Characterization of molecular hemoglobin C and beta thalassemia

M.H. Lagares[1,2,3], M.S. Rabelo[1,2,3], C.G.B. Dias-penna[1,2,3], R.S. Mascarenhas[1,2], K.S.F. Silva[2,3] and K.K.V.O. Moura[1,2,3]

[1]Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil

[2]Núcleo de Pesquisa Replicon, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil

[3]Departamento de Biomedicina, Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil

Corresponding author: K.S.F. e Silva

E-mail: smallbinho@hotmail.com

Genet. Mol. Res. 16 (4): gmr16039851

Received October 14, 2017

Accepted November 08, 2017

Published December 15, 2017

DOI http://dx.doi.org/10.4238/gmr16039851

ABSTRACT. The hemoglobinopathies are a group of hereditary alterations prevalent in many parts of the world, but significantly affect the Brazilian population for its abundant miscegenation. These alterations in structural genes that cause the formation of hemoglobin variants, and/or regulatory genes, causing thalassemias. Currently, the number of identified abnormal hemoglobin has increased due to improvement in methods of analysis, however, many routine laboratories are not prepared for the correct identification of these changes. In the present study aimed to assess the prevalence of hemoglobinopathies by classical methods and perform the molecular characterization of mutations S, C, beta thalassemia IVS-110, IVS-1, IVS-6 and CD-39 by gene amplification using the PCR technique – AE. The molecular study used specific primers that bind promptly at the position of the mutated allele in position and the normal allele, and thus can perform gene allele specific amplification. We collected 200 samples of peripheral blood of patients in clinical laboratories at PUC-Goiás from July/2012 to December 2012. We observed two patients (1%) AC one (0.5%) AS, two (1%) with IVS-6 mutation and one (0.5%) IVS-6. The codon 39 and IVS-110 were not detected in any of the investigated patients. We concluded that our molecular methodology in the characterization of hemoglobin mutations is useful and it can be used to identify hemoglobinopathies.

Key words: Hemoglobinopathies; Thalassemias; PCR.

INTRODUCTION

The hemoglobinopathies are a type of recessive hereditary disorders consisting of thalassemia and hemoglobin (HB) variants. Mutations occur in specific regions are often caused by ethnic and geographic distributions, justifying control programs of these mutations and genetic counselling. Mutations can be diagnosed by several methods, but the method of choice involves care methodology applied and the population group to be studied. The Brazilian population is characterized by having wide genetic heterogeneity, reflecting the polymorphism of hemoglobin (Chinelato-Fernandes and Bonini-Domingos, 2005; Alexandre and Marine, 2013). The hemoglobinopathies may reflect mutations affecting regulatory genes, causing an imbalance in the quantitative content of polypeptide chains, causing thalassemias, or even changes involving structural genes which cause the formation of Hb molecules with different characteristics also called HB variants (Ondei et al., 2005) (Manual do Ministério da Saúde de Brasília 2016).

The number of Hb variants described in the literature has increased significantly. We have been catalogued 1.194 Hb variants in Hemoglobin Variant Data Base (access 09/24/2014). Most of them are caused by amino acid substitutions resulting from changes in the sequences of nucleotides (Chinelato-Fernandes; Bonini-Domingos, 2006). The World Health Organization (WHO) estimates that 5% of the population is in possession of some type of inherited anemia. The diagnosis of these variants has been carried out by various methods, but the laboratory experience makes clear the lack of sufficient resolution for the correct characterization, changes confirmation and the establishment of a follow-up protocol of patients in the methodologies most used for routine monitoring. The introduction of molecular approach can contribute to safe and correct manner, characterizing the type of mutation responsible for the disease. Screening tests alone are not specific to any hemoglobinopathies, but together, they can help in the diagnosis and drive subsequent procedures, assisting in the understanding of the pathophysiological processes related to the genetic disorder in question (Chinelato-Fernandes and Bonini-Domingos 2006; Giovelli et al., 2011).

MATERIAL AND METHODS

Sample collection

Two hundred blood samples with EDTA were collected after informed consent from patients in clinical laboratories of the Catholic University of Goiás, regardless of sex, over 18 years old, ethnicity or social class. This work is in accordance with the guidelines of Resolution 196/96 of the National Health Council/MS and with a favorable opinion of the Ethics Committee of the Catholic University of Goiás, under protocol number 76165.
From erythrogram data (MCV, MCH, MCHC and RDW) and HPLC performed with 200 samples were selected 44 samples showed abnormalities as reference values (Failace, 2003) for investigation and analysis specific allele gene amplification (PCR-AE), but 2 samples showed no amplification. The samples of DNA extraction and molecular analysis of mutations were carried out in the Replicon Laboratory of the Catholic University of Goiás.
They were adopted as inclusion criteria patients with exams sent to the Clinical Laboratory at PUC-Goiás, 18 years old and both sexes.

