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► To cite this version:

Tossea A Kouï, Ernest Sery Gonedele-Bi, Eric Gbessi Adjì, Albert A Gnon djui, Berenger Ako Ako, et al.. Genotyping of S and C Mutated Beta Globin Gene: Development of a Set of Primers for Use with DfV'VnG PCR Systems. *Biochemistry & Molecular Biology Journal*, 2022, 8 (12), pp.108-114. 10.36648/2471-8084.8.12.108 . pasteur-04665643

HAL Id: pasteur-04665643

<https://pasteur.hal.science/pasteur-04665643v1>

Submitted on 31 Jul 2024

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Genotyping of S and C Mutated Beta Globin Gene: Development of a Set of Primers for Use with Different PCR Systems

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ABSTRACT

Sickle cell disease is a genetic disorder that affects nearly 5% of world population. In Ivory Coast, SCD is a real problem of health and screening is not systematic after birth. Here, we designed a set of primers to detect abnormal hemoglobin S and C which can be used both in conventional and quantitative PCR (by curves combinations analysed). A total of 60 blood samples including 13 AA, 23 AS, 9 SS, 12 SC and 3 CC hemoglobin type were screened using hemoglobin electrophoresis and PCR. The universal control primer HBU/R4 was successfully amplified for all of 60 samples. In conventional PCR, for typing of allele S sensibility and specificity of primers were respectively 86.36% and 87.50%. For allele C, sensibility and specificity of this pair were respectively 53.33% and 91.11%. In qPCR, specificity and sensitivity of primers were greater than 85% for allele S and C specific primers.

Keywords: Sickle cell disease; PCR; Ivory coast

Abbreviations: SCD: Sickle Cell Disease; PCR: Polymerase Chain Reaction; qPCR: Quantitative PCR

INTRODUCTION

Sickle cell disease is a genetic disorder that affects nearly 5% of world population [1,2]. There are several hemoglobin disorders known, but in Africa, most of the diseases emerge from mutations of the codon 6 of exon 1 of the beta chain of hemoglobin is involved in this disease. Mutations of this codon are related to C and S types of hemoglobin associated with the most severe disease when expressed as homozygote

SS, CC or as hybrid SC. The mortality and morbidity associated with SCD can be reduced if appropriate preventive measures are undertaken. Electrophoresis of Hemoglobin (EH) is the routine method for the diagnosis of SCD in most medical centers. In developing countries, capillary electrophoresis is becoming the gold standard, but this equipment is rarely used in low income countries. Standard electrophoresis is difficult to use because this method is invasive and not applicable after 06 months after birth due to large amount of fetal hemoglobin [3,4]. Thus, diagnosis of SCD at maternity ward is

Received:	12-March-2022	Manuscript No:	IPBMBJ-22-12662
Editor assigned:	15-March-2022	PreQC No:	IPBMBJ-22-12662 (PQ)
Reviewed:	29-March-2022	QC No:	IPBMBJ-22-12662
Revised:	10-October-2022	Manuscript No:	IPBMBJ-22-12662 (R)
Published:	17-October-2022	DOI:	10.36648/2471-8084.8.12.108

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Citation Stephane KTA, Gonedele-Bi ES, Adji EG, Gnonjdjui AA, Ako BA, et al. (2022) Genotyping of S and C Mutated Beta Globin Gene: Development of a Set of Primers for use with Different PCR Systems. *Biochem Mol Biol J*. 8:108.

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not conducted for babies, which hinders health care. In Ivory coast, SCD is a real problem of health and screening is not systematic after born. People are not interested in the first to be their diagnostic cause of coast generally or the non-disponible materiel in proximity hospital. The lack of equipment, and the cost of the test when available, further increases the risk for missing affected persons. Some rapid antigenic test is on validation, but cannot give reliable information when used in newborns. The routine method for the diagnostic of SCD is Electrophoresis of Hemoglobin (EH) in Cote d'Ivoire medicals centers. Molecular approaches are more reliable, robust and non-invasive as they can be conducted with all types of cells [5-9]. In regards to the advantages of the molecular techniques, several authors have used these methods to screen SCD. Here, we designed a set of primers to detect normal hemoglobin A and abnormal hemoglobin S and C which can be used both in conventional and quantitative PCR (by curves combinations analysed). These primers can be optimized for the development of rapid isothermal molecular test.

