Molecular analysis of the *GYPB* gene to infer S, s, and U phenotypes in an admixed population of Minas Gerais, Brazil

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Objective: *To implement genotyping for S, s and U antigens of the MNS blood group system at the Fundação Hemominas and to evaluate the occurrence of GYPB gene polymorphisms associated with the U- and U+var phenotypes and deletion of the GYPB gene for the first time in an admixed population of Minas Gerais, Brazil. The S, s and U antigens can cause transfusion reactions and perinatal hemolytic disease. Genotyping is a useful tool in immunohematology, especially when phenotyping cannot be performed.*

Methods: *Ninety-six samples from blood donors and patients with sickle cell disease previously phenotyped for the S, s and U antigens were selected. Allele-specific primer polymerase chain reaction (ASP-PCR) and polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) assays were employed to identify the GYPB*S and GYPB*s alleles and the GYPB(P2) and GYPB(NY) variants, as well as deletion of the GYPB gene.*

Results: *The results of allele-specific genotyping (GYPB*S and GYPB*s) were totally in agreement with the phenotyping of S+ (n = 56), s+ (n = 60) and s- (n = 35) samples. However, the GYPB*S allele, in association with the GYPB(P2) variant, was detected in 17.5% of the S- samples (n = 40), which shows the importance of assessing this variant in the Brazilian population. Of the S-s- samples (n = 10), 60% had the deletion of the GYPB gene and 40% were homozygous or hemizygous for the GYPB(P2) variant.*

Conclusion: *Genotyping was an effective strategy to infer the S, s, and U phenotypes in the admixed population from Minas Gerais (Brazil) and may contribute to transfusion safety.*

Keywords: *MNSs blood-group system; Molecular biology; African continental ancestry group; Brazil*

Introduction

The MNS is a highly complex immune blood group system which comprises 46 known antigens[1]. The main antigens of this system are M and N, present in the glycophorin A protein (GPA), and S, s, and U, present in glycophorin B (GPB). The *GYPA* and *GYPB* genes, that encode for the GPA and GPB proteins, respectively represent two closely linked loci located on chromosome 4 (4p28-q31)[2]. Single nucleotide polymorphisms (SNPs) are responsible for the S/s and M/N allelic variants. Individuals who have the *GYPB* gene deletion are negative for the S, s and U antigens. However, the S-s-U+var phenotype, characterized by a weak expression of the U antigen and absence of the S and s antigens on the surface of red blood cells, is associated with mutations in exon 5 [*GYPB(NY)* variant] or in intron 5 [*GYPB(P2)* variant] of the *GYPB* gene. These changes are found in African or Afro-descendant populations and are associated, respectively, with the complete or partial omission of the expression of exon 5 of the *GYPB* gene[3].

The antigens of the MNS system are important in the clinical practice as they are able to provoke transfusion reactions and perinatal hemolytic disease[4]. Patients who were recently submitted to transfusions and those with autoimmune hemolytic anemia may not always be phenotyped and in these cases genotyping has been successfully employed[5]. S-s-U- or S-s-U+var individuals, when exposed to U+ red blood cells, can produce anti-U alloantibodies which may cause severe transfusion reactions. As donors with U- phenotypes are rare in many populations, and good anti-U serums are scarce, the compatibilization of this antigen is often a challenge in the transfusion practice. Thus, the use of molecular biology tools together with knowledge on genetic bases and the expression of antigen variants is very important in the routine of immunohematology laboratories[6,7].

The State of Minas Gerais comprises a large region in the southwest of Brazil and has a highly admixed population[8] as a result of intense migration from several regions of Africa due to the slave trade during the colonial period[9]. This demographic scenario suggests that *GYPB* variants described in African and Afro-descent populations may also be observed in the population of the state of Minas Gerais, Brazil. The aims of this study were: to implement genotyping for the S, s and U antigens of the MNS blood group system at Fundação Hemominas and to evaluate, for the first time, the occurrence of mutations of intron 5 and exon 5 of the *GYPB* gene related to the U- and U+var phenotypes in an admixed population from Minas Gerais, Brazil.

Methods

Samples

In order to implement and validate the genotyping assays for the S, s and U antigens, 96 DNA samples from blood donors and patients with sickle cell disease from the Fundação Hemominas with known MNS phenotypes were analyzed. The samples had the following phenotypes: S+s+ (n = 30), S-s+ (n = 30), S+s- (n = 26) and S-s- (n = 10).

