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Molecular Characterization of RHD in Rh-Negative Blood Donors in Congo Brazzaville

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Abstract

Background: The D antigen is the most important and immunogenic antigen of the Rh blood group; its correct screening prevents any risk of alloimmunization in the RHD negative recipient. The D negative phenotype is characterized by the absence of the D antigen (RH1) on the surface of the erythrocyte. Three main mechanisms can generate this absence: total or partial deletion of the RHD gene, insertion of base pairs within the said gene and gene conversion. The objective of this study was to report the first data on RHD genotyping in RHD negative congolese blood donors. **Materials and Methods:** Blood samples came from regular RHD-negative blood donors selected from the blood transfusion stations in Brazzaville and Pointe-Noire. They were analyzed individually by conventional PCR, targeting exons 4, 5, 7 and 10 of the RHD gene. **Results:** Fifty-nine regular RHD negative blood donors were selected for this study. The immuno-hematological profile was determined individually, and the *dccee* phenotype was the most frequent (n = 52; 88.1%). The search for the weak D antigen was negative for all donors. Exons 4, 5, 7 and 10 of the RHD gene were amplified in the following respective proportions: 89.8%, 81.4%, 6.8% and 42.4%. Moreover, (1) donor was found to carry all four specific exons sought. **Conclusion:** Conventional PCR amplification allowed to study the presence of specific exons of the targeted D gene. At least one exon was detected in the entire study population. These results suggest that the RHD gene is indeed present in the donors studied and that the deletion cannot be considered as the main mechanism causing the RH-1 (D negative) phenotype in this sample.

Keywords

Molecular Characterization, Rhesus, RHD Gene, Blood Donors

1. Introduction

The RH system is the most complex blood group system and the most important in terms of transfusion safety after the ABO system. It includes about fifty antigens of a polypeptide nature, but only 5 of them are of clinical interest in transfusion medicine. These are the antigens: RH1 (D), RH2 (C), RH3 (E), RH4 (c), and RH5 (e), which are carried by two highly homologous proteins, encoded by two RHD and RHCE genes located on chromosome 1. These genes encompass 10 exons and their structures are highly homologous [1] [2].

The D antigen or standard rhesus factor is the most involved in transfusion and obstetric incompatibilities, and this antigen has a strong immunizing power. Indeed, once introduced into an organism that does not possess it, alloimmunization follows which could be responsible for a severe acute or delayed hemolytic transfusion reaction and hemolytic disease of the newborn. [3] [4]. Depending on the presence or absence of the D antigen on the membrane of red blood cells, they are called Rh-positive (RH: 1) or Rh-negative (RH: −1) [5]. The RH: −1 phenotype characterized by the absence of the D antigen is caused by several mechanisms. It can be, on the one hand, a partial deletion interrupting the reading frame of the RHD gene, or a deletion of the entire RHD gene, which is the most frequent molecular mechanism in European and Chinese populations. On the other hand, the RHD gene is present but does not encode the RH1 antigen following mutations, insertions or gene exchanges making this RHD gene particular. It is therefore unable to synthesize the RH1 (D) antigen and finally, gene conversions lead to the formation of hybrid genes contributing to the RH-1 phenotype [6]-[11].

Furthermore, there are some aberrant RHD alleles (RH:1 variants) resulting from point mutations or gene rearrangements between the RHD and RHCE gene, leading to reduced expression of the D antigen on the surface of red blood cells that can be wrongly typed as rhesus negative (RH: −1) by standard serological methods including the indirect antiglobulin test (IAT) and be at the origin of anti-D alloimmunizations, once transfused to rhesus negative recipients, hence the interest in molecular typing to circumvent the limitations of serological methods, resolve discrepancies and identify RHD variants; by PCR tests on blood samples [12].

