Case Report

Severe perinatal hemolytic disease due to anti-e

G. Soler-Noda, Y. Romero-Díaz, L. Orbeal-Aldama, and S. Aquino-Rojas

Maternal antibody-mediated fetal red blood cell destruction secondary to non-D Rh system antibodies is a significant cause of hemolytic disease of the fetus and newborn. Here, we report a rare case of severe perinatal hemolytic disease associated with maternal antibody to the e antigen. In addition to severe anemia, the infant developed hyperbilirubinemia. Resolution of the infant's anemia and hyperbilirubinemia occurred after treatment with phototherapy, intravenous immunoglobulin, and transfusion. ***Immunohematology*** **2021;37:72–77. DOI: 10.21307/immunohematology-2021-011.**

Key Words: hemolytic disease of the fetus and newborn, HDFN, postnatal diagnosis, Rh blood group system, Rhe, Rh5

Hemolytic disease of the fetus and newborn (HDFN) is an alloimmune condition in which shortened survival of the red blood cells (RBCs) of the fetus or the newborn is caused by maternal antibodies passing through the placenta and binding to the corresponding antigen on the fetal RBCs.

The pathogenesis of HDFN is based on a blood group incompatibility between the mother and fetus, where the fetal RBCs have antigens of paternal origin that are lacking on the mother's RBCs. If the mother has an immune response to these antigens, the resulting immunoglobulin (Ig)G class antibody can cross the placenta and attach to and facilitate hemolysis of the antigen-positive fetal RBCs. An exception to this process is ABO-HDFN, in which the maternal IgG antibodies are preformed and do not depend on paternal origin for stimulation.

For this condition to occur, it is also necessary that the antigen encoded by the inherited paternal gene is fully expressed on the fetal RBCs and stimulates the formation of an IgG antibody. The passage of these antibodies through the placenta causes a diversity of symptoms in an antigen-positive fetus or newborn, ranging from mild anemia to death.[1]

Alloimmunization against D during pregnancy is the most frequent cause of severe HDFN, but antibodies to other specificities within the Rh and other blood group systems can also give rise to HDFN.

We report a case of HDFN in which solely anti-e was shown to cause severe symptoms.

Case Report

A white male neonate was born at 39.5 weeks' gestation to a 39-year-old group A, D+ woman. She had a total of 10 pregnancies: nine abortions and the current full-term pregnancy, which had progressed without obstetric complications. She reported a history of prior RBC transfusion.

The infant showing a cephalic presentation was born by vaginal delivery without fetal distress and had a normal and attached placenta, clear amniotic fluid, 9/9 APGAR, and a birth weight of 3100 g. The physical examination at birth revealed good general condition, moist skin, and mucous membranes without infiltrates; audible sounds in both lungs (respiratory rate 44 per minute without rales; normal 44 per minute); rhythmic heart sounds with good intensity and no murmurs (heart rate 136 beats per minutes; normal range 120–160 beats per minutes); and a soft and predictable abdomen that was not painful on palpation. Complete blood count, reticulocyte count (CR), total bilirubin, and ABO and D typing were performed with the following results: hematocrit (Hct): 60 percent (reference range 42–60%); CR: 3 percent (reference range 2–6%); total bilirubin: 55 μmol/L (normal <103 μmol/L); and phenotype group A, D+.

At 8 hours after delivery, the baby maintained a good general condition but had slight jaundice, so it was decided to perform a direct antiglobulin test (DAT) and repeat the Hct, CR, and bilirubin. Phototherapy was also initiated. The results of the additional tests showed the following results: decrease in Hct from 60 to 38 percent, accompanied by increased reticulocytosis (217%), total bilirubin of 292 μmol/L, unconjugated bilirubin of 204 μmol/L, and a positive DAT.

This abrupt decrease in Hct accompanied by reticulocytosis, hyperbilirubinemia, and positive DAT raised a strong suspicion of HDFN due to an antibody directed at antigens other than ABO or D. Peripheral blood samples from both parents and the baby were sent to the Laboratory of Immunohematology at the National Institute of Hematology and Immunology (Havana, Cuba) for immunohematologic investigation.

The treatment was initiated with intravenous immunoglobulin (IVIG) at a dose of 500 mg/kg/day and phototherapy during the first and second day. Total bilirubin levels remained stable (between 254 and 300 mmol/L; unconjugated bilirubin levels were between 166 and 212 mmol/L), and CR decreased to 10 percent. Jaundice, however, remained in covered areas of the body, and Hct values decreased to 30 percent. Increasing the phototherapy to twice daily and the IVIG dose to 1 g/kg/day caused a decrease in total bilirubin after 3 days. After transfusion of 62 mL RBC concentrate at a rate of 10 mL/kg, Hct levels rose to 53 percent. The newborn remained under strict medical control until the normalization of the laboratory complementary values.

Materials and Methods

Serologic Study

ABO, Rh, and K1 typings of RBC samples from both parents and newborn were performed by standard tube testing with monoclonal reagents according to manufacturers' instructions (Anti-A, Anti-B, Anti-A,B, and Anti-D; ior Hemo-CIM SC, LABEX, Santiago de Cuba, Cuba; Anti-C, Anti-c, Anti-E, Anti-e, and Anti-K1; SPINREACT, SA, Girona, Spain).

The DAT was performed using polyspecific antihuman globulin (AHG) and monospecific anti-IgG and anti-C3d reagents (Anti-IgG/Anti-C3d, Anti-IgG, and Anti-C3d; Bio-Rad Medical Diagnostics, Cressier, Switzerland) after manually washing a sample of the infant's RBCs three times.