Serological tests

All the samples collected from peripheral blood had previously been submitted to phenotyping in the Immunohematology Laboratory of the Fundação Hemominas. The S and s antigens were determined by the column agglutination technique using ID-LISS/Coombs gel (containing polyspecific human antiglobulin) and polyclonal anti-S and anti-s human antibodies (DiaMed AG, Switzerland). The S-s- samples were phenotyped for the U antigen by the column agglutination technique using ID-LISS/Coombs gel and serum from alloimmunized patients containing previously identified anti-U antibodies.

Genomic DNA extraction

The purification of genomic DNA was achieved using whole blood with the QIAamp Blood DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions.

Genotyping of *GYPB*S* and *GYPB*s* - allele-specific primer polymerase chain reaction

All the 96 samples evaluated in this study were submitted to the allele-specific primer polymerase chain reaction (ASP-PCR) assay to genotype the *GYPB*S* and *GYPB*s* alleles. The tests were carried out using 100 ng genomic DNA in a 20 μL reaction containing 20 mM Tris-HCl (pH 8.4), 2.0 mM MgCl$_2$ for *GYPB*S* and 2.5 mM for *GYPB*s*, 200 μM of each deoxynucleotide triphosphate, 0.8 U Platinum Taq DNA polymerase (Invitrogen, USA) and 2 pmol of each primer GPB 1640 and GPBS or GPBs (Table 1). Polymerase chain reaction (PCR) was carried out in a thermocycler (model TC-412, Techne, United Kingdom), under the following conditions: an initial phase at 95°C for 5 min, followed by 35 cycles of 95°C for 30 seconds, an annealing phase for 30 seconds at 60°C for *GYPB*S* and 63°C for *GYPB*s*, 72°C for 30 seconds and a final extension phase of 72°C for 5 minutes. The amplified products were submitted to 2% agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide.

The detection of a product with 207 pb after the *GYPB*S* and *GYPB*s* genotyping reactions indicates the presence of alleles corresponding to the S and s antigens, respectively.

Molecular analysis to evaluate polymorphisms in exon 5 and intron 5 of the *GYPB* gene or its deletion

The S-s- phenotype samples (n = 10) and the samples phenotyped as S-s+ that showed the *GYPB*S* allele in the ASP-PCR described above (n = 3) were further submitted to a combination of the ASP-PCR and PCR-RFLP assays. This approach identifies the deletion of the *GYPB* gene and also detects polymorphisms in exon 5 and intron 5 associated to the loss of S antigen expression and weakening of the U antigen expression in red blood cells [*GYPB(P2)* and *GYPB(NY)* variants].

For the combined ASP-PCR and PCR-RFLP test, multiplex PCR was performed using 100 ng genomic DNA in a reaction with a final volume of 20 μL [20 mM Tris-HCl (pH 8.4), 2.0 mM MgCl$_2$, 200 μM each deoxynucleotide triphosphate, 4 pmol of the GBP 4/5 and GBP IVS5 primers, 6 pmol of the GBP 5T primer (Table 1) and 0.8 U of Platinum Taq DNA polymerase (Invitrogen, USA)]. One pmol of each human growth hormone (HGH) sense and antisense primers were also used as a positive control of amplification (Table 1). The PCR reaction was carried out under the same conditions as outlined above for *GYPB*S,* but with the annealing phase at 55°C. The amplified products were analyzed by 8% polyacrylamide gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide.

The absence of the 260 bp product amplification in samples phenotyped as S-s- identifies the S-s-U- phenotype related to *GYPB* gene deletion. The presence of an allele-specific product of 145 bp indicates the presence of the 230 C>T mutation in exon 5 related to the *GYPB(NY)* variant. A 260 bp product indicates that the *GYPB* gene is present and, in these cases, it is necessary to investigate the occurrence of the +5 g>t mutation in intron 5 of the gene by PCR-RFLP as this identifies the *GYPB(P2)* variant.