The samples were encoded to preserve the identity of individuals, and subjected to specific tests for the determination of hemoglobin profile. The evaluation of haematological parameters was performed by automated procedures, following the quality control standards. The evaluated haematological parameters were Hematocrit (Ht), hemoglobin (Hb), total count of red blood cells (RBC) mean corpuscular volume (VCM), men corpuscular hemoglobin (MCH), concentration, mean corpuscular hemoglobin (MCHC) and anisocytosis index (RDW) at the automated equipment Sysmex XE (Siemens®) Sysmex Corporation, Kope, Japão.

The red cell morphology was observed in the blood smear by a simple optical microscope after staining dye fast panótipo (Newprov®). The size, color and variations of erythrocyte forms were carefully analyzed as morphological reference Failace (2003). Then the samples were analyzed by high performance liquid chromatography (HPLC) Variant II Automated System from Bio-Rad® assay kit with beta thalassemia heterozygous (Beta-Short-Thal) (Bio-Rad, 2014). Of the 200 samples analyzed 42 were subjected to molecular analysis that began with the extraction and quantification of genomic DNA.

Extraction and quantification of genomic DNA

DNA extraction of 44 peripheral blood samples was carried out according to Kaswi® (*Genomic DNA Purification Kit*) instructions. Therefore, were subjected to the quantification NanoVue ™ Plus spectrophotometer (GE, Cambridge, UK) according to the manufacturer's instructions, taking only the most relevant result of the sample whose quantization with respect to DNA concentration was higher than a 5ng/µl.

Genomic DNA amplification

Amplification of the genetic material to identify the genotype of hemoglobin S, hemoglobin C and four beta thalassemia mutations codon 39, IVS I-110, IVS I-6 and IVS I-1 was carried out by gene amplification technique allele specific (PCR-AE) according to Bertholo & Moreira protocols (2006). Amplification reactions were performed in a thermocycler Mini Cycler from MJ Research TM, according to the algorithm established for the equipamentoe in dependence DNA fragment to be amplified and the Tm of primers used.

Identification of genotypes AA, AS, SS, AC, DC, and SC

In determining genotypic 3 tubes used for each DNA sample for a final volume of 100 uL reaction by adding 20 mmol/L of buffer, 0.2 mmol/L of each dNTP; 1.5 mmol/L $MgCl_2$; 1.25 U of Taq DNA polymerase (Promega ™ GoTaq ™ Flexi DNA Polymerase); 50.0 100.0 ng genomic DNA and 0.2 mmol/L each of primer, the tube 1 being added to the primers 5'-Beta, Beta, Beta 5'-Ab-b; on the tube 2 the primers 5'- Beta, Beta, Beta 5' Cb and tube 3 the primers 5'-Beta,Beta, Beta 5'-be Sd. The amplification process was carried out by a prior denaturation 95°C for 5 min, increased by 35 cycles of 94 for 1 min, 55°C 1 min, 65°C for 2 min followed by a final extension at 65 for 7 min. Analysis of the products was checked on agarose gel 2% (Bertholo & Moreira, 2006 Adapted - final extension temperature adjusted from 5 to 7 min).

Identification of mutation IVS I-6 and IVS I-110

For mutation IVS I-6 and IVS I-110 was used 2 tubes for each DNA sample. For IVS I-6 AE PCR reaction was performed in a final volume of 100 uL of reaction: 10 mmol/L of buffer, 0.2 mmol/L each dNTP, 1.5 mmol/L $MgCl_2$; 1.25 U of Taq polymerase; 100.0 50.0 ng of the genomic DNA and 0.2 mmol/L of each primer, and the tube 1 pCO1 added primers, and PCO9 IVSI6W tube 2 and the pCO1 primers PCO9 and IVSI6M. The amplification process was carried out by a prior denaturation 95°C for 7 min increased by 32 cycles of 94 for 50 seconds, 54°C for 50 seconds, 72°C for 50 seconds, and a final extension at 72°C for 7 min. The LA-PCR reaction for IVS I-110 was performed under the same conditions as above using the primers pCO1, and PCO9 TB110W the tube 1 and pCO1, PCO9 TB110M and the second tube; the amplification process the hybridisation temperature was 55°C, performing 30 cycles. Analysis of the products was checked on agarose gel 2% (Bertholo & Moreira, 2006).

