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Research Article
Comparison Between Three Molecular Diagnostics for the Identification of Heterozygous Hemoglobin E

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Abstract

Background and Objectives: Hemoglobin E is a variant hemoglobin caused due to the base substitution G→A at codon 26 in the β-globin-coding gene that is followed by the alteration of glutamic acid (GAG) to lysine (AAG). Various types of molecular analysis methods such as tetra-primer amplification refractory mutation system (T-ARMS-PCR), Tm-shift real-time polymerase chain reaction (Tm-shift qPCR) and high-resolution melting analysis (HRMA) are commonly used to detect several mutations in the β-globin-coding gene. This study was conducted to compare the detection result of Cd 26 (G→A) mutation in the β-globin-coding gene of heterozygous HbE between the above-mentioned methods. **Materials and Methods:** DNA samples were isolated from blood archive of heterozygous HbE and analyzed for the detection of the mutation using HRMA and Tm-shift on a real-time PCR instrument, whereas T-ARMS analysis was performed on a conventional PCR equipment. High resolution melt v3.1 software and Bio-Rad CFX Manager software were used to analyze the result of HRMA and Tm-shift qPCR, whereas the T-ARMS-PCR result was analyzed by observing the number and size of DNA bands on gel electrophoresis. **Results:** Among 21 samples, the Cd 26 mutation was detected in numbers 18, 19 and 21 by HRMA, Tm-shift qPCR and T-ARMS-PCR. DNA Sequencing confirmed Cd 26 mutation on 5 ambiguous samples and revealed two homozygous mutation. **Conclusion:** The Cd 26 (G→A) mutation was detected in proportions 100, 91 and 86% by T-ARMS-PCR, Tm-shift qPCR and HRMA, respectively.

Key words: Cd 26 (G→A), HbE mutation, HRMA, T-ARMS-PCR, Tm-shift qPCR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Blood disorders can occur in the cellular component of blood and tissue, or in components that are found in blood cells such as hemoglobin in the erythrocyte. Hemoglobin disorders affect the quality and quantity of blood production in the body[1]. Disorders are caused due to a decrease in hemoglobin synthesis in the erythrocyte are known as thalassemia or hemoglobinopathy[2]. Hemoglobinopathy is a disorder that is caused due to the presence of a hemoglobin variant in the erythrocyte[3]. This disorder results in an alteration in the amount and type of globin molecule[4]. An example of hemoglobinopathy is hemoglobin E (HbE), which is caused due to a mutation in codon 26 of the β-globin gene. It causes substitution of glutamic acid (GAG) for lysine (AAG)[5]. Point substitution is the common mutation found in the β-globin gene[6].

The Cd 26 mutation is distinct in several Asian countries such as Indonesia, Malaysia and China[7]. In addition, the alteration in the nitrogenous base of the DNA sequence could provide information regarding the mutation defect[8]. Hemoglobin E could appear as heterozygous (HbE carrier) or homozygous (HbE major) mutation[9]. A Research conducted by Viprakasit *et al.*[10] reveals about 33% prevalence of HbE carriers in Indonesia. So, it encourages the effort to manage the number of HbE major mutations by blood screening, genetic counseling and prenatal diagnosis. The initial step in determining the status of the participants being screened is hematological analysis. The samples that are hematologically identified as heterozygous HbE must be confirmed by molecular analysis.

Various types of molecular analysis methods such as tetra-primer amplification refractory mutation system (T-ARMS-PCR), Tm-shift real-time polymerase chain reaction (Tm-shift qPCR) and high-resolution melting analysis (HRMA) have been applied to detect the type of mutation in the β-globin-coding gene. T-ARMS-PCR is a flexible and low-cost method that can be performed on a conventional PCR system for SNP detection. The amplification of normal and mutant alleles is performed in a single reaction using a pair of outer primers[11,12]. The T-ARMS-PCR primers have mismatches in the middle of the specific allele that is at the 3'- and the -2' end before the 3'-end[4].

Single nucleotide polymorphism (SNP) can be determined using the Tm-shift qPCR method. It requires using a fluorescent dye such as SYBR Green 1, instead of a specific probe. Tm-shift qPCR is less time-consuming and can be performed on a large population sample. The genotype collected from the sample can be determined in a single tube by assessing the difference in the melting temperature. The PCR product can also be observed in real time and hence, this method decreases the possibility of cross-contamination from post PCR manipulation, such as electrophoresis analysis. This method is also less expensive compared to HRM assay[13-15].

HRMA is a method that is based on DNA double-stranded separation or melting point that is produced from PCR. The basis of HRMA is the difference in the melting temperature and the melting curve of the DNA samples[16]. The melting point of the amplification product depends on the GC content and the length of the double strands. In addition to these factors, the melting temperature is influenced by the heterozygosity of the samples, the fluorescence concentration and the rate of temperature exchange on the thermal cycler[16,17]. The exchange of a single nucleotide could alter the melting profile[18]. HRMA is a rapid and accurate method for the detection of mutation. This method can be used to detect the mutation of a gene by the difference plot of the melting curve and melting temperature[19]. HRMA is a time-saving method and produces high-throughput data and data errors can be minimalized because the samples are analyzed in a closed-tube analyzed the fluorescence directly added to the thermal cycler and has a high specificity and sensitivity with low operational cost[10,19,20].

Twenty-one blood archives stored in the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada were detected as HbE carriers and HbE/β-thalassemia carriers based on the hematological data-sheet but the Cd 26 mutation is not detected yet. All the above-mentioned methods can be applied to the molecular diagnosis of hemoglobinopathy. Therefore, it becomes necessary to confirm and compare the detection result of the codon 26 (G→A) mutation in the β-globin-coding gene using T-ARMS-PCR, Tm-shift qPCR and HRMA performed on the blood archives that were hematologically identified as heterozygous HbE.