Table 1 - Primers used for ASP-PCR and ASP-PCR/RFLP to genotype the S, s and U antigens[3]

Primer	Direction	Allele	Sequence (5' to 3')	Product
GPB 1640	Sense	*GYPB*S* *GYPB*s*	GGTAAGACTGACACATTACCTCAC	
GPB S	Antisense	*GYPB*S*	AGTGAAACGATGGACAAGTTCTCCCA	207 bp
GPB s	Antisense	*GYPB*s*	AGTGAAACATGGACAAGTTCTCCCG	
GPB 4/5	Sense	*GYPB*	CTGTCTTATTTTTCTATTGCTATG	260 bp*
GPB IVS5	Antisense		CTGTTTCTCTTTTGAGTTTAACTG	
GPB 5T	Antisense	*GYPB(NY)*	ACTGTAAGAAATTAAGAGGCTCA	145 bp
HGH	Sense	HGH	TGCCTTCCCAACCATTCCCTTA	434 bp
HGH	Antisense		CCACTCACGGATTTCTGTTGTGTTTC	

*This product indicates the presence of the *GYPB* gene

All the samples with amplification of the 260 bp were further tested for the *GYPB(P2)* using PCR-RFLP. In this reaction, the digestion of the 260 bp PCR product by the EcoRI enzyme (Promega, USA) generates fragments of 92 bp and 168 bp that identify the *GYPB* wild type, while the absence of digestion indicates the presence of the +5 g>t mutation[3].

Results

Allele-specific PCR – *GYPB*S* and *GYPB*s*

All samples were subjected to the ASP-PCR technique for *GYPB*S* and *GYPB*s* genotyping. The expected 207 bp amplification product which identifies the allele that encodes for the S antigen was found in the 56 samples phenotyped as S+ (30 S+s+ samples and 26 S+s- samples) (Table 2). From the 40 samples phenotyped as S- (30 S-s+ samples and 10 S-s- samples), seven (17.5%) showed the presence of the *GYPB*S* allele (Table 2). Of these seven samples, three had been phenotyped as S-s+ and four as S-s- (Table 2).

The seven samples phenotyped as S- that were amplified by ASP-PCR as *GYPB*S* were analyzed by ASP-PCR and PCR-RFLP[3] to identify mutations in intron 5 and exon 5 of the *GYPB* gene; these mutations prevent the expression of the S antigen on the red blood cell surface (see below).

On the other hand, the results of genotyping for the *GYPB*s* allele confirmed the phenotype results, i.e. all 60 s+ samples (30 S-s+ samples and 30 S+s+ samples) had the expected 207 pb amplification product, whereas the 35 samples phenotyped as s- (25 S+s- samples and 10 S-s- samples) were negative for *GYPB*s* (Figure 1).
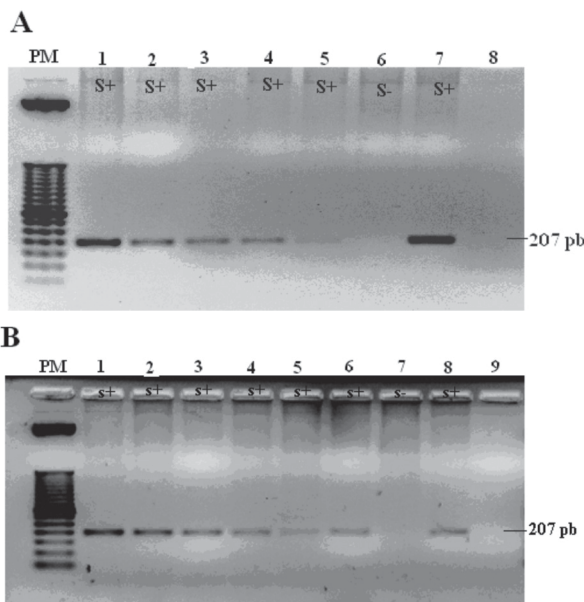

Figure 1 - *GYPB*S* and *GYPB*s* genotyping - 2% agarose Gel.
(A): Genotyping of the *GYPB*S* allele. PM: 50 bp DNA ladder. 1 to 5: samples phenotyped as S+. 6: negative control. 7: positive control. 8: water – "blank control"
(B) Genotyping of the *GYPB*s* allele. PM: 50 bp DNA ladder. 1 to 6: samples phenotyped as s+. 7: negative control. 8: positive control. 9: water – "blank control"

Molecular analysis to evaluate polymorphisms in exon 5 and intron 5 of the *GYPB* gene or its deletion

The ten samples phenotyped as S-s- were investigated in respect to *GYPB* gene deletion by ASP-PCR and PCR-RFLP. Four of these samples presented the 260 pb product in this reaction, thus indicating the presence of the gene. The other six samples should be considered S-s-U- as a result of deletion of the *GYPB* gene.