Identification of mutations IVS I-1 and CD 39

For mutation IVS I-1 and CD 39 was used 2 tubes for each DNA sample. For CD39, the LA-PCR reaction was performed in a final volume of 100 uL of reaction: 10 mmol/L of buffer, 0.2 mmol/L of each dNTP; 1.5 mmol/L $MgCl_2$, 1.25 U of Go Taq DNA polymerase (Promega); 100.0 to 50.0 ng of genomic DNA and 0.2 mmol/L each primer, and the tube 1 pCO1 primers PCO9 PS39W tube 2 and the pCO1 primers PCO9 and PS39M. The amplification process was carried out by a prior denaturation 95°C for 7 min increased by 32 cycles of 94 for 50 seconds, 54°C for 50 seconds, 72°C for 50 seconds, and a final extension at 72°C for 7 min. The reaction PCR-AE for to IVS I-1 was performed under the same conditions up with minor changes: use of pCO1 primers, PCO9 and IVSI1W the tube 1 and pCO1, PCO9 IVSI1M the second tube; the amplification process the hybridisation temperature was also 54°C, performing 35 cycles. Analysis of the products was verified on agarose gel 2% (Bertholo & Moreira, 2006). In the 200 samples analyzed 16.5% (33/200) showed abnormalities in erythrocyte in both sexes, and RBC, Hb, HT and MCV (Figure 1) when compared with reference values (Table 1) for the relevant gender.

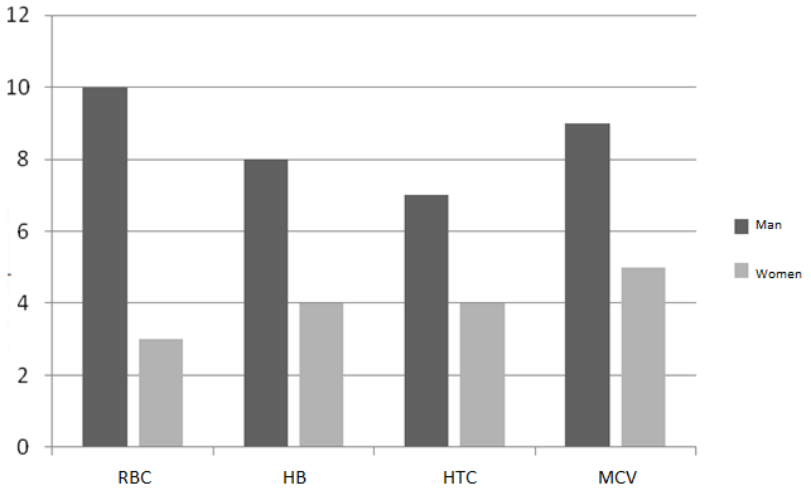

Figure 1. Changes in erythrocyte. Number of patients by gender to change s parameters in erythrocytes (RBC), hemoglobin (Hb), Hematocrit (Ht) and mean corpuscular volume (MCV).

When analyzed the parameter RBC were found 5% (10/200) patients in the reference value. For parameter Hb found 4% (8/200) patients out of the reference value. When we analyzed the Ht parameter were found 3.5% (7/200) of the patients in the reference value. For parameter MCV were found 4.5% (9/200) of the patients in the reference value. By means of HPLC were identified with a sample hemoglobinic profile AS (Figure 2), presenting 64.5% of Hb S, 19.6% Hb F and 7.0% of A2. 02 samples with hemoglobinic profile AC, 22 samples with increased Hb A2 and 10 with increasing fetal Hb (HbF), suggesting beta thalassemia (Figure 3).

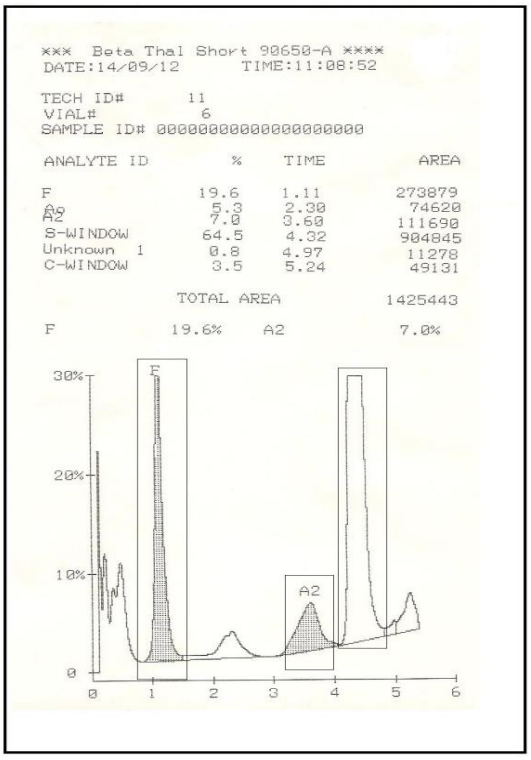

Figure 2. Chromatographic profile observed by HPLC with Variant Bio-rad equipment, and diagnostic kit for beta thalassemia, showing 64.5% Hb S, 19.6% Hb F and 7.0% of Hb A2.