MATERIALS AND METHODS

Population, Sites of Study and Sampling

Patients were recruited at Yopougon University Hospital and Institute Pasteur Cote d'Ivoire (IPCI). After informed consent, hemoglobin status of the patients was determined using standard acetate electrophoresis. The electrophoresis of hemoglobin was realized with sebia electrophoresis kit according fabricant's protocols. For this, the sample was total blood collected in a tube with Ethylene Diamine tetraacetic

Acid (EDTA). To be including in this study, the patents will be confirmed to sickle cell disease by the Electrophoresis of hemoglobin (routine test). For molecular screening, the sample was Dried Blood Spot (DBS). Sample was prepared with 50 μ L of total blood on 5 M whatman paper followed by drying for 24 hours at room temperature. The dried spot was stored in zip locked bag contained silicate gel for conservation until use.

Molecular Procedure

DNA purification: DNA purification was realized with DNA Blood and tissues extraction kit (Qiagen) according manufacturing protocols.

Primers Design

The sequence of human chromosome 11 (accession number NC_000011.10: c5227071-5225466 in NCBI) was used as reference sequence. The software used was Serial Clone 2.6.1 and Bio Edit 7.2.5. As positive control, a forward primer without mismatch named HBU (Hemoglobin Universal: CCTCAAACAGACACCATGGTGCA (T/C) CTGACTCCTGA) was used. To amplified specific allele A, S and C, we designed specific forward primers called SSA (CCTCAAACAGACACCATGGTGATC), HBS (CCTCAAACAGAACCATGGTGCA (T/C) CTGACTCCTAT) and HBC (CCTCAAACAGACACCATGGTGCA (T/C) CTGACTCCAA). In order to make strongly the specificity of the primers for each specific allele A, S and C, supplementary mismatches were introduced for S and C sequences primers (**Table 1**).

Table 1: Sequences of primers designed.

Primer name	Sequence	Primer length (pb)	Nucleotide substitution (accession number : NC_000011.10)	Primer position (Conventional nomenclature)
HBU	CCTCAAACAGACACCA TGGTGCA(T/ C)CTGACTCCTGA	32	N/A	261 to 292
HBS	CCTCAAACAGACACCA TGGTGCA(T/ C)CTGACTCCTAT	35	G16A A17T	261 to 292
HBC	CCTCAAACAGACACCA TGGTGCA(T/ C)CTGACTCCAA	35	T15C G16A	261 to 295
SSA	CCTCAAACAGACACCA TGGTGATC	25	N/A	276 to 295
R4	GGCAGAGAGATCAG TGCCTATCAGAAACCC AAGA	35		261 to 285
R2	GGCAGAGAGATCAG TGCCTATCAGAAACCC AAGATCTT	40		459 to 493

The reverse primer R4 designed without mismatch was used as universal reverse with HBS and HBC; SSA primer was used with R3 reverse primer. All primers were used both to

conventional and quantitative PCR amplification (polymerase chain reaction).

Conventional Amplification

Conventional amplification was performed in conventional PCR machine (applied bio system 9700). For HBS and HBC primers, amplification was conducted in 20 μ l with 0.25 μ M of each primer (specific primer/R4), molecular water, 5 x Master Mix Enzyme Ready to Load 12.5 μ g MgCl₂ (Solis Bio dyne and 5 μ l of DNA template. Typing with SSA primer was performed in 20 μ l with 2.5 mM of MgCl₂, 0.25 μ M of primers (SSA/R3), 0.5 mM of DNTPs and 1 U of polymerase. For each sample, four separated amplifications were conducted with SSA, HBS, HBC and HBU primers. The Initial denaturation step was performed at 95°C during 10 min followed by 35 cycles with 96°C denaturation during 10's. Primers hybridization was conducted for 15's at 55°C, 53°C, 56°C for HBU and HBS, SSA, and HBC respectively. The elongation was conducted at 72°C during 20's; and 72°C during 7 min and 72°C during 7 min for the final elongation. PCR products were analysed on 2% agarose gel at 80 volts for 1 hour and visualized using geldoc imager (BIORAD).