MATERIALS AND METHODS

This research was held on February to May, 2018 at Laboratory of Genetics and Breeding Faculty of Biology, Universitas Gadjah Mada, Center of Veterinary Wates, Yogyakarta and Integrated Research and Testing Laboratory, Universitas Gadjah Mada.

The samples used in this study were the blood archives stored in the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada. A total of 17 samples were detected as HbE carriers and 2 samples as HbE/β-thalassemia carriers based on the hematological data-sheet, specifically

Table 1: Primers sequences of three methods used for the amplification of Cd 26 (G→A) mutation

Methods	Primers	Primer sequences (5"→3")	Product length (bp)
T-ARMS-PCR	Inner forward (mutant)	GTGAACGTGGATGAAGTTGGTGTTA	462
	Inner reverse (normal)	GATACCAACCTGCCCAGGGCATC	276
	Outer forward (1F)	CCAAGGACAGGTACGGCTGTCATC	704
	Outer reverse (5R)	CCTTCCTATGACATGAACTTAACCAT	
Tm-shift qPCR	Forward	AGAAGTCTGCCGTTACTGCC	
	Normal reverse	GCGGGCAGGGCGGCCAACCTGCCCAGGGCCTC	88
	Mutant reverse	GCGGGCCAACCTGCCCAGGGCCTT	80
HRMA	Forward	CCTGAGGAGAAGTCTGCCGTT	138
	Reverse	GTCTCCACATGCCCAGTTTCT	

Underlined nucleotides represent the GC tails and the bolded nucleotide represents a mismatch

from the level of HbA2 that is >13%. Two additional samples were used as a positive control (HbE heterozygous based on PCR-RFLP) and a negative control (normal/wild type).

Three primary steps, i.e., DNA isolation, mutation detection and data analysis, were followed in this study and the details of each step are mentioned below.

DNA isolation: DNA isolation was performed using the Gene Aid Genomic DNA Mini Kit for Blood or Cultured Cell ISO9001:2008QMS procedure (GeneAid, Taiwan).

Mutation detection: Detection of Cd 26 (G→A) mutation was performed using the following three methods: T-ARMS-PCR, Tm-shift qPCR and HRMA. DNA sequencing was performed to identify double mutations in HbE/β-thalassemia and confirm the detection results. Table 1 shows the primer design of each method.

Tetra primer amplification refractory mutation system: DNA amplification using T-ARMS-PCR referred to Ye and Rawangkran[21,22] research with modification in primer and PCR condition. T-ARMS-PCR was performed in a volume of 25 µL containing 0.5 µL of each primer outer forward 1F and outer reverse (0.2 µM), 0.75 µL of each primer inner forward and inner reverse (0.3 µM), 7 µL of ddH_2O, 12.5 µL of 2x MyTaq™ HS Red Mix (Bioline, Philadelphia, Pennsylvania); and 3 µL of DNA template. Amplification was performed on T100™ Biorad Thermal Cycler (Biorad, Hercules, California). The PCR conditions were as follows: Enzyme activation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 58.2°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min.

Tm-shift real-time polymerase chain reaction: DNA amplification using Tm-shift qPCR referred to Ghaissani research with modification in primer and PCR condition[23]. Tm-shift qPCR was performed in a volume of 20 µL containing 0.3 µL of forward primer (0.15 µM), 0.175 µL of normal reverse primer (0.085 µM), 0.2 µL of mutant reverse primer (0.1 µM), 7.325 µL of ddH_2O, 10 µL of 2x THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Japan) and 2 µL of DNA template. Amplification was performed on real-time PCR Biorad CFX96 (Biorad, Hercules, California) under the following conditions: Pre-denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 62°C for 30 sec. The melting process was performed from 65-95°C with increment 0.2°C every 0.05 sec.

High resolution melting analysis: DNA amplification using HRMA referred to Hidayati research with modification in primer and PCR condition[24]. HRMA was performed in a volume of 20 µL containing 0.8 µL of each forward and reverse primer (10 µM), 10 µL of 25 mM $MgCl_2$, 5.6 µL of ddH_2O, 10 µL of 2x SensiFAST HRM Master Mix (Bioline, Philadelphia, Pennsylvania) and 2 µL of DNA template. HRMA was performed on a real-time PCR Applied Biosystem 7500 Fast (Thermofisher, Waltham, Massachusetts) under the following conditions: Enzyme activation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 64°C for 30 sec. The melting curve analysis protocol consisted of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, HRM at 95°C for 15 sec and annealing at 60°C for 15 sec. The ramp rate of each step was 100%, except for HRM step, which was 1%.

DNA sequencing: DNA sequencing was performed by the Sanger method with the 1st Base DNA Sequencing Services using the outer primers of T-ARMS-PCR as a confirmatory test.

Data analysis: The result of T-ARMS-PCR were analyzed by observing the number and size of DNA bands on 2% gel electrophoresis. The result of HRMA and Tm-shift qPCR were analyzed using the high resolution melt software v3.1 and the Bio-Rad CFX Manager software, respectively. Using the HRM software, the aligned melt curve and the difference plot of each sample were compared with the

negative and positive control. Similar to the HRMA result, the results of the melting peak on Tm-shift qPCR were also compared with the controls. DNA The DNA sequencing result was analyzed and assembled using the Gene Studio Software and then aligned using Clustal W in the MEGA 6.0 Software.