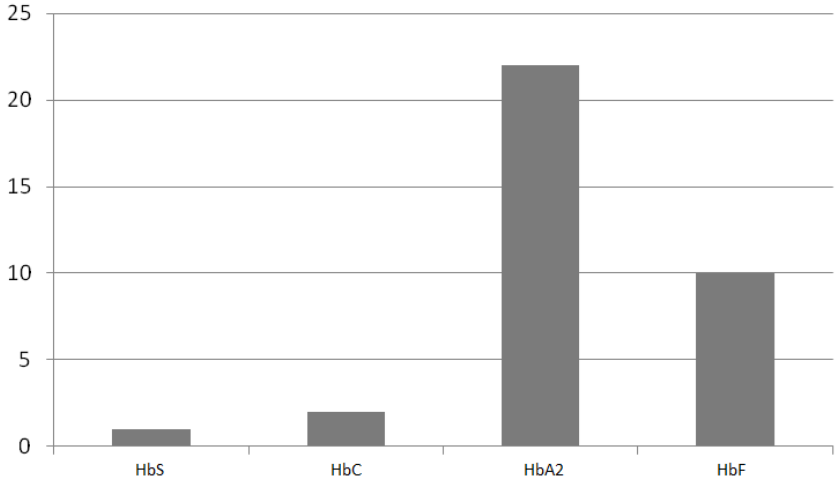

Figure 3. Number of patients with hemoglobin S, C or increasing the concentration of Hb A2 and HbF. Source: Rabelo, 2014.

The total of 42 samples were subjected to molecular analysis to determine the genotype AS, SS and AC and analysis of IVS I-1, IVS I-6, IVS I-110 and CD 39, beta thalassemia mutations. For the identification of genotypes AA, AS, SS, AC, CC and SC was observed as an amplification product, a band of 660 bp characterizing the validity and effectiveness of the reaction, representing the positive control of the same and should be present in 3 tubes while a 216 bp band appeared depending on the primer used, S (Figure 4) and C (Figure 5), allowing the characterization of the different genotypes.

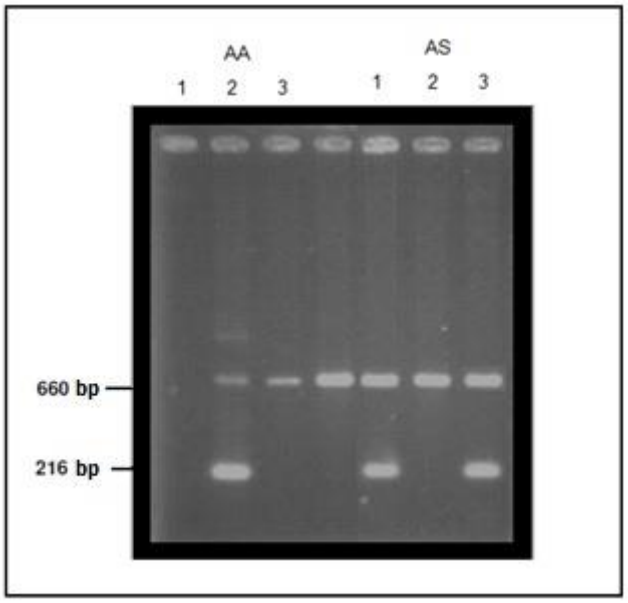

Figure 4. Allele specific PCR for identification of AA and AS genotypes. Amplification products observed by electrophoresis in 2% agarose gel in TBE 1X buffer pH 8.5, showing bands of 660 and 216 bp depending on the genotype analysis. Source: Rabelo, 2014.

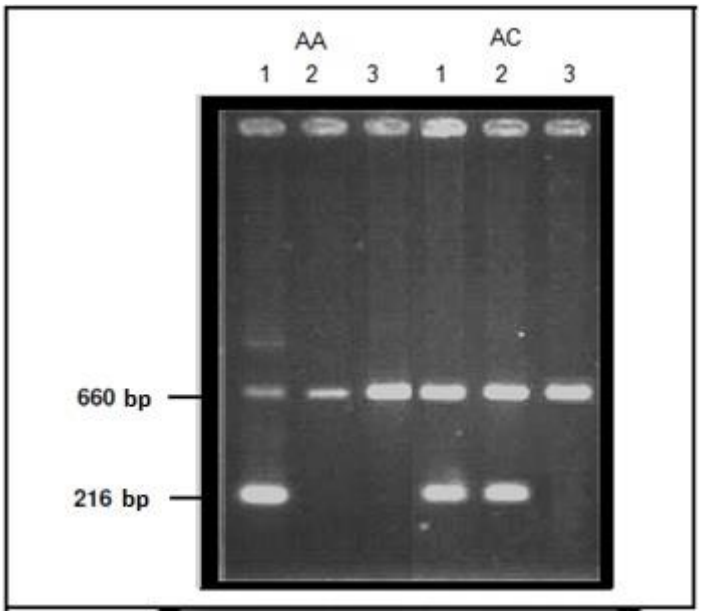

Figure 5. PCR-allele specific to identity AA and AC genotypes. Amplification products observed by electrophoresis in 2% agarose gel in TBE 1X buffer pH 8.5, showing bands of 660 and 216 bp depending on the genotype analysis. Source: Rabelo, 2014.