In Congo-Brazzaville, pre-transfusion immuno-hematological compatibility is limited to ABO/RHD blood grouping and cross-matching. Research for weak D, partial D or Del antigens is not carried out. Blood transfusion is then carried out with a common and possible risk of alloimmunization [13]. Knowing the importance and the involvement of the RHD antigen and its antibodies (anti-D) in transfusion incompatibilities, it is justified to develop new strategies to complement these standard serological methods which are very limited. Molecular techniques make it possible to avoid potential alloimmunization in rhesus negative recipients by highlighting, in the blood units to be transfused labeled rhesus negative (RH: −1), the variants of the RHD gene, expressing the D antigen, which can cause anti-D alloimmunization.

All these difficulties mentioned above do not guarantee transfusion safety. With a view to improving the daily practice of blood transfusions, we undertook this preliminary study in Congo Brazzaville, with the aim of highlighting by conventional PCR the exons of the RHD gene in our blood samples.

2. Materials and Methods

2.1. Blood Collection

A descriptive cross-sectional study was conducted on 59 regular RHD-negative blood donors, recruited from the database of the National Blood Transfusion Center (CNTS) in Brazzaville and the Interdepartmental Blood Transfusion Center (CIDTS) in Pointe-Noire, during the period from February 1, 2018 to November 30, 2018. From each participant, approximately 5 ml of blood was collected by phlebotomy from the vein of the elbow crease into an EDTA (Ethylenediaminetetraacetate) tube, in compliance with good practices and asepsis.

2.2. Determination of the Phenotype

RHD RhC, RhE, Rhc and Rhe phenotypes of red blood cells were determined using monoclonal antibodies IgM, anti-D, anti-C, anti-E, anti-c and anti-e, respectively, according to the manufacturer's recommendations (CYPRESS Diagnostics®, Belgium).

Subsequently, a search for weak D antigen was carried out on all samples using the indirect Combs technique with polyvalent human antiglobulin in a tube and on a cassette (Ortho Biovue® France). For the Tube Technique 50 µl of a 5% suspension of washed red blood cells was placed in a hemolysis tube with 50 µl of Anti-D Blend serum (IgM + IgG). This mixture was incubated at 37˚C for 15 minutes, then washed three times with 0.9% physiological serum. Then after aspirating the last supernatant, the red blood cells were resuspended with 100 µl of antihuman globulin (AHG), then centrifuged at 1000 rpm for one minute. The appearance of agglutinations, indicating the presence of weak D antigen, was checked macroscopically by gently shaking the tube [14]. On the other hand, for the cassette technique, following the manufacturer's instructions, 50 µl of 5% suspension of the red blood cells to be tested were placed in each appropriate reaction chamber (containing the antiglobulin), with 50 µl of Anti-D Blend serum (IgM + IgG, CYPRESS Diagnostics®, Belgium). Then the cassette was incubated at 37˚C for 30 minutes. Finally, after incubation, the cassette was centrifuged at 1000 rpm for five minutes using the Ortho Biovue System centrifuge (Ortho Bioview Systhem® France). Reading both sides of the columns made it possible to look for an agglutination reaction. The adsorption-elution test was not carried out in this study.

2.3. Determination of RHD Genotype

2.3.1. DNA Extraction

Total DNA extraction from the different samples was performed using the

MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit: mini Kit according to the conditions specified by the suppliers (Applied systems, Germany). The extracted DNA was stored at −20˚C for further analysis after verification of the concentration using the Nanodrop® (one/onec, Thermo Fisher scientific inc, USA).

2.3.2. Characterization of D Genes by Standard PCR

Conventional PCR performed using a T100 thermocycler (Bio-Rad, France), was used to study the presence of four exons 4, 5, 7 and 10 of the RHD gene, using the primer pairs specific to each sequence (Table 1).

Table 1. Primer pairs used for the amplification of specific exons of the D gene.