RESULTS

Molecular analysis of the gene, especially in term of mutation detection, was performed to observe the effect of the mutation and to decide the appropriate treatment. That molecular analyses of the gene for mutation detection such as T-ARMS-PCR, Tm-shift qPCR and HRMA have been applied to detect several mutations in the β-globin-coding gene. In this study, the results of these molecular diagnostic methods for investigating Cd 26 (G→A) mutation in the β-globin gene to identify HbE cases. Each method was performed using the specific instrument and primers, so that it was able to produce accurate data. These results are presented in figures and tables as follow:

T-ARMS-PCR results: The results of the T-ARMS-PCR method are represented by the electrophoregram of DNA amplicon fragments (Fig. 1) with several product sizes. Normal samples exhibited 704 and 276 bp bands, whereas heterozygous HbE showed an additional band size of 462 bp. Based n the electrophoregram, the Cd 26 (G→A) mutation was detected in all the samples confirmed by the presence of 462 band. In addition to the existence of the Cd 26 (G→A) mutation, two samples (A6 and B1) showed a thick non-target band size of 600 bp that indicated another mutation in the β-globin gene.

Tm-shift qPCR results: The Tm-shift qPCR result is represented as the melt peak of negative and positive controls (Fig. 2),

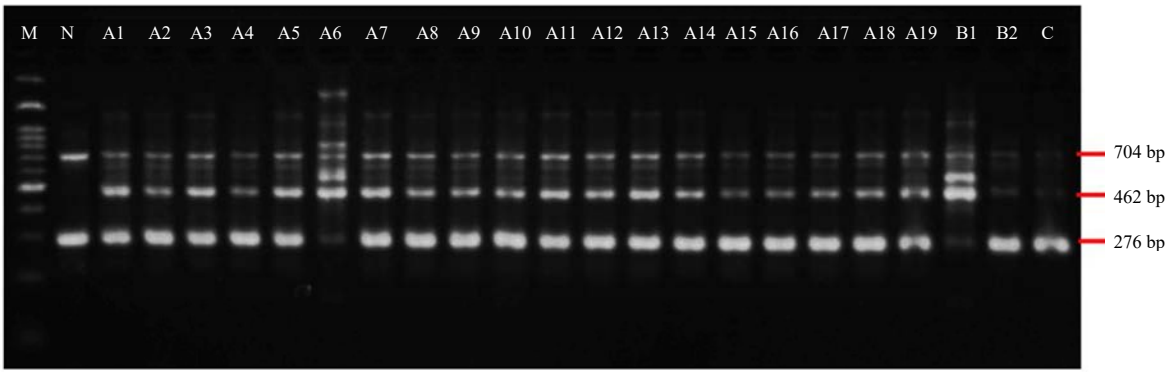

Fig. 1: Electrophoregram of all samples on 2% agarose gel
N: Normal (negative control), C: Heterozygous HbE based on RFLP (positive control), A1-A19, B1-B2: Examined samples, M: 100 bp DNA ladder

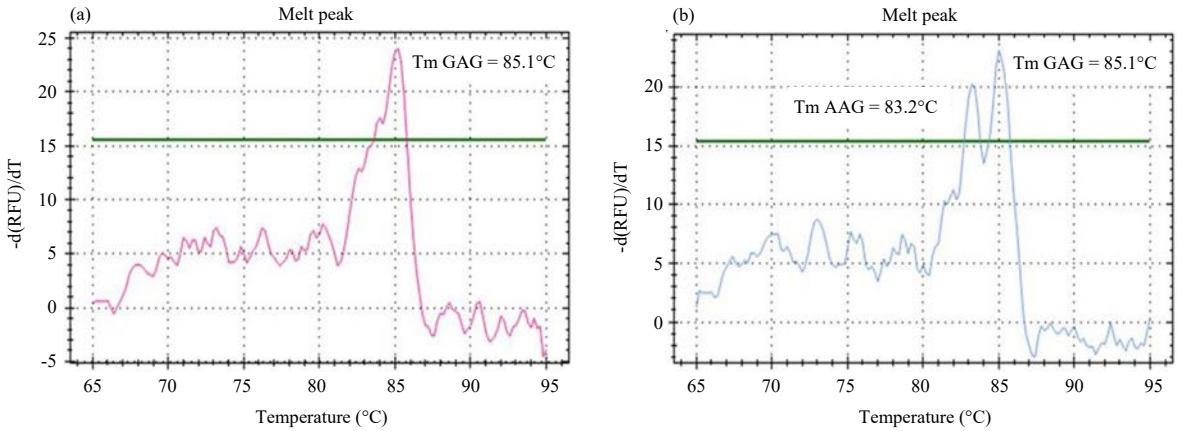

Fig. 2(a-b): Melt curve of (a) Negative control and (b) Positive control

completed by all the samples (Fig. 3). The Tm for the wild-type allele was 85.1±0.12°C. The wild-type sample had only one allele (-G); thus, there was only one single peak in the melt curve. The heterozygous HbE had two alleles, one wild-type (-G) and one mutant (-A) and had two Tm values, 85.1±0.12°C for the -G allele and 83.2±0.19°C for the -A allele, so that two peaks were formed. Almost all samples show heterozygous genotype proved by two peaks, 85.1±0.12 and 83.2±0.19°C, but there were two samples that were not found to have the Cd 26 (G→A) mutation (A6 and A9).

HRMA results: The results of HRMA are shown in the aligned melt curve (Fig. 4a) and the difference plot (Fig. 4b and 5). The aligned melt curve showed in Fig. 4a