Quantitative Amplification (qPCR)

Quantitative amplification (qPCR) was performed with rotor gene Q (Qiagen) in 20 μ l containing 0.25 μ M of each primer, molecular water, 5 x hotfirepol evergreen qPCR mix plus no rox (Solis Bio dyne and 5 μ l of DNA template. The primer HBU was used as a positive amplification control. The PCR process started with an initial denaturation at 95°C during 10 min followed by 40 cycles with a denaturation at 96°C during 10's. Primers hybridization was conducted for 20's at 65°C; and elongation at 72°C during 20's. Both amplification and melting curves were analysed for each sample and each target (allele A, S and C). Quality of the amplification was controlled by migration of PCR product on 2% agarose gel.

Sanger Sequencing

To confirm electrophoresis of hemoglobin and PCR typing,

Table 2: Amplifications parameters in this study compared to the gold standard test.

	Hemoglobin electrophoresis*		Parameters		
	Presence of HbA	Absence of HbA	Sensibility	Specificity	
Conventional PCR (cHBB)					
	Presence of allele A	33	19	91.67% (77.53%-98.25%)	20.83% (7.13%-42.15%)
	Absence of allele A	3	5		
	Presence of allele C	8	4	53.33% (26.59%-78.73%)	91.11% (78.78%-97.52%)
(cHBB)	Absence of allele C	7	41		
	Presence of HbS				
	Presence of allele S	38	2	86.36% (72.65%-94.83%)	87.5% (61.65%-98.45%)
	Absence of allele S	6	14		

one third of the samples were sequenced on the two DNA's strains using Sanger method (Genewiz Company). The sequencing results were analyzed using bio edit 7.2.5 software and data were submitted on NCBI for comparison with reference sequence using BLAST.

RESULTS

A total of 60 blood samples including 13 AA, 23 AS, 9 SS, 12 SC and 3 CC hemoglobin type were screened using hemoglobin electrophoresis and PCR. In this study, we considered only the presence or not of an allele. In conventional PCR, the universal control primer HBU/R4 was successfully amplified for all of 60 samples. The fragment length is 233 pb (Figure 1). 33/60 samples were amplified by the specific primer SSA/R2. The sensibility and specificity of this primer were respectively 91.67% and 20.63%. Using HBS/R4 specific primers, 38/60 of the samples were amplified. The sensibility and specificity of these primers were respectively 86.36% and 87.50% (Table 2).

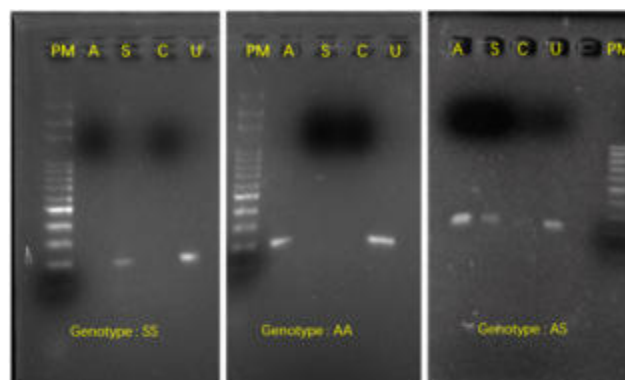


Figure 1: Amplification of the A,S and C alleles by the specific primers SSA, HBS and HBC (respectively).

