this study.

法とSYBR法）を確立し報告した[7][8]．

今回，これら2法の輸血前検査への導入の有用性を確認するために患者検体について検査を実施し，結果の比較検討をおこなった．

方　法

1. 輸血前血液

サンプルは，2009年1月4日から2010年3月31日まで，久留米大学病院臨床検査部輸血検査室で輸血前のルーチン検査のために採血された血液の一部を用いた．患者血液が45サンプル集まった時点で，約300 μlを分注し *HP*^{del} 診断用に用いた．96 well plateに45サンプルずつduplicateで調整した．既報のとおり条件検討をおこない，全血を100倍希釈したものをPCR反応の鋳型として用いた[7][8]．全患者に対して輸血副作用に関する検査を行う旨の包括的同意を文章で得ている．なお本研究計画の実施は久留米大学倫理委員会の承認を得て実施した．

2. TaqMan法

HP エクソン1の上流域（HP5'，内在性コントロール領域）と *HP*^{del} の欠失点を含む領域（HP-del）を増幅するプライマー，プローブセットを用いた[7]．設計，合成はバイオサーチテクノロジー社（Tokyo, Japan）に依頼した．プライマー，プローブの相対的な位置，配列をFig. 1，Table 1に示す．

PCR反応液（20μl）は鋳型の100倍希釈血液を1μl，10μlの *Premix Ex Taq*™（Perfect Real Time; Takara, Shiga, Japan），HP5'-F and -R primers（150nmol/l），HP5'-probe（42nmol/l）（HP5'検出用），HPdel-F and -R primers（300nmol/l），HPdel-probe（83nmol/l）（HP-del検出用）を加え調整した．0.5ngのゲノムDNAを用いたポジティブコントロール，鋳型を入れないネガティブコントロールを調整した．492/516nm（FAM），585/610nm（CAL Fluor Red 610）の励起/蛍光フィルターを装着したリアルタイムPCR機器Mx3000P system（Agilent Technologies Japan, Tokyo, Japan）を用いて95℃-30sec：1サイクル，95℃-5sec，60℃-30sec：40サイクルのPCR反応と蛍光シグナルの測定をおこない，MxPro™ Software（version 4.00, Agilent Technologies）を使ってdataの解析をおこなった[7]．

3. SYBR法

HP^{del} の欠失点と，*HP* のエクソン1上流域の2領域を増幅する為に，*HP*^{del} に特異的なforward primer（HPdel-690F）と健常（非欠失）アリル特異的forward primer（HPR-F）および共通のreverse primer（HPdel-823R）の3プライマーを用いた[8]．プライマーはPrimer 3 software（http://primer3.sourceforge.net/）を用い設計しオペロンバイオテクノロジー社（Tokyo, Japan）に合成を依頼した．プライマーの相対的な位置と配列を

Table 1 Sequences and locations of TaqMan probes and primers used in real-time PCR for HP^{del} detection. The breakpoint of HP^{del} locates at 782-783 bp of GenBank no. AB025320.

	Sequence	Position
HP5'-F	5'-CACATTTACTGATTTCAGGCTGGA-3'	513–536 bp, GenBank no. M10935
HP5'-R	5'-CCTTTTCACAGTAATTTTCTCCACCT-3'	571–596 bp (reverse), GenBank no. M10935
HP5'-Taqman-probe	5'-CAL Fluor Red 610 -AGCTTTTAAGCAATAGGGAGATGGCCACA-BHQ2-3'	538–566bp, GenBank no. M10935
HPdel-F	5'-TCTTTATGGCACTGGGGAACA-3'	694–714 bp, GenBank no. AB025320
HPdel-R	5'-AGCAAGACACTCGTGAGTGGAA-3'	822–801 bp (reverse), GenBank no. AB025320
HPdel-Taqman-probe	5'-FAM-TGTGCAAGAGCCTTTCCAATTTTGATCABHQ1-3'	772–799 bp (reverse), GenBank no. AB025320
HPdel-690F	5'-TATTTCTTTATGGCACTGGGGAACA-3'	690–714 bp, GeneBank no. AB025320
HPdel-823R	5'-GAGCAAGACACTCGTGAGTGGAAAT-3	823–799bp (reverse), GeneBank no. AB025320 and 13,067–13,043 bp (reverse), GeneBank no. NC_000016.9
HPR-F	5'-CTGCAACTATTGGAAATGAGATCAGC-3'	12,920–12,945 bp, GeneBank no. NC_000016.9, located in the 3' end of the deleted region in HPdel. Intron 4 of the HPR