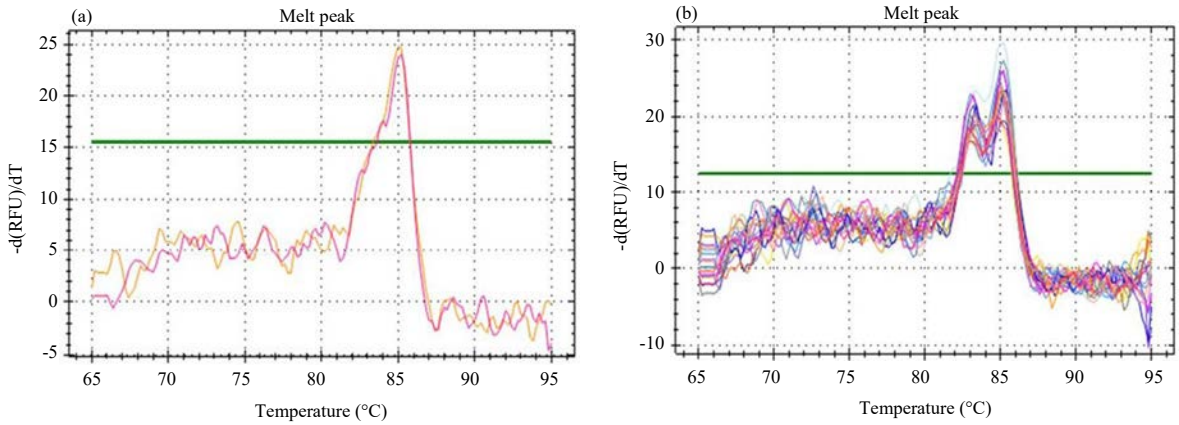

Fig. 3(a-b): Melt curve of all samples, (a) Normal samples and (b) Heterozygous HbE samples

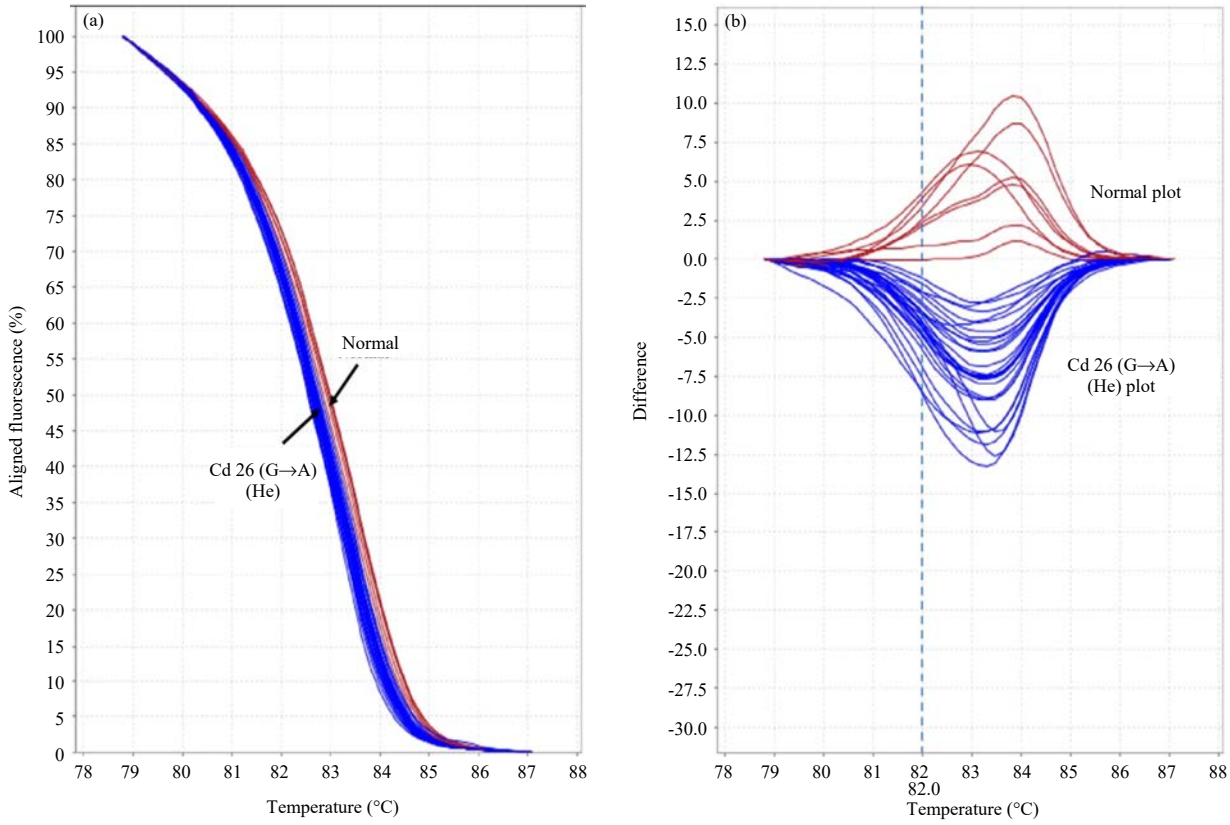

Fig. 4(a-b): (a) Aligned melt curve and (b) Difference plot from all the samples
He: Heterozygous