**Quantitative PCR
(qHBB)**

	Presence of HbS	Absence of HbS	
Presence of allele S	40	2	90.91% (78.33%-97.47%)
Absence of allele S	4	14	87.5% (61.65%-98.45%)
	Presence of HbC	Absence of HbC	
Presence of allele C	15	0	1 (92.13%-100.00%)
Absence of allele C	0	45	(78.2%-100.0%)

*Gold standard; ()=IC 95%; cHBB=Hemoglobin conventional PCR; qHBB=Hemoglobin quantitative PCR

Relative to allele C typing, 8/60 samples were amplified by specific primer HBC/R4. The sensibility and specificity of this pair were respectively 53.33% and 91.11%. When using qPCR, all the samples (60) were amplified by the primer HBU used as a control (Figure 2). When amplification was conducted for less than 21 cycles melting curves differed were 88°C. The amplification rate was therefore 100% for the universal primer HBU as confirmed by agarose electrophoresis of PCR products. For amplification with primer HBS of samples with S allele, Ct value was less than 25 cycles and hybridization temperature was 87.5°C. 40/60 of the S containing samples was amplified (Figure 3). In the same line, amplification and melting curves of the primer HBC designed for abnormal allele C, showed Ct value less than 25 cycles and hybridization temperature at 87.3°C for the samples having at least one C allele. Amplification was 15/60 of samples with C allele (Figure 4). For S and C amplification, migration of PCR products on agarose gel confirmed that only samples with Ct less than 25 cycles harbored a true PCR band. Ct more than 25 is thus not specific. Specificity and sensitivity of primers were greater than 85%. For the primer SSA targeting allele A, the same problem occurred. Ct value below 20 cycles are observed in patients with at least one A allele, but when Ct was more than 20 cycles amplification was not specific. Specificity of the couple SSA/R3 was than very low and needs to be optimized. Analyse of Sanger sequences showed correlation between electrophoresis results and phenotypical results.

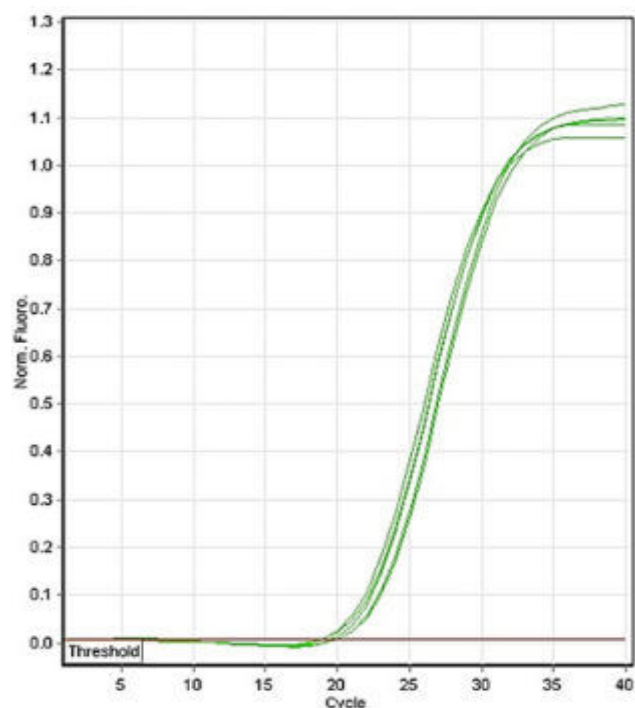


Figure 2: Amplification curve with HBU.

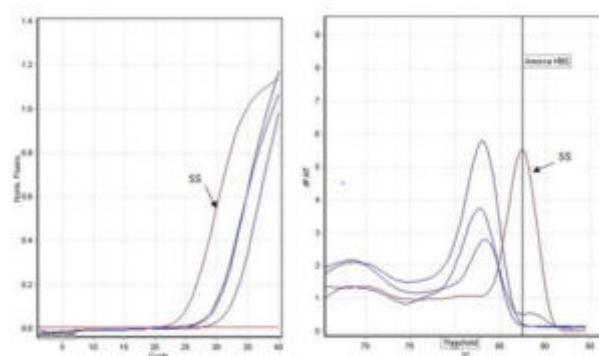


Figure 3: Amplification plot with HBS.

At left, amplification curves. Before 25 cycles, we have the Ct of the positive sample (SS); after 25 cycles we have sample without allele S. At right, we have melt curves. Sample with allele S have a melt at 87.3°C and samples without allele S have different melt temperatures.