To determine the genotype of beta thalassemia, was observed as an amplification product, a band of 687 bp as the positive control of reaction, while a band 331pb to IVS I-6 Genotype (Figure 6) and 326 bp for IVS I-1. The IVS I-110 and CD 39 mutations were not seen in the sample group.

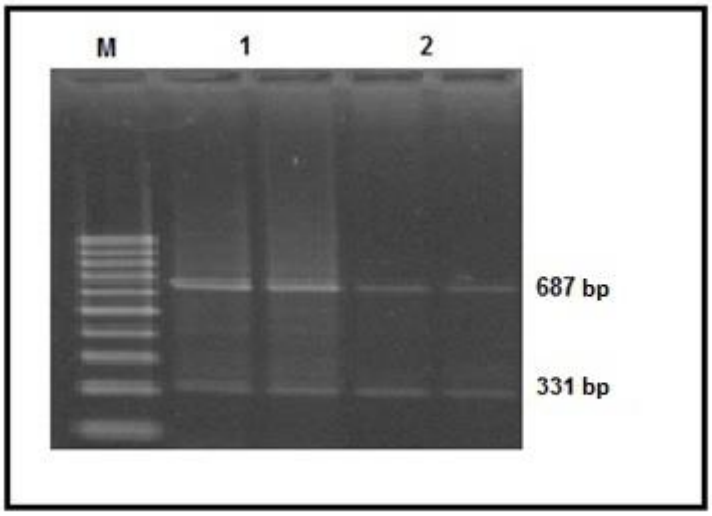

Figure 6. Allele specific PCR for identification of IVS I-6 genotypes. Amplification products observed by electrophoresis in 2.0% agarose gel TBE 1X buffer pH 8.5, showing bands of 687 and 331 bp depending on the genotype analysis. Source: Rabelo, 2014.

In all reactions polymerase chain specific allele carried out to identify the of hemoglobinopathies this study, internal control was being successfully amplified. Forty-two AE-PCR was performed, and it was delected one AS genotype two AC genotype, one IVS-1 mutation and two IVSI-6 mutation. Three ethnic groups were observed in the study, 33.5% (67/200) self-classified as mulato, 18.5% (37/200) as black and 48% (96/200) as white (Figure 7).

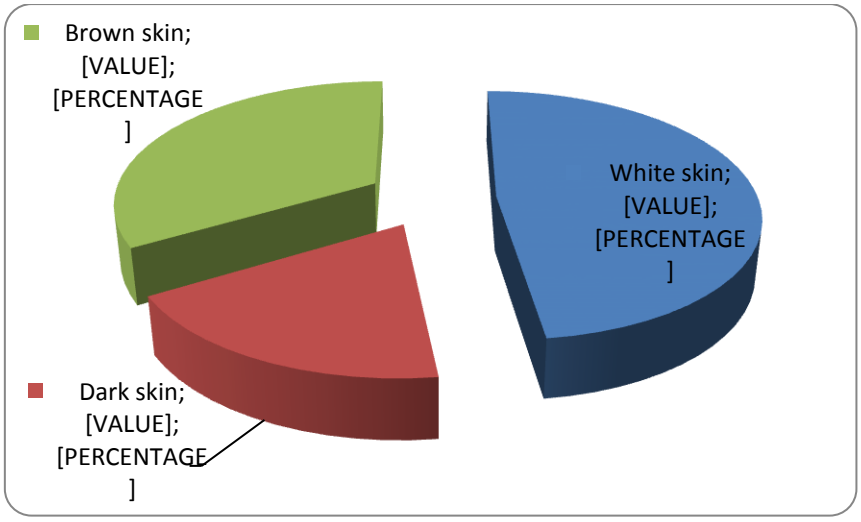

Figure 7. Percentage of individuals in ethnic groups.

Considering the age range of the patients was observed patients with at least 18 years old and maximum 89 years old and average age of 45.9 years old.

Table 1. Age range of patients

Age Range	N. of patients
	N %
18 - 27 years	35(18%)
28 - 37 years	51(26%)
38 - 47 years	31(16%)
48 - 57 years	28(14%)
58 - 67 years	30(15%)
68 - 77 years	13(6%)
78 - 89 years	12(5%)
Total	200 (100%)

The prevalence of individuals with heterozygous profile for Hb S in the study population was 0.5% (1/200) for heterozygous profile of Hb C were observed 1% (2/200). The prevalence of patients with beta thalassemia was 0.5% (1/200) for IVS I-1 mutation and and 1% (2/200) for IVS I-6 mutation. The presence of Hb S was observed in ethnic black patient, as the presence Hb C was observed in brown colored patients. As for beta thalassemia was observed 67% (2/3) with Caucasian and 33% (1/3) with brown ethnicity.