Exons	Sequence 5' → 3	Size (pb)	References
4	F:5'-CCACATGAACATGATGCACA-3' R:5'-CAAACTGGGTATCGTTGCTG-3'	127	Banseok Kim, *et al.* 2017 (Korea)
5	F: 5'-CGCCCTCTTCTTGTGGATG-3' R:5'-GAACACGGCATTCTTCCTTTC-3'	82	Behzad Nazel, 2019 (Iran)
7	F:5'-GTTGTAACCGAGTGCTGGGGATT-3' R: 5'-TGCCGGCTCCGACGGTATC-3'	123	Banseok Kim, 2018 (Core)
10	F:5'-CCTCTCACTGTTGCCTGCATT-3' R 5' -AGTGCCTGCGCGAACATT-3'	74	Behzad Nazel, 2019 (Iran)

Classical PCR was performed in a 25 µL reaction mixture containing: 6 µL of sterile distilled water, 0.5 µL of each primer (20 µM), 12.5 µL of Quantitect Probe PCR Invitrogen master mix, 0.5 µL of Taq DNA polymerase (Eurogentec, Belgium) and 5 µL of DNA extract. A positive RHD sample was used as a positive control for exon amplification. Amplification conditions are shown in Table 2.

Table 2. Conditions for reactions to the PCR standard for different exons (4, 5, 7 and 10).

Parameters	Conditions/Duration			
	Exon 4	Exon 5	Exon 7	Exon 10
Initial Denaturation	95˚C/10 min	95˚C/10 min	95˚C/10 min	94˚C/2 min
Denaturation	95˚C/30 sec	92˚C/20 sec	95˚C/30 sec	95˚C/30 sec
Pairing	50˚C/30 sec	64˚C/30 sec	60˚C/30 sec	55˚C/30 sec
Elongation	72˚C/30 sec	68˚C/3 min	72˚C/30 sec	72˚C/30 sec
Final Elongation	72˚C/5 min	72˚C/5 min	72˚C/5 min	72˚C/5 min
Number of Cycles	**35**	**35**	**35**	**30**

Sec: seconds; min: minutes; ˚C: degrees Celsius.

Amplification products were detected by electrophoresis using 2% agarose gel after ethidium bromide (BET) staining, together with a DNA molecular weight marker (BenchTop pGEM® DNAMarker, Promega, Madison, Wisconsin,

USA). Images were digitized using the Viviliber photodocumentation system (E-Box).

3. Results

In the present study the *dccee* phenotype is the most represented with a high frequency (88.13%) followed by the *dCcee* phenotype (10.17%) and finally the dCcEe (1.7%) (Table 3).

Table 3. Distribution of C, E, c, e antigens in the study population.

RhCE Phenotype	Staff (n)	Percentage (%)
dccee	52	88.1
dCcee	6	10.2
dCcEe	1	1.7
Total	**59**	**100**

The search for weak D antigen by indirect Coombs test with polyvalent antihuman globulin was carried out on samples blood from 59 donors, the result was negative in all donors.

Fifty-nine (59) DNA extracts from donors were analyzed by conventional PCR technique. Exons 4 and 5 were highlighted in the majority of donors in our study with rates of 89.8%. and 81.4% respectively, followed by exon 10 with 42.4%, while exon 7 was detected in only 6.8% of the study population (Table 4).

Table 4. Results of the amplification of the four specific exons of the RHD gene in the study population.

Results	Exon 4	Exon 5	Exon 7	Exon 10
	n (%)			
Presence	53 (89.8)	48 (81.4)	04 (6.8)	25 (42.4)
Absence	6 (10.2)	11 (18.6)	55 (93.2)	34 (57.6)
Total	**59 (100)**	**59 (100)**	**59 (100)**	**59 (100)**

Only one (1) donor was found to carry all four specific exons sought. Donors with the *ddccee* phenotype are predominantly carriers of all four target exons (Table 5).

Table 5. Results of amplification of the four specific exons of the RHD gene in donors according to the RHCE phenotype.

RhCE Phenotype	ISBT Phenotype	Exon 4 + (%)	Exon 5 + (%)	Exon 7 + (%)	Exon 10 + (%)
ddccee (n = 52)	Rh: −1, −2, −3, 4, 5	44 (84.6)	35 (67.3)	4 (7.7)	20 (38.5)
dCcee (n = 6)	Rh: −1, 2, −3, 4, 5	3 (50)	1 (16.7)	0	4 (66.7)
dCcEe (n = 1)	Rh: −1, 2, 3, 4, 5	1 (100)	1 (100)	0	1 (100)

Figure 1 and Figure 2 show two examples of the bands obtained after amplification of exons 4 and 5.