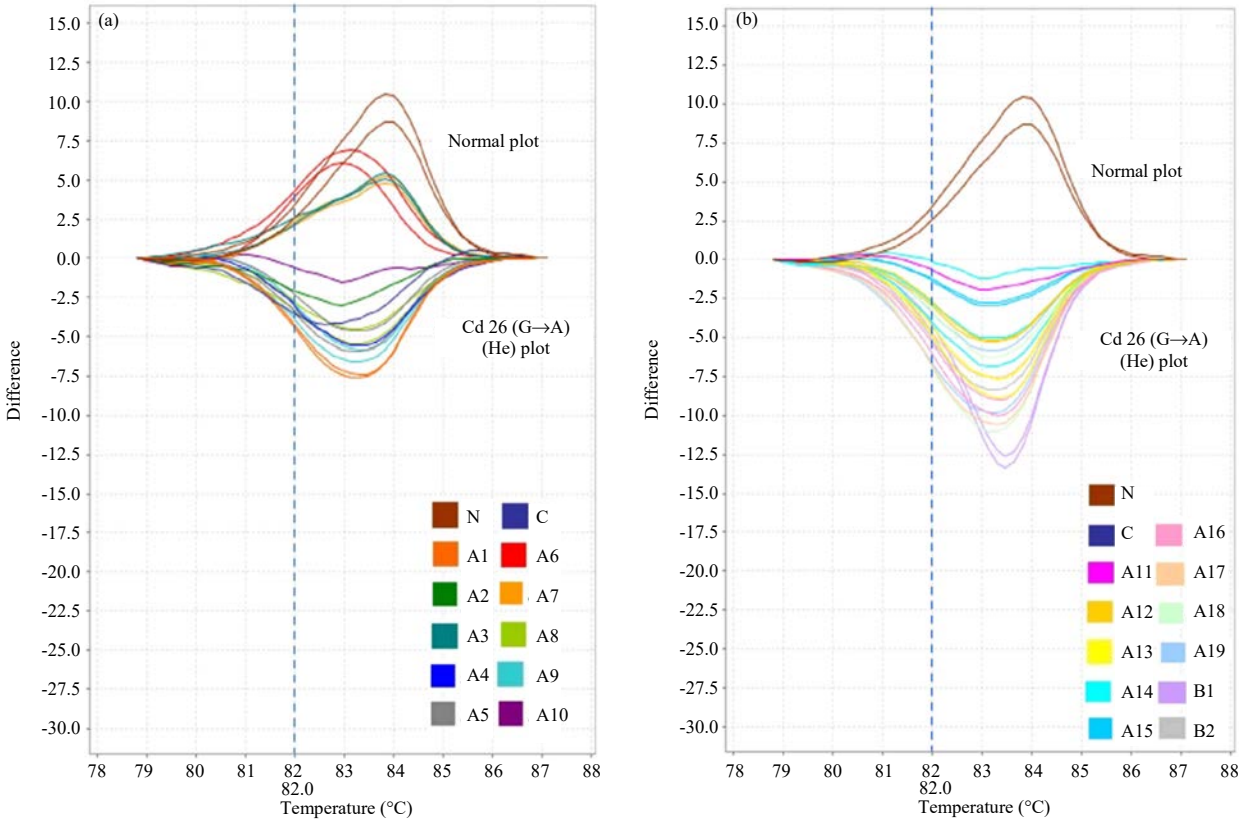

Fig. 5(a-b): Difference plot from all the samples

He: Heterozygous, N: Normal (negative control), C: Heterozygous HbE based on RFLP (positive control), A1-A19, B1-B2: Examined samples

indicates that there were two distinct groups. The normal and heterozygous samples shown a red and a blue color melting curve, respectively. The melting temperature of normal samples were higher than that of heterozygous samples.

The difference plot represents the clustering of the samples. Each plot (normal and Cd 26 (G→A) heterozygous plot) represented the controls to the study samples. The difference plots of Cd 26 (G→A) heterozygous mutation based on normal and heterozygous curves are shown in Fig. 4b. The plots of normal and heterozygous samples are shown in red and blue colors, respectively. The sample-based difference plot (Fig. 5) demonstrated that almost all of a single clustered in the heterozygous plot, but there were three samples that clustered to the normal plot, indicating that the Cd 26 (G→A) mutation was not present. The other samples clustered to the positive control plot, confirming that these samples were heterozygous HbE.

DNA sequencing results: DNA sequencing was performed in samples that demonstrated inconsistent results in each of the abovementioned detection methods (Table 2) and also in two samples of HbE/β-thalassemia carriers. In total, five samples were sequenced to confirm the existence of the Cd 26 (G→A) mutation and other types of mutation. The results are shown in Fig. 6 and 7.

The presence of Cd 26 (G→A) mutation was confirmed in all the 5 samples (Fig. 6). In the chromatogram, the Cd 26 site was located at nucleotide number 238. Heterozygous HbE were found in A7, A9 and B2 confirmed by double peaks from -G allele (black) to -A allele (green). Meanwhile, homozygous HbE was found in A6 and B1 confirmed by single peak of -A allele.

The DNA alignment results shown in Fig. 7 demonstrates the representative DNA sequence comparison results of the beta-globin gene from all five samples. In addition to the mutation in Cd 26, there were three other sites of mutation detected within the samples, which were Cd 2, Cd 35 and IVS-II-16.

Comparison of the mutation detection techniques: Table 2 shows a summary of the results of the three ambiguous samples and two double mutation samples, along with the haematological condition (carrier or normal condition based

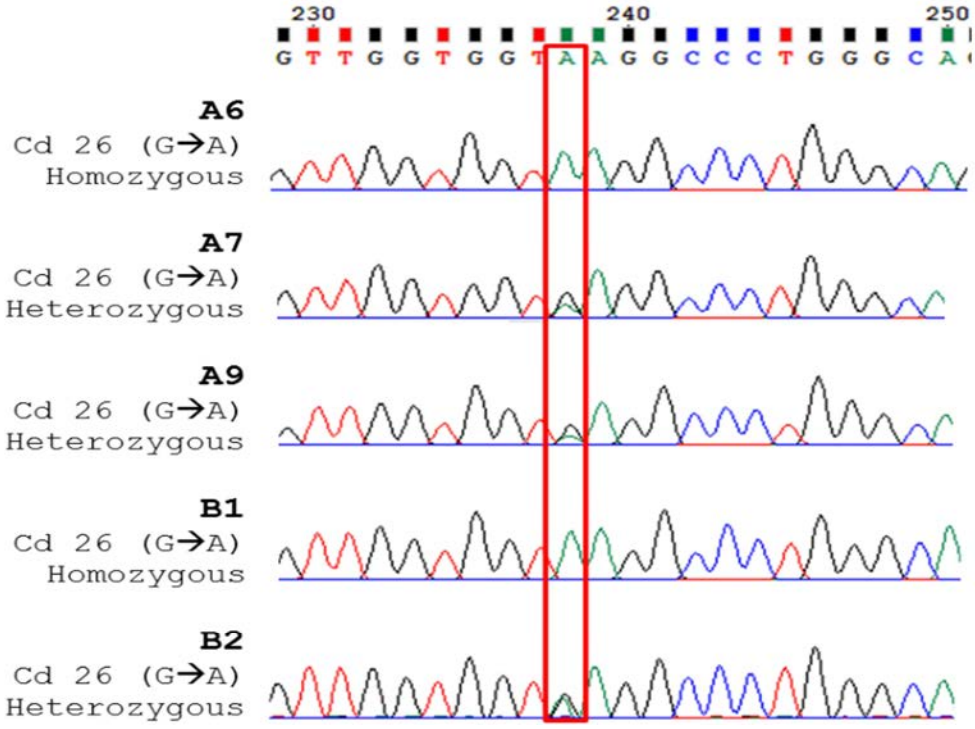

Fig. 6: Sequencing chromatogram of 5 samples with information about the Cd 26 (G→A) mutation