DISCUSSION

The set of laboratory results, both the classical methods, the molecular contribute to the knowledge of hemoglobin in Brazil, elucidating interactions and assisting in genetic and educational counselling (Naoum PC and Bonini-Domingos, 2007). According to the study Chinelato-Fernandes and Bonini-Domingos (2005) in July 2000 to February 2003 were analyzed 220 blood samples from individuals suspected of carrying hemoglobin variants, from eight states of Brazil. Of this total, 98 samples showed a hemoglobinic fraction with migration pattern like Hb S in alkaline electrophoresis in cellulose acetate. In this study, Hb was not observed with similar migration pattern of Hb S, whereas did not use the methodology electrophoresis on cellulose acetate.

Population studies allow the diagnosis of heterozygotes and genetic counselling providing support for individuals to decide consciously about their offspring, as well as improving the quality of life of patients (Orlando GM, 2000). In this context it is possible to carry out preventive studies of hemoglobinopathies, especially in areas where the incidence of such changes achieved remarkable frequencies to public health, focusing on the best of the population and the type of hemoglobinopathy that affects. The development of prevention programs requires the support of official health agencies, skilled personnel training for diagnosis, genetic and clinical counselling of patients and risk couples (World Health Organization, 1989; Orlando GM, 2000).Kessler (1989), reviewing the subsequent literature to 1979 about the educational and reproductive aspects of genetic counselling programs found that, despite the methodological differences, these programs

usually reach their goals with regard to educational aspects, information on the diagnosis and genetic risk. Now about reproductive decisions after genetic counselling, they do not show a similar efficiency.

According to the World Health Organization (WHO), 270 million people on every continent carry genes that determine the presence of abnormal hemoglobin (Sanctis et al., 2017). As a measure to prevention and control these diseases, the Ministry of Health included, in 2001, testing for hemoglobinopathies in the National Neonatal Screening Program (PNTN). Since then, from the diagnosis of children, surveys to identify this pathology have been carried out in the country, which found such genetic abnormalities.

The World Health Organization recommends the implementation of programs for prevention and control hemoglobinopathies in Latin America, especially in Brazil (Thomas C and Lothar T, 2002). The organization of a prevention program requires support of official health agencies, skilled personnel training for diagnosis, genetic counselling and clinical patients (Orlando GM, 2000). The success of these programs depends on the responsiveness, availability and interest of the population being studied (Zamaro et al., 2002). The detection of individuals with the imperceptible forms of hemoglobinopathies, heterozygotes, are extremely important for public health because, in addition to representing a source of new heterozygotes, can, through marriages between carriers, give homozygous and double heterozygous individuals, for example, SC of hemoglobin (Hb SC), which manifest a clinical form (Orlando GM, 2000). Thus, we emphasize that the fundamentals of preventive hemoglobinopathies program comprises the dissemination of information to the public, recognition of heterozygotes, neonatal diagnosis and genetic counselling. This includes enlightenment activities bearer of change clearly shall be carried out by specialist or geneticist with specific educational information (Zamaro et al., 2002).

It is possible to infer that the molecular diagnosis of hemoglobin variants and thalassemia should be included in routine laboratory as complementary and necessary examination in the identification of these genetic disorders in Brazil since the Brazilian population has genes for abnormal hemoglobin with variable frequencies and influenced by their groups racial trainers. Therefore, the detection of carriers of these genetic alterations is of importance to public health, since they represent sources of new heterozygotes and homozygotes possible (Viana-Baracioli et al., 2001).

The hemoglobin S and D have similar pattern on electrophoresis gel, leaving inaccurate diagnosis. But the treatment for both is the same, with no significant difference in the conduct of clinical (Zamaro et al., 2002).
Published data show that giagnosis, especially at birth, and appropriate treatment, dramatically improve the survival rate and quality of life of patients. In sickle cell anemia the simples heme of vaccination against pneumococcus and Haemophilus influenza, accompanied by prophylactic penicillin terapy decreases the number of deaths in the critical period, situated between six months and three years old. However, the diagnosis is difficult in some methodologies used, such as electrophoretic migration in alkaline pH, o it has similarities with other hemoglobin, and additional studies for its proper characterization are needed. In patients with Hb S the Hb A2 values increased because when measured by HPLC of the HbS changed raise the perk A2 (Gulbis B and Vertongen MVF, 1999), this makes Hb A2 dosage unfeasible by this method, becoming required quantification by methodology.

The control of hemoglobinopathies has been possible through genetic counselling and early diagnosis. Clinical follow of homozygotes, heterozygotes of clarification and in particularly of risk couples may help to prevent the birth of children with a genetic disorder, often lethal (Viana-Baracioli et al., 2001). PCR is also a versatile technique, because as modifications in the reaction conditions (primers, temperature, buffers) can be amplified and various DNA sequences thus identifying the different genes, with or without the mutation. These attributes have led to the development of a wide variety of methods based on PCR, both for research and for routine clinical diagnosis (Zamaro et al., 2003). The investigation of genotypes A, S, C and mutations for beta thalassemia were possible in this work from the use of specific primers and other PCR constituents under specific conditions and temperature cycling.