Figure 1. 2% agarose gel electrophoresis of standard PCR amplification products of the D-Exon-4 gene.

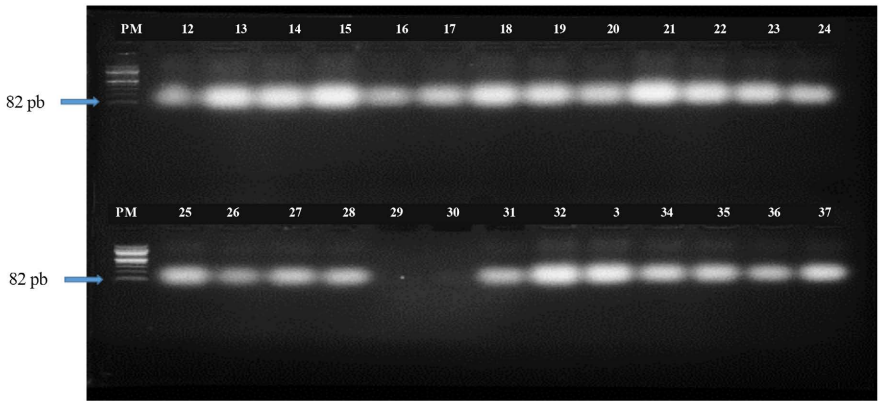

Figure 2. 2% agarose gel electrophoresis of PCR amplification products of exon 5 (samples 12 to 37). MW: molecular weight marker; bp: base pair.

4. Discussion

Every blood bank must ensure that Rh-negative blood units are correctly labelled, free of all traces of immunising elements (D antigen) in order to prevent anti-D alloimmunisation and to ensure the immunological transfusion safety of Rh-negative recipients.

We undertook a prospective cross-sectional study with a descriptive aim to study for the first time the presence of the RHD gene in RHD negative Congolese blood donors. The conventional PCR technique was used to amplify the different specific exons of the RHD gene. We conducted this study to complement current serological methods because it is documented that alloimmunization (allo or auto) due to blood group antigens compromises immunological transfusion safety.

Four specific exons of the RHD gene (4, 5, 7 and 10) were then amplified in order to verify whether the RH: -1 phenotype is associated with the presence or total absence of the RHD gene in Congolese blood donors typed RHD negative. Indeed, in the presence of a deletion of the D gene, none of these exons are amplified. However, detection of a single exon is associated with the presence of

either a weak D allele or another particular allele (variant of the D gene) incapable of synthesizing the D antigen, resulting in the RHD negative phenotype (RH: −1) [15] [16].

This research is justified since the D antigen is the most immunogenic of the RH system, its correct identification is very important to ensure that the blood units to be transfused are free of any trace of D antigen, in order to prevent any risk of anti-D alloimmunization (transfusional and obstetrical). Standard serology is the routinely used technique for detecting the D antigen, but it has its limitations. Thus, RHD genotyping using molecular biology techniques, in this case standard PCR, makes it possible to circumvent these limitations. Indeed, certain RH:1 variants (weak D, partial D and Del) characterized by quantitative or qualitative modifications of the RH1 protein can be falsely typed negative by conventional serological methods [17]-[20].

In our study population, the phenotype (*dccee*) (RH: −1, −2, −3, 4, 5) was the most frequent with 88.1%, followed by the phenotype (*dCcee*) (RH: −1, 2, −3, 4, 5) with a frequency of 10.2%, these results are close to the data reported by other studies [21]-[25]. Other studies, on the other hand, have reported lower frequencies: 0.4% in Morocco, 2.26% in Algeria and 6.45% in Ghana [14] [22] [26] [27].