Fig. 1. Table 1 に示す．PCR 反応は，鋳型の 100 倍希釈血液を 1μl，10μl の 2×SYBR® *Premix Ex Taq*™ II (Perfect Real Time；Takara)，あるいは SYBR® Premix DimerEraser® (Perfect Real Time；Takara) と，500 nmol/l HPdel-690F，250nmol/l HPdel-823R，75nmol/l HPR-F を含む 20μl の反応液を調整した．コントロール反応は TaqMan 法と同様に調整した．SYBR® *Premix Ex Taq*™ II は 95℃-30sec：1 サイクル，95℃-5sec, 65℃-30sec：40 サイクル，SYBR® Premix DimerEraser® は 95℃-30sec：1 サイクル，95℃-5sec，55℃-30sec，72℃-30sec：40 サイクルの PCR 反応をおこなった．さらに同機で，95℃-1min，70℃-30sec，の処理後 0.2℃/sec で 90℃ まで加温し，その蛍光シグナルを FAM filter を用いて測定し，MxPro™ Software で融解曲線解析をおこなった[8]．

結　果

2009 年 1 月 4 日から 2010 年 3 月 31 日までに輸血前患者 2,954 名の血液について TaqMan 法および SYBR 法の 2 法を用い HP^{del} 診断をおこなった．

条件検討時には蒸留水で 100 倍希釈した血液を直接鋳型とし良好な結果が得られていたが，多数の患者検体の解析を始めてみると，結果の再現性（蛍光シグナル強度）にばらつきが認められた．そこで希釈液を 50 mM NaOH 溶液とし血液を 100 倍に希釈後 95℃，5 分処理し鋳型とした結果，顕著な向上が認められた(data not shown)．

TaqMan probe 法では，健常者（*HP*/*HP*）では CAL Fluor Red 610 のシグナルのみ，*HP*/HP^{del} では CAL Fluor Red 610 と FAM シグナルの両方，HP^{del}/HP^{del} では FAM シグナルのみが検出され，結果は反応中にリアルタイムで予想可能であった．更に，遺伝子型をグラフ表示することが可能であり，サンプル数が多い場合にも瞬時に *HP*/HP^{del} を同定出来ることが示された．

一方，SYBR 法では，当初 SYBR® *Premix Ex Taq*™ II を試薬に用い，融解曲線解析の結果，*HP*/*HP* では Tm 値が約 84.5℃ の HPR intron 4 由来の産物(148 bp)ピークのみが，*HP*/HP^{del} では Tm 値が約 80.3℃ の HP^{del} 由来の増幅産物（134 bp）と約 84.5℃2 つのピークが，HP^{del}/HP^{del} では Tm 値が約 80.3℃ のピークのみが検出された(Fig. 2A)．ところが解析を続ける中で negative control の 10% 程度に増幅シグナルが観測され，融解曲線解析の結果このシグナルは目的の増幅産物の何れとも異なる Tm 値のピーク（77℃ 程度）を有しており，電気泳動の結果，サイズからプライマーダイマーであることが示唆された．そこで，試薬を SYBR® Premix DimerEraser® (Perfect Real Time, Takara) に変更した結果，ダイマーと思われる増幅シグナルは出現しなかった．このシグナルは，鋳型以外の反応液を分注して冷凍保存した PCR プレートを用いた場合でも出現しなかった．なお，増幅産物の Tm 値はどちらも約 1.5℃ 低くなった(Fig. 2B)．一方，ダイマー形成の軽減を目的とした本試薬を用いると，SYBR® *Premix Ex Taq*™ II を用いる場合より判定までに要する時間が 20 分程度長くなること，SYBR® *Premix Ex Taq*™ II でも常に primer dimer の増幅が認められるわけではないこと，融解曲線解析の Tm 値から非特異的産物であることが判定可能であること，さらに希釈血液試料では出現しないことなどを考慮すると試薬の選択は難しい問題であり今後の検討課題である．

2010 年 3 月 31 日現在，2 法で結果が矛盾する症例は無く，両法の正確性が確認された．検査対象者 2,954

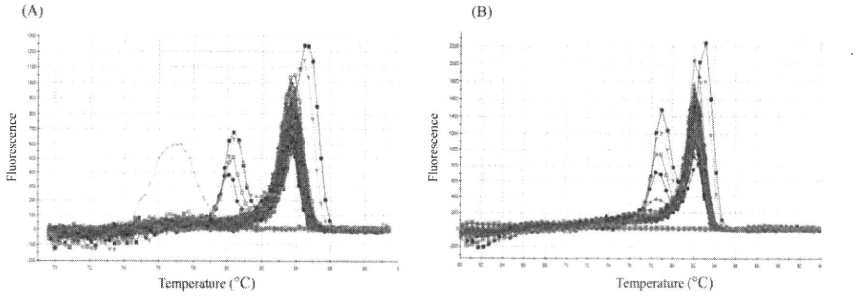

Fig. 2　Results of SYBR Green I-based real-time PCR for distinguishing HP^{del} and HP alleles. Results obtained by using SYBR® *Premix Ex Taq*™ II (A) and SYBR® Premix DimerEraser® (B).