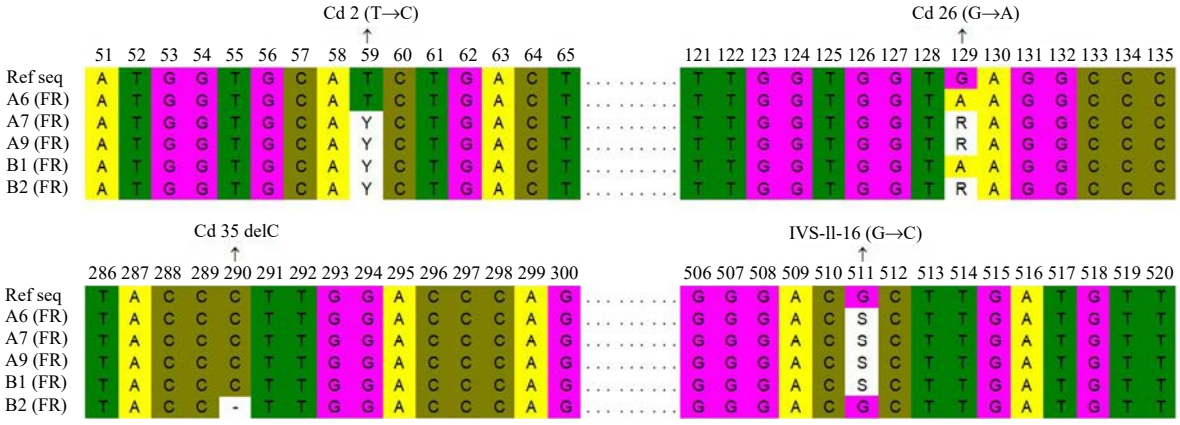

Fig. 7: DNA alignment of β-globin gene sequences from 5 samples

Ref seq: Reference sequence (NC_000011.10 accessed from NCBI), FR: Assembled DNA sequence from forward and reverse primers, Cd: Codon, IVS: Intervening sequence, the numbers above the letters indicate the nucleotide sequence number in the β-globin gene

Table 2: Results of comparison of the three mutation detection techniques and DNA sequencing confirmation

		Mutation detection			
Sample code	Hematological classification	T-ARMS-PCR	HRMA	Tm-Shift qPCR	DNA sequencing
A6	HbE carrier	+/+	-/-	-/-	+/+
A7	HbE carrier	+/-	-/-	+/-	+/-
A9	HbE carrier	+/-	-/-	-/-	+/-
B1	HbE/β-thalassemia carrier	+/+	+/-	+/-	+/+
B2	HbE/β-thalassemia carrier	+/-	+/-	+/-	+/-

+/+: Homozygous HbE, +/-: Heterozygous HbE, -/-: Homozygous normal

on blood profile) and the type of mutation based on the results of DNA sequencing. From the table, it is known that among 21 samples, the Cd 26 mutation was detected in numbers 18, 19 and 21 by HRMA, Tm-shift qPCR and T-ARMS-PCR.

DISCUSSION

This study was performed to compare the detection results of the Cd 26 (G→A) mutation of heterozygous. Figure 1 clearly showed that twenty-one samples were confirmed to Cd26 mutation using T-ARMS-PCR, but there are non-specific-bands appears in sample A6 and B1. Previous research has demonstrated that several factors, including the melting temperature and primer balancing are involved in the T-ARMS-PCR technique[25]. The primers used for this method had a GC content ranging from 38.46% (outer reverse) to 60.87% (inner reverse). The concentrations of dNTPs, Taq polymerase and $MgCl_2$ in the PCR assay could have an influence on the specificity and sensitivity of this method due to the non-specific-product formed. Betaine reagent could be added to PCR to decrease non-specific-bands and $MgCl_2$ to balance the PCR assay[25].

The T-ARMS-PCR method has the advantages of being less time-consuming and a relatively low-cost because it does not require enzyme incubation time also detection of the mutant and normal alleles can be performed simultaneously in a single tube[4,26-28]. The difficulties of using the T-ARMS-PCR method including the determination of the melting temperature primers and a considerable time interval in the electrophoresis step[29].

Figure 3 showed almost all samples show heterozygous genotype proved by two peaks, 85.1±0.12 and 83.2±0.19°C, but there were two samples that were not found to have the Cd 26 (G→A) mutation (A6 and A9). In this method, a long GC tail of 5″-GCGGGCAGGGCGGC-3″-(14 bp) and a short GC tail of 5″-GCGGGC-3″-(6 bp) were added to R(N) and R primer. The long GC tail was attached to the reverse primer for the wild-type allele, which has a higher Tm base and the short GC tail was attached to the reverse primer for the mutant allele, which has a lower Tm base. A Mismatch was also added to R, so that the mutant allele could be recognized[13,30].

The Tm-shift genotyping method has several advantages. Product amplification can be performed in real-time and less time-consuming and can be performed on a large population sample. This method is also less expensive compared with the HRM assay and does not require a probe, so that it is easy to apply[13-15]. However, SYBR Green 1 is a highly sensitive fluorescent dye. In addition, it is necessary to consider the primer design to be used as it would affect the specificity and discrimination ability of the PCR products[31].