Table 2 - Results of AS-PCR for *GYPB*S* and *GYPB*s* and evaluation of *GYPB* gene deletion

Phenotype	Genotyped samples (n)	Amplification of *GYPB*S*	Amplification of *GYPB*s*	Presence of GYPB gene
S+s+	30	30	30	Not performed
S+s-	26	26	0	Not performed
S-s+	30	3	30	Not performed
S-s-	10	4	0	4

The three samples phenotyped as S-s+ that presented an amplification product by *GYPB*S* by ASP-PCR and the four samples phenotyped as S-s- which showed the presence of *GYPB* gene were investigated for polymorphisms in intron 5 and exon 5 of the *GYPB* gene (Figure 2).
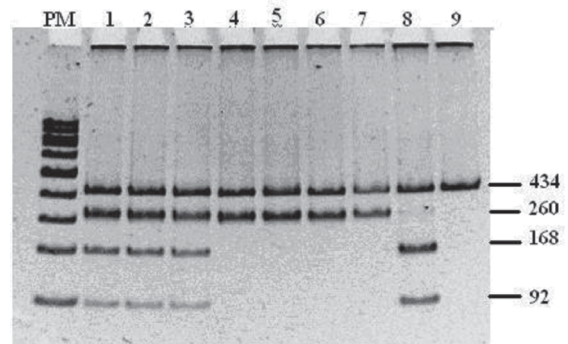

Figure 2 - Analysis of *GYPB* exon 5 and intron 5 by a combination of ASP-PCR and PCR-RFLP - 8% polyacrylamide gel. PM: 100 bp DNA ladder. 1 to 3: S+s- samples genotyped as heterozygous for the *GYPB(P2)* variant and *GYPB* wild-type. 4 to 7: S-s- samples genotyped as homozygous or hemizygous for the *GYPB(P2)* variant 8: positive control (U+ sample, homozygous for the *GYPB* wild-type). 9: negative control (S-s-U- sample due to deletion of the *GYPB* gene).

These investigations revealed that the four samples phenotyped as S-s- were homozygous or hemizygous for the mutation related to the *GYPB(P2)* variant (Samples 4-7, Table 3).

Table 3 - Results of molecular analysis of *GYPB* exon 5 and intron 5 in samples S-s+ and S-s- with presence of the *GYPB*S* allele

Sample	Phenotype	Analysis of exon 5 and intron 5
1	S-s+	+5 intron 5 (g>t) / *GYPB* wild-type
2	S-s+	+5 intron 5 (g>t) / *GYPB* wild-type
3	S-s+	+5 intron 5 (g>t) / *GYPB* wild-type
4	S-s-	+5 intron 5 (g>t)
5	S-s-	+5 intron 5 (g>t)
6	S-s-	+5 intron 5 (g>t)
7	S-s-	+5 intron 5 (g>t)

The three samples phenotyped as S-s+ that presented amplification for *GYPB*S* by ASP-PCR were heterozygous for the allele corresponding to the *GYPB(P2)* variant and another wild-type allele corresponding to *GYPB* (Samples 1-3, Table 3).

Discussion

To evaluate the effectiveness of the genotyping techniques employed, ASP-PCR was performed to identify the *GYPB*S* and *GYPB*s* alleles in 96 samples which had been phenotyped as follows: S+s+ (n = 30), S+s- (n = 26), S-s+ (n = 30) and S-s- (n = 10).

The results of *GYPB*S* and *GYPB*s* genotyping were consistent with the hemagglutination tests for samples phenotyped as S+s- or S+s+.

However, 10% of samples phenotyped as S-s+ (n = 30) had positive amplification products with ASP-PCR, suggesting the presence of the *GYPB*S* allele. This value represents a higher percentage of cases than previously reported for African descent individuals from the northeast of Brazil, where the presence of the *GYPB*S* allele was identified in 7.4% of samples phenotyped as S-s+[6] and the 5% discrepancy between phenotyping and genotyping of the S and s antigens reported in Brazilians from the Amazon region[10].

The analysis of intron 5 and exon 5 of the *GYPB* gene showed that the three samples phenotyped as S-s+ with amplification for the *GYPB*S* allele presented the silencing mutation associated with the *GYPB(P2)* variant in heterozygosis with the wild *GYPB* gene. In these cases, the deduced genotype would be *GYPB(P2)*S/GYPB*s*.

Of the ten S-s- samples, six (60%) presented deletion of the *GYPB* gene. Previous studies carried out in Brazil show a prevalence of 76.5% (13/17) of this deletion in samples phenotyped as S-s-[6].