名中，91名のHP^{del}ヘテロ接合体と1名のホモ接合体が検出された．この結果から福岡地区におけるHP^{del}の遺伝子頻度は約1.6%程度であると推定される．なお，今回検査をおこなった全患者に重篤な輸血副作用の報告は無く，1名のHP^{del}/HP^{del}の輸血予定患者への輸血は必要性が無くなったため実施されなかった．

考　察

希釈血液を直接鋳型としTaqMan probeとSYBR Green Iを指標としたリアルタイムPCR2法を用い輸血前患者血の遺伝子診断を実施し臨床現場での利用の可能性について検討をおこなった．輸血前診断を開始してみると，条件検討時にはみられなかった再現性の低いサンプルやプライマーダイマーの出現などの問題が発生したが，50mM NaOHの使用，試薬の変更により大幅に改善された．

いずれの方法も，DNA抽出を必要とせず希釈血液を用いることが可能でありHP^{del}の接合性を1サンプルにつき1本のチューブで判定可能で，採血から凡そ1時間半で診断結果が得られる．操作が簡便で診断結果が明確であるため，遺伝子解析専門の技術者がいない医療現場での利用も可能であると考えられる．さらに従来法と比較して増幅後チューブの開閉を必要としない閉鎖系で産物のキャリーオーバーによるコンタミネーションの危険性が軽減されること，ハイスループット解析に適すること，などメリットが大きい．

2法を比較すると，TaqMan法では，前述の通りリアルタイムに結果が観察・予想できること，遺伝子型のグラフ化によりHP^{del}/HP^{del}の同定が容易であり，よりハイスループット解析に適しており規模の大きな施設での輸血前診断や，献血者のスクリーニング検査によりHP欠損者のドナープールの作製にも有用なものと評価される．一方SYBR法は，蛍光が1波長で比較的安価な装置で解析が可能であること，一般的なPCR法に用いる試薬のみで十分であり準備や調整が容易で幅広い臨床現場に導入しやすい方法であると位置づけられる．

経費については，TaqMan法では1検体あたり約130円，SYBR法では約110円と試算される．

HP^{del}に起因するHP欠損の輸血前診断は，出現頻度を考慮すると費用対効果は必ずしも高いものとは言い難く，遺伝子診断を実施する為の倫理面の問題などクリアすべき問題は少なくない．一方，因果関係が明確であるアナフィラキシーショックの割合は低いが，抗IgAを始めとする同定されているものについては可能であれば輸血前の検査が実施されることが望ましい．またオーダーメイド医療の観点からも遺伝的欠損症については生涯に一度の検査で十分であり有用な検査項目であると考えられる．今後も検討を継続し，個々の現場環境に即した診断法を確立することにより，将来的には低頻度ながらHP欠損による輸血副作用を予防し安全な輸血が実施されることを期待したい．

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EVALUATION OF TWO REAL-TIME PCR METHODS FOR DETECTION OF HP^{del}, A RISK MUTATION FOR ANAPHYLACTIC SHOCK, BEFORE TRANSFUSION

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Abstract:

Anhaptoglobinemia is one of the risk factors for severe anaphylactic transfusion reaction because the patient produces serum haptoglobin antibodies. The causal mutation of anhaptoglobinemia in Japan is the haptoglobin deletion allele (HP^{del}), and the frequency of HP^{del} homozygotes is estimated to be one out of 4,000 individuals. The clinical detection of HP^{del} before transfusion is important to prevent anaphylactic shock. In order to facilitate safe blood transfusion, we recently developed two rapid and simple methods for detection of HP^{del}, TaqMan probe- and SYBR green I-based real-time PCR, using diluted blood samples directly as templates. Both methods need only one tube per sample and about one and a half hours to obtain results. In this study, we evaluated these two methods for detection of HP^{del} in patients who were scheduled for blood transfusion at Kurume University Hospital. The genotypes of 2,954 patients as determined by the two real-time PCR methods from January 2009 to March 2010 were fully concordant, and 91 HP/HP^{del} and one HP^{del}/HP^{del} were detected. We can predict the results in real time by monitoring the raw data during the reaction and easily detect the HP^{del}/HP^{del} in many samples analyzed by a dual-scatter plot in the TaqMan-based method. This method seems to be suitable for high-throughput analyses such as detection before transfusion at large institutions or screening for HP^{del} in a large population study. On the other hand, the SYBR Green I-based method is appropriate for relatively small institutions due to its low initial cost and analyzability using economical real-time PCR machines.

Keywords:
anhaptoglobinemia, anaphylactic transfusion reactions, diagnosis before transfusion

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