Table 1. Primers used for *RH* PCR-SSP

Exon	Name of primers	DNA sequence of primers (5´-3´)	Resulting specificity
2	D-2-201(sense)	GCTTGGGCTTCC TCA CCT CG	*RHD/C*
	D-2-307(anti-sense)	CAG TGT GAT GAC CAC CTT CCC AGA	
3	D-3-383(sense)	TGTCGGTGCTGATCT CAG TGG A	*RHD*
	D-3-451 (anti-sense)	ACT GAT GAC CAT CCT CAG GTT GCC	
4	D-4-527 (sense)	ACA TGATGC ACA TCTACGTGTTCG C	*RHD*
	D-4-602 (anti-sense)	CAG ACA AACTGG GTA TCG TTG CTG	
5	D-5-676 (sense)	ATGTTCTGGCCAAGTGTCAACTCT G	*RHD*
	D-5-787 (anti-sense)	CTG CTC ACC TTG CTG ATC TTC CC	
6	D-6-826 (sense)	TTATGTGCA CAG TGCGGTGTT GG	*RHD*
	D-6-916 (anti-sense)	CAG GTA CTTGGCTCC CCC GAC	
7	D-7-967 (sense)	GTT GTA ACC GAG TGCTGGGGATTC	*RHD*
	D-7-1048 (anti-sense)	TGCCGG CTC CGACGG TAT C	
9	D-9-1168 (sense)	TAT GCATTT AAA CAG GTTTGCTCCTAA ATC	*RHD*
	D-9-1193 (anti-sense)	AGA AAA CTTGGT CAT CAA AAT ATT TAG CCT	
10	D-10-1255 (sense)	TCC TCA TTTGGCTGTTGG ATT TTA AG	*RHD*
	D-10-1358 (anti-sense)	CAG TGCCTGCGCGAA CAT TG	
C/c	C-1-(-24) (sense)	GAT GCC TGGTGCTGGTGGAAC	*RHC/c* (cyt 48)
	C-1-48 (anti-sense)	GCTGCTTCCAGT GTA GGG CG	
c	C-2-201(sense)	GGC TTG GGCTTC CTC ACC TCA	*RHc/c* (cyt 48)
	C-2-307 (anti-sense)	AGT GTG ATG ACC ACCTTCCCA GG	
E/E	E-5-676 (sense)	GAT GTTCTG GCC AAGTGT CAA CTC TC	*RHE*
5	D-5-676 (sense)	ATGTTCTGGCCAAGTGTCAACTCT G	*RHe*
e/e	E-5-787 (anti-sense)	CTG CTC ACC ATGCTG ATC TTC CT	
C/C	C-IN2-s	5′-GCTCTGTTGCCCAGTCTGAAGTG-3′	
	C-IN2-as	5′-CCACTGGGAAGTGACAAAGGGC-3′	

For all primers, the respective exons, their specificity for *RHD* and *RHCE* alleles, and their position from the 3´ end on the *RHD* and *RHCE* coding sequences are given.

PCR-SSP = polymerase chain reaction using sequence-specific primers.

Table 2. Determination of the specificity of maternal alloantibody

Panel	C	c	D	E	e	K	k	Kp^a	Fy^a	Fy^b	M	N	S	s	P1	Le^a	Le^b	Lu^a	Lu^b	Jk^a	Jk^b	Wr^a	Di^a	Xg^a	Peculiarities	IAT	ENZ*
I	+	+	+	+	+	0	+	+	0	0	+	+	+	+	+	+	0	0	+	0	+	+	+	+		1+	2+
II	+	+	+	0	+	+	+	0	0	+	+	+	+	0	0	0	0	0	+	+	0	0	0	0		1+	2+
III	+	+	+	0	+	0	+	0	+	0	0	+	0	+	0	0	+	0	+	0	+	0	0	0		1+	2+
																									AUTO	0	
1	+	+	+	+	+	0	+	0	0	0	+	+	+	+	+	+	0	0	+	0	+	0	0	+		1+	2+
2	+	+	+	0	+	+	+	0	0	+	+	+	+	0	0	0	0	0	+	+	0	0	0	0		1+	1+
3	+	+	+	0	0	0	+	0	+	0	0	+	0	+	0	0	+	0	+	0	+	+	0	0		0	0
4	+	+	+	0	+	+	+	+	0	0	+	+	+	+	0	0	0	+	0	+	0	0	0	0		1+	2+
5	0	0	+	0	+	0	+	0	+	0	+	0	+	+	+	0	0	0	+	0	+	0	0	+		1+	2+
6	+	0	+	0	+	0	+	0	+	0	+	+	0	+	0	0	0	0	+	+	+	0	0	+		2+	3+
7	0	+	0	+	0	0	+	0	+	+	+	+	+	+	0	0	+	0	+	0	+	0	+	0		0	0
8	0	+	0	0	+	0	+	0	+	0	+	+	0	+	+	+	0	0	+	0	+	0	0	0		1+	2+
9	0	+	0	0	+	0	+	0	0	0	0	+	0	+	+	?	0	+	0	+	+	0	0	+	C^w(+), Js(a+)	1+	1+
10	0	+	0	0	+	0	+	0	0	0	+	+	+	0	0	+	0	0	+	+	+	0	0	0		1+	2+

*Papain-treated red blood cells. Red font highlights reactivity with e+ and e– panel red blood cells.
IAT = indirect antiglobulin test; AUTO = autocontrol.

A postpartum maternal serum sample was used for antibody detection, identification, and titration studies. The antibody specificity was determined using an antibody identification RBC panel; this RBC panel was obtained from voluntary blood donors and prepared in the immunohematology laboratory of our institution (Institute of Hematology and Immunology, Havana, Cuba). The serum was also tested against papain-treated RBCs (