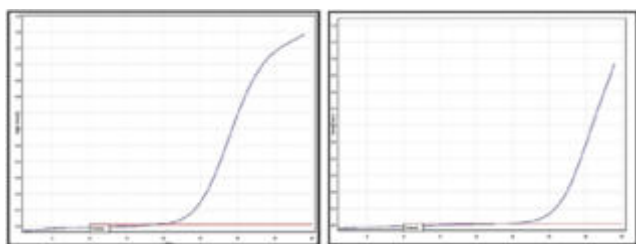


Figure 4: Amplification plot with HBC.

At left, amplification curve of positive sample (CC). The threshold is before 25 cycles, At right, amplification curve of sample without allele C. The threshold is after 25 cycles.

DISCUSSION

Primers design and detection of SNPs located on the same codon

The aim of this study was to develop a unique molecular primer set to be use in all PCR systems. We optimized SCD molecular typing protocols from the designed primers. The major difficulty was that the mutations leading to S and C genotypes were located in the same codon. Conventional PCR amplification can detect alleles S and C with high sensitivity and specificity. However difficulties rose from the specificity of primer SSA (for the typing of allele A) as it amplified all hemoglobin allele types. That difficulty is probably caused by a small number of mismatches between normal and abnormal alleles which can be corrected by DNA polymerase during amplification process. In order to increase the specificity of primers by keeping mismatches, it could be better to use enzymes of low fidelity which will not be able to correct these mismatches. In the same line using master mix cannot allow to decrease $MgCl_2$

to increase specificity. To increase specificity of the primers, mismatches were inserted in the sequences to decrease stability of hybridization on the target DNA. Several authors have tested the influence of mismatches at specific positions on the primer on amplification and according as well as position of the mutations in a primer sequence could negatively influence gene amplification [10-12]. They concluded that SNPs at the 5' end of the primer and in middle of the sequence have very little influence on the quality of the amplification while mutations at the 3' end negatively affected amplification or even inhibit it. Mutations introduced at the 3' end and another ten bases forward of the end were the most effective. In qPCR, in order to determine the genotypes of individuals for the S and C alleles, it was possible to encounter both amplification curve and melting curve. Therefore, a melting step should be included when editing the amplification program of amplification. However, only samples with Ct value less than 25 cycles must be considered. Although primer designs vary between amplification methods [13–15], we have shown here that the same set of primers can be used for several PCR amplification methods. Results of the two methods were consistent with each other and with hemoglobin electrophoresis.

Phenotypic versus genotypic technics, what to prefer?

Several phenotypic methods can be used as an alternative of electrophoresis of hemoglobin used tandem Mass Spectrometry (MS/MS) for the typing of sickle cell disease in newborns, whereas concluded that HPLC typing was the most efficient method for this typing. However, mass spectrometry remains limited by a relatively low sensitivity [16]. The HPLC method needs technical platforms that are not usually available in routine laboratories. Several studies have also focused on the molecular typing of abnormal hemoglobin used real time PCR (FRET) to detect foetal blood contamination by the maternal blood to assign the right genotype of the child used the same method for retrospective typing of SCD in population with malaria in Cote d'Ivoire. Although effective, that technique requires advanced equipment and a qualified technician. In opposite, devices for classical PCR are now available in most of clinical laboratory and have used classical PCR only for alleles A and S typing. The comparison of our conventional PCR and qPCR results indicated no difference between the sensitivity and the specificity of allele S typing (*Chi-square* test, $p=0.9952$); but difference existed for the sensitivity and specificity for allele C typing (*Chi-square* test, $p=0.01455$). For this abnormal allele (allele C), qPCR typing present good results. These results indicate that our primers set are suitable for molecular typing of SCD.

CONCLUSION

We designed a set of primers for conventional and quantitative amplification. These primers correctly discriminates S and C alleles in conventional PCR and qPCR. We recommend the use of HBS and HBC primers for the discrimination of allele S and allele C. HBU primer can be used as amplification control primer. The next step will be to develop a point of care molecular test that could be perform at room temperature and read optically. Based on these first results, a RPA test is already in design to reach this goal.

CONFLICT OF INTEREST

There are no conflicts of interest

ACKNOWLEDGEMENTS

We thank people and institutions who contributed to the realisation of this work.

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