Figure 5 showed 3 of 21 samples were not detected for Cd 26 mutation. The aligned melt curve is a normalized melt curve calculated by the HRM software to unify the melt curve points of all samples at 100 and 0% relative fluorescence intensities[17]. Figure 4 presented the presence of Cd 26 substitution mutation by the exchange of guanine (GAG) to adenine (AAG) resulted in a decrease in the melting temperature. Due to the sensitivity of this method, any error in the experiment could lead to the problem of melt curve clustering. It also could not analyze the significant polymorphism that lies in the mutation fragment[32].

HRMA is a time-efficient method, high-throughput data, has high sensitivity and specificity with low operational cost and has been applied to detect mutations in several gene spectrums[20,28,33,34]. Although HRMA is an effective method for genotyping, HRMA could not detect insertion and large deletion mutation. This method requires a high-quality and precision instrument. Routine clinical use of HRMA is limited by the high price of the instrument, the lack of protocol understanding and the requirement of extensive and comprehensive optimation[18,35-37].

There were 3 ambiguous samples and 2 double mutation samples were confirmed using DNA sequencing. From sequencing method, It is known that all the other mutations were heterozygous that included a were single nucleotide substitution from tyrosine to cytosine in Cd 2 detected in all samples except A6, a single cytosine deletion in Cd 25 identified in B2 and a guanine-to-cytosine substitution in IVS-II-16 found in all samples except B2. In addition, one of the two HbE/β-thalassemia carriers were successfully identified as a double mutation (B2) carrying Cd 26 (G→A) and Cd 35 del C, whereas the other one (B1) was homozygous HbE. The mutations in Cd 2 and IVS-II-16 were silent mutation and simply SNP, respectively and thus both did not affect the molecular diagnosis.

This study discovered the advantages and disadvantages of three molecular detection to detect Cd 26 mutation that can be beneficial to develop and improve novel methods for mutation detection. This study will help the researchers to compare three molecular methods to know the advancement and limitation that many researchers were not able to explore. Thus, a new theory on molecular detection of Cd 26 mutation may be arrived at the instrument that is used in the study, the type of mutation in the samples and possibility of SNP.

CONCLUSION

Based on the molecular detection results of 21 samples in this study, the Cd 26 (G→A) mutation was detected in proportions of 100, 91 and 86% by T-ARMS-PCR, Tm-shift qPCR and HRMA, respectively. Deciding the right method of molecular detection for genetic disease have to consider the mutation location and any possibility of SNP in amplification area.

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REFERENCES

1. Davies, S.C., E. Cronin, M. Gill, P. Greengross, M. Hickman and C. Normand, 2000. Screening for sickle cell disease and thalassaemia: A systematic review with supplementary research. Health Technol. Assess., Vol. 4, No. 3. 10.3310/hta4030.
2. Jia, S.Q., J. Li, Q.H. Mo, C. Liao, L.Y. Li and X.M. Xu, 2004. α^0 Thalassaemia as a result of a novel 11.1 kb deletion eliminating both of the duplicated α globin genes. J. Clin. Pathol., 57: 164-167.
3. Rogers, K., 2011. Blood: Physiology and Circulation. 1st Edn., Britannica Educational Publishing, New York, USA., ISBN-13: 9781615301218, Pages: 240.
4. Hedrick, P.W., 2011. Selection and mutation for α thalassemia in nonmalarial and malarial environments. Ann. Hum. Genet., 75: 468-474.
5. Fahim, F. and M.G.S. Ahmed, 2010. Genetic haemoglobin disorders. Haematol. Updates, 16: 21-22.
6. Koh, D.X.R., R.Z.A.R. Sabudin, M.M. Yusoff, N.H. Hussin, R. Ahmad, A. Othman and E. Ismail, 2017. Molecular characterisation of *α*- and *β*-thalassaemia among indigenous Senoi Orang Asli communities in Peninsular Malaysia. Ann. Hum. Genet., 81: 205-212.
7. Handayani, N.S.N., R. Purwanto, 2015. Cd35 (del C) frameshift mutation in exon 2 of β-globin gene on β-thalassemia carriers. Biomed. Eng., 1: 19-23.
8. Handayani, N.S.N., N. Husna and I. Sanka, 2017. α-globin alteration in α-thalassemia disorder: Prediction and interaction defect. Pak. J. Biol. Sci., 20: 343-349.
9. Uddin, M.K., M.A. Aziz, M.H. Sardar, M.Z. Hossain and M.F. Bhuya *et al*., 2010. Electrophoretic pattern of hereditary haemoglobin disorders in Bangladesh. J. Dhaka Med. Coll., 19: 39-42.
10. Viprakasit, V., C. Lee-Lee, Q.T. Chong, K.H. Lin and A. Khuhapinant, 2009. Iron chelation therapy in the management of thalassemia: The Asian perspectives. Int. J. Hematol., 90: 435-445.
11. Alyethodi, R.R., U. Singh, S. Kumar, R. Alex and R. Deb *et al*., 2018. T-ARMS PCR genotyping of SNP rs445709131 using thermostable strand displacement polymerase. BMC Res. Notes, Vol. 11. 10.1186/s13104-018-3236-6.
12. Heidar, M.M. and M. Khatami, 2017. Designing and validation of one-step T-ARMS-PCR for genotyping the eNOS rs1799983 SNP. Iran. J. Biotechnol., 15: 208-212.
13. Navarro, L., C. Gongora and P. Benavides, 2010. Single nucleotide polymorphism detection at the *Hypothenemus hampei Rdl* gene by allele-specific PCR amplification with T_m-shift primers. Pestic. Biochem. Physiol., 97: 204-208.
14. Pan, W., Y. Fu, A.Y. Abdullahi, M. Wang and X. Shi *et al*., 2017. Development of T_m-shift genotyping method for detection of cat-derived *Giardia lamblia*. Parasitol. Res., 116: 1151-1157.
15. Zhou, S.H., M. Liu, W.X. An, X.H. Liang, W.J. Yu, B.L. Gong and F.Y. Piao, 2012. Genotyping of human platelet antigen-15 by single closed-tube T_m-shift method. Int. J. Lab. Hematol., 34: 41-46.
16. Perng, C.L., H.Y. Chen, T.S. Chiueh, W.Y. Wang, C.T. Huang and J.R. Sun, 2012. Identification of non-tuberculous mycobacteria by real-time PCR coupled with a high-resolution melting system. J. Med. Microbiol., 61: 944-951.
17. Ruskova, L. and V. Raclavsky, 2011. The potential of High Resolution Melting Analysis (HRMA) to streamline, facilitate and enrich routine diagnostics in medical microbiology. Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub., 155: 239-152.
18. Traeger-Synodinos, J. and C.L. Harteveld, 2014. Advances in technologies for screening and diagnosis of hemoglobinopathies. Biomarkers Med., 8: 119-131.
19. Reed, G.H., J.O. Kent and C.T. Wittwer, 2007. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics, 8: 597-608.
20. Ramezanzadeh, M., M. Salehi and R. Salehi, 2016. Assessment of high resolution melt analysis feasibility for evaluation of beta-globin gene mutations as a reproducible, cost-efficient and fast alternative to the present conventional method. Adv. Biomed. Res., Vol. 5. 10.4103/2277-9175.180640.