On identification by molecular methods several authors report the use of allele-specific primers and PCR followed by restriction enzyme for verifying changes in hemoglobin molecule S and C, and interactions (Bertholo LC and Moreira HW, 2006). In this work we used the chain reaction allele-specific polymerase (PCR-AE), allowing direct detection of the normal allele or mutant in genomic DNA, without additional evidence of hybridization, binding or cleavage with restriction enzyme or the use of radioactivity, still ensuring a lower operational cost. By PCR-AE was possible to distinguish the mutant alleles S and C heterozygous as well as differentiate them from the normal allele. It has been estimated that approximately 7% of all people in the world are suffering from beta thalassemia, and to 300,000 to 500,000 children are born each year with the severe form

of the disease (Premawardhena et al., 2001). In Brazil, beta thalassemia has been studied, however, because of limitations such as lack of standardization of laboratory diagnostic methodologies; the large sample size, ethnic diversity, and the high degree of racial mixture of country, data on the actual prevalence of beta thalassemia are still scarce and fragmented. In the Brazilian population we find four type of hemoglobin mutations. The codon represents them 39, IVS I-1, IVS I-6 and IVS I-110. The first two being the type β - and the other two, the type β + (MARTINS, 2010)

CONCLUSION

In the study by Freitas et al., (2010) mutations IVS I-6, CD-39 IVS I-1 and beta-thalassemia have been characterized in 68,2% of cases of thalassemia. The most frequent mutation was IVS I-6 which was present in 14 of 44 alleles studied, i.e., 45.5%, then CD 39 (18.2%) and IVS I-1 (4.5%). Researchers have shown in Brazil, different profiles of mutations for beta thalassemia most often IVS I-110, CD 39 in the South (Reichert, 2008), IVS I-6 in the northeast (Araújo KI et al., 2007), IVS I-110 and CD 39 in the south-eastern region of the country (Fonseca SF et al., 1998).

Reichert et al. (2008) show a significant difference in VCM variables, MCH, MCHC and Hb A2 among patients with IVS I-6 mutation for other mutations (IVS I-1, IVS I-110 and codon 39). Bertuzzo CS and Sonati MF (1997), demonstrate that high levels of HCM and Hb A2 can be associated with the presence of mild mutations thalassemia as in the case of heterozygotes for IVS I-6 when compared to heterozygotes for CD 39 and IVS mutations I-1. Prevention programs collaborate to raise awareness of heterozygous providing information, so they can decide responsibly on the future of his descendants; favours the clinical direction in mild cases and indicates the appropriate follow-up to the holders of severe forms of the disease. Appropriate technology for diagnosis, using various selective laboratory tests, and confirmatory clinical data and familial study, as well as the training of skilled personnel to the correct laboratory diagnosis is important (Orlando et al., 2000).

REFERENCES

A Database of Human Hemoglobin Variantes And Thalassemias [Internet]. Editado em: 05/09/2014. Available on: HbVar, http://globin.cse.psu.edu/globin/hbvar/

Alexandre JM and Marini Dc. Conhecendo e tratando as hemoglobinopatias: anemia falciforme e beta-talassemia. FOCO - Ano 4 - Nº 5 - Julho/Dezembro 2013.

Araújo KI, Cappellini MD, Rachmilewitz EA (2007). Beta-thalassaemia and sickle cell anaemia as paradigms of hypercoagulability. Br. J. Haematol. 139: 3-13.

Bertholo LC, Moreira HW (2006). Amplificação gênica alelo-específica na caracterização das hemoglobinas S, C e D e as interações entre elas e talassemias beta.J. Bras. Patol. Med. Lab. 42:4. https://doi.org/10.1590/s1676-24442006000400004

Bertuzzo CS, Sonati MF, Costa FF (1997). Hematological phenotype and the type of beta thalassemia mutation in Brazil. Braz. J. Gen. 2:319-321. https://doi.org/10.1590/s0100-84551997000200024

Bio-Rad Laboratories. Disponível em: www.bio-rad.com/diagnostics [Acesso em: 29/09/2014]

Chinelato-Fernandes A R, Bonini-Domingos C R (2005). Contribuição do estudo molecular de hemoglobinas S-like para o conhecimento da diversidade genética da população brasileira. Rev. Bras. Hematol. Hemoter. 3: 208-210. https://doi.org/10.1590/s1516-84842005000300014

Chinelato-Fernandes AR, Bonini-Domingos CR (2006). Metodologias laboratoriais para o diagnóstico de hemoglobinas variantes. São José do Rio Preto. Rev. Bras. Hematol. Hemoter. 28:1. https://doi.org/10.1590/s1516-84842006000100015

Failace R (2003). Manual De Hematologia. Ed. ArtMed, (4thedn).