The search for weak D antigen by indirect Coombs test with anti-globulin performed on samples from fifty-nine RHD negative blood donors was negative, although we did not use the adsorption-elution test, no weakened expression of D antigen was noted. This result is similar to that reported by Behzad *et al.*, in Iran [20].

Fifty-nine DNA extracts from RHD negative blood donors were analyzed individually from the original FTA paper, exons 4, 5, 7 and 10 were amplified in 53 (89.8%), 48 (81.4%), 04 (6.8%) and 25 (42.4%) cases respectively. These results reveal the presence of the D gene in our analyzed samples despite the absence of a weakened expression of the D antigen, and suggest that the RHD negative profile of these donors is probably not related to a total deletion of the RHD gene, could it be non-functional RHD genes or pseudo RHD genes? Given the importance of this highly immunogenic marker and its implication in transfusion incompatibilities, further molecular investigations are necessary to study these genes in depth.

Although our sample size is small, the results of this preliminary study deserve some comments and some comparisons with other authors.

In the present study, we limited ourselves to the detection of specific exons of the RHD gene due to the high costs of molecular techniques, other authors having at their disposal other molecular techniques identifying alleles (variants) of the RHD gene, reported the following results: Banzeok *et al.*, had found 21 donors carrying a variant of the RHD gene, and a total deletion of the RHD gene in 74 other donors, St-Louis M., *et al.* in Quebec on the other hand reported only thirteen D negative donors out of 115, carriers of a variant of the RHD gene (0.33%), including nine: RHD * Ψ negative (haplotype *Ce*), and four RHD * Ψ positive (*ce* haplotype) [28] [29]. Ouchari *et al.* (2013) [30] in Tunisia reported no amplification

of the exons of the RHD gene from the DNA of 39 RHD negative donors, which supports a total deletion of the RHD gene.

We noted an amplification of these four specific exons in only one (1) donor. Could he be carrying a variant of the D gene? Other molecular investigations could answer this question. The study carried out by Behzad *et al.* also reports the presence of exons 5, 7 and 10 of the RHD gene in two (2) donors (out of 200 samples analyzed), one was found to be a carrier of the three exons and the other was only positive for exon 10 [20].

In the present study, phenotype donors (*dccee*) (RH: −1, −2, −3, 4, 5) were predominant with 88.1%, and mostly carriers of RHD gene exons. These results reveal the presence of the D gene in the majority of our samples. Discussions with blood transfusion specialists would allow us to assess the relevance of molecular analyses on Rh-negative blood donations, in order to facilitate the search for compatible blood for Rh-negative recipients. This trend has also been observed by other studies, Mariza Mota *et al.* in Brazil (2012) on the one hand and Eiman Hussein *et al.* (2013) on the other hand, the latter even suggested that D gene variants are much more found in RH blood donors: −1 phenotyped (*dccee*). This trend is different from that observed by Hiroshi *et al.*, in Japan who report that all RHD negative donors carrying the RHD gene, were *CC* or *Cc* phenotype [12] [23] [25].

The results of the present study would not exclude the possibility of the existence of the "partial D or weak D" variants in our study population, knowing that the donors expressed the specific exons of the RHD gene, hence the interest of PCR genotyping in the laboratories of blood transfusion centers to ensure that the D negative blood units intended for transfusion are free of any trace of D antigen because some alleles such as DEL and weak D may not be detected by conventional serological methods and be considered as RHD negative by omission.

5. Conclusion

Our study reports the first data on the use of conventional PCR to study the genetic background of the D negative phenotype (RH: −1), in RHD negative congolese blood donors. The results obtained demonstrate the importance of the molecular approach in addition to conventional serological methods in immunohematology laboratories for efficient screening of blood units labeled Rh negative. Further studies on larger samples could be interesting to better assess the genetic background of the RHD negative phenotype, and thus contribute to the immunological transfusion safety of RHD negative recipients.

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Authors' Contributions

All authors contributed to the completion of the study, as well as the writing and correction of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest in relation to this study.

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