21. Rawangkran, A., N. Janwithee, P. Wong and N. Jermnim, 2013. Prevalence of thalassemia trait from screening program in pregnant women in the lower Northern region of Thailand. Thai J. Genet., 1: 156-159.
22. Ye, S., S. Dhillon, X. Ke, A.R. Collins and I.N. Day, 2001. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res., Vol. 29, No. 17. 10.1093/nar/29.17.e88.
23. Ghaissani, S.S., 2016. Optimalization of T_m shift SYBR green I real time PCR method for genotyping SNP Ile655Val HER-2 on breast cancer patients. Undergraduate Thesis, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia.
24. Hidayati, N.I., 2017. Detection IVSI-5 (G→A) and Cd 35 (Del C) mutation in the β-globin-coding gene on β-thalassemia carriers using high resolution melting analysis. Undergraduate Thesis, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia.
25. Medrano, R.F.V. and C.A. de Oliveira, 2014. Guidelines for the tetra-primer ARMS-PCR technique development. Mol. Biotechnol., 56: 599-608.
26. Ahlawat, S., R. Sharma, A. Maitra, M. Roy and M.S. Tantia, 2014. Designing, optimization and validation of tetra-primer ARMS PCR protocol for genotyping mutations in caprine *Fec* genes. Meta Gene, 2: 439-449.
27. Li, M., X. Sun, J. Jiang, Y. Sun and X. Lan *et al*., 2014. Tetra-primer ARMS-PCR is an efficient SNP genotyping method: An example from SIRT2. Anal. Methods, 6: 1835-1840.
28. Xiong, F., Q. Huang, X. Chen, Y. Zhou and X. Zhang *et al*., 2011. A melting curve analysis-based PCR assay for one-step genotyping of β-thalassemia mutations: A multicenter validation. J. Mol. Diagn., 13: 427-435.
29. Zhang, C., Y. Liu, B.Z. Ring, K. Nie and M. Yang *et al*., 2013. A novel multiplex tetra-primer ARMS-PCR for the simultaneous genotyping of six single nucleotide polymorphisms associated with female cancers. PLoS ONE, Vol. 8. 10.1371/journal.pone.0062126.
30. Wang, J., K. Chuang, M. Ahluwalia, S. Patel and N. Umblas *et al*., 2005. High-throughput SNP genotyping by single-tube PCR with T_m-shift primers. BioTechniques, 39: 885-893.
31. Fukuoka, H., K. Miyatake, S. Negoro, T. Nunome, A. Ohyama and H. Yamaguchi, 2008. Development of a routine procedure for single nucleotide polymorphism marker design based on the T_m-shift genotyping method. Breed. Sci., 58: 461-464.
32. Slomka, M., M. Sobalska-Kwapis, M. Wachulec, G. Bartosz and D. Strapagiel, 2017. High Resolution Melting (HRM) for high-throughput genotyping-limitations and caveats in practical case studies. Int. J. Mol. Sci., Vol. 18, No. 11. 10.3390/ijms18112316.
33. Lin, M., J.W. Jiao, X.H. Zhan, X.F. Zhan and M.C. Pan *et al*., 2014. High resolution melting analysis: A rapid screening and typing tool for common β-thalassemia mutation in Chinese population. PLoS ONE, Vol. 9. 10.1371/journal.pone.0102243.
34. Vossen, R.H.A.M., E. Aten, A. Roos and J.T. den Dunnen, 2009. High-Resolution Melting Analysis (HRMA)—More than just sequence variant screening. Hum. Mutat., 30: 860-866.
35. Chang, Y.C., Y.S. Chang, C.C. Chang, T.C. Liu and Y.C. Ko *et al*., 2016. Development of a high-resolution melting method for the screening of *TNFAIP3* gene mutations. Oncol. Rep., 35: 2936-2942.
36. Wittwer, C.T., 2009. High-resolution DNA melting analysis: Advancements and limitations. Hum. Mutat., 30: 857-859.
37. Yang, M., Y.J. Yue, T.T. Guo, J.L. Han and J.B. Liu *et al*., 2014. Limitation of high-resolution melting curve analysis for genotyping simple sequence repeats in sheep. Genet. Mol. Res., 13: 2645-2653.