Fonseca SF (1998). Genetic analysis of beta-thalassemia major and beta-thalassemia intermediate in Brazil. Hemoglobin. 22: 197-207.

Freitas MF, Goncalves RP (2010). Caracterização clínica, hematológica e molecular dos adultos com beta talassemia no Ceará. Dissertação (Mestrado). Fortaleza (CE): Universidade Federal do Ceará, Departamento de Pós-Graduação em Patologia.

Giovelli LL, Karina D, Bortolotto AN Mastella AK (2011). Estudo comparativo entre metodologias de triagem para detecção de hemoglobina S em bancos de sangue. J Bras Patol Med Lab. 47:137-140. https://doi.org/10.1590/s1676-24442011000200007

Gulbis B, Vertongen VMF (1999). Interference of Hemoglobin D in Hemoglobin A_2 Measurement by Cation-Exchange HPLC. Clinical Chemistry. 8: 1317-1318.

Kessler S (1989). Pyschological aspects of genetic conseling: a critical review of literature dealing with education and reproduction. Am J Med Genet. 34: 340-53. https://doi.org/10.1002/ajmg.1320340310

Manual técnico sobre triagem neonatal biológica". Brasília – DF 2016. http://bvsms.saude.gov.br/bvs/publicacoes/triagem_neonatal_biologica_manual_tecnico

Naoum PC, Bonini-Domingos CR (2007). Dificuldades no diagnóstico laboratorial das hemoglobinopatias. Rev. Bras. Hematol. Hemoter. 3:226-228. https://doi.org/10.1590/s1516-84842007000300007

Naoum PC (1997). Hemoglobinopatias e talassemias. São Paulo. Ed. Sarvier. 171.

Ondei LS, Zamaro PJA, Bonini-Domingos CR (2005). A importância do diagnóstico laboratorial clássico na identificação de variantes de hemoglobinas. São José do Rio Preto. Rev. Bras. Hematol. Hemoter. 27:1. https://doi.org/10.1590/s1516-84842005000100016

Orlando GM, Naoum PC, Siqueira FAM, Bonini-Domingos CR (2000). Diagnóstico laboratorial de hemoglobinopatias em populações diferenciadas. São José do Rio Preto. Rev. Bras. Hematol. Hemoter. 22: 2.

Premawardhena A, Fisher CA, Fathiu F, Silva S (2001). Genetic determinants of Jaundice and Gallstones in Haemoglobin E Beta Thalassemia. Lancet. 357: 1945-1946. https://doi.org/10.1016/s0140-6736(00)05082-0

Reichert VCD, Castro SM, Wagner, SC Albuquerque DM (2008). Identification of beta thalassemia mutatios in Shouth Brazilians. Ann. Hematol. 5: 381-384.

Sanctis V, Kattamis C, Canatan D et al. (2017). β-Thalassemia Distribution in the Old World: an Ancient Disease Seen from a Historical Standpoint. Mediterr J Hematol Infect Dis. 9; e 2017018. https://doi.org/10.4084/mjhid.2017.018

Thomas C, Lothar T (2002). Biochemical Markers and Hematologic Indices in the Diagnosis of Functional Iron Deficiency. Clinical Chemistry. 7:1066-1076. https://doi.org/10.2298/jmh0403235t

Viana-Baracioli LMS, Bonini-Domingos CR, Pagliusi RA, Naoum PC (2001). Prevenção de hemoglobinopatias a partir do estudo em gestantes. Rev bras Hematol hemoter. 23,1: 31-39. https://doi.org/10.1590/s1516-84842001000100005

World Health Organization. In: Annual Meeting of the WHO, Cagliari. Report: working group on the feasibility study on hereditary disease community control programes (hereditary anaemies), 1989.

World Health Organization. Sickle cell anaemia. Report by the secretariat, Executive Board 117 th session. 2005; 5f.

Zamaro Pja, Canalli AA, Júnior WAS,Domingos CRB (2002). Diagnóstico laboratorial de hemoglobinas semelhantes à HbS. Rio de Janeiro. Jornal Brasileiro de Patologia e Medicina Laboratorial. 38: 4. https://doi.org/10.1590/s1676-24442002000400003

Zamaro PJA, Hidalgo CA, Bonini-Domingos CR (2003). Análise quantitativa e molecular de hemoglobina fetal em indivíduos da população brasileira, Rev. Bras. Hematol. Hemoter. 4:223-229.

Zamaro PJA (2002). Análise quantitativa e molecular de hemoglobina fetal em doadores de sangue. Rev Bras Hematol. Hemoter. 4:312-313. https://doi.org/10.1590/s1516-84842002000400012