The four samples phenotyped as S-s- for which the *GYPB* gene was detected were also subjected to ASP-PCR and PCR-RFLP to analyze mutations in exon 5 and intron 5. All of these samples were homozygous or hemizygous for the *GYPB(P2)* variant.

The +5 a>g mutation in intron 5 coupled with the *GYPB(P2)* variant was shown as the most common mechanism associated to the silencing of the S antigen and the occurrence of the S-s-U+var phenotype in Brazilian and North American populations as showed in previous studies. Among 17 S-s- samples from Afro-Brazilians assessed in the northeast of the country, four had the *GYPB* gene; two were homozygous and one was heterozygous for the *GYPB(P2)* variant[6]. Among Afro-Americans this variant was reported in 83% of S-s-U+var samples[3].

Although no cases of the *GYPB(NY)* variant were found in this study, other authors who investigated Brazilian blood donors reported that two out of four individuals with the S-s-U+var phenotype and three of 11 S-s+ individuals who amplified for *GYPB*S* were positive for the *GYPB(NY)* allele[6]. Among Afro-Americans from the New York Blood Center in the USA, 7 of 41 S-s-U+var individuals presented the *GYPB(NY)* variant in homozygosis (n = 4) or in heterozygosis with *GYPB(P2)* (n = 3)[3].

The five centuries of miscegenation in the state of Minas Gerais has made this population highly admixed and extremely variable at ancestral levels, even within so-called traditional phenotypic categories such as "White", "Black" or "Afro-descendant"[11]. Considering this demographic process, the selection of samples regardless of ethnic profile is interesting and able to effectively portray the population and avoid the subjectivity of the process of ethnic self-denomination in admixed populations.

The prevalence of the S-s- phenotype is 2 to 8% in the African population and has been described among Pygmies (20%), in Western Africa (up to 37%) and Afro-Americans (1%)[12-15]. The existence of polymorphisms of the *GYPB* gene in individuals of African descent has led to the speculation that these variants may have been selected as a result of the relative resistance that they confer against *P. falciparum* malaria[10].

The African admixture in the Brazilian population is expressive; it is as high as 34.1% in blood donors and 45.5% in patients with sickle cell disease from Minas Gerais[8]. Thus, it is not only expected that the S-s-U- and S-s-U+var phenotypes should be found in Brazil, but also that their frequencies would vary between different regions of the country, as the levels of admixture varies considerably[8,16-21].

Considering all the 40 S- samples in this study, 17.5% were positive in the PCR for *GYPB*S* and for the presence of the *GYPB(P2)* silencing variant. These data highlight the significance of this polymorphism in the population of Minas Gerais and are a clear example of the importance of analysis of currently known variants when genotyping is used to infer the S, s and U antigen phenotypes of the MNS system. The identification of other variants is a challenge for future studies.

Although analyses were carried out on samples with previously known phenotypes, genotyping of S, s, and U might be very useful in cases where serologic tests to determine the antigens cannot be performed. In these cases, known polymorphisms that change the *GYPB* expression should be investigated.

The implications of molecular changes involving the U+var phenotype on transfusion medicine remain unknown, although it is well known that U+var individuals are capable of producing anti-U antibodies when in contact with the antigen.

Transfusions to alloimmunized patients requiring S-s-U- red blood cells still post a challenge in any transfusion service due to lack of well-characterized good quality serologic reagents[22]. It is difficult to obtain anti-U antibodies with good reactivity in an amount sufficient to allow the phenotyping of patients and potential blood donors[6]. To overcome these difficulties, immunohematology laboratories in Brazil and other parts of the world occasionally use anti-U serum from alloimmunized patients to phenotype red blood cells of donors and patients. However, these antibodies are not well characterized and may not detect the U antigen variant, weakly expressed on the surface of red blood cells. This can lead to false-negative results (U+var individuals phenotyped as U-). This probably was the cause of the inaccurate phenotyping in four of the ten S-s- samples (Samples 4 to 7 - Table 3) which were initially phenotyped as S-s-U- and, following the detection of variant *GYPB(P2)* by genotyping, were reclassified as S-s-U+var. The false-negative phenotyping of the U antigen is especially important in donors as their red blood cells might be transfused in U- patients.

Thus, the use of molecular biology tools to determine S, s and U antigens in blood donors and patients is essential to minimize the problems encountered in immunohematology, such as the accurate identification of S-s-U- and S-s+U+var individuals. The genotyping of S, s and U antigens could lead to a reduction in the risk of potentially severe transfusion reactions involving these antigens, especially in multitransfused patients such as individuals with sickle cell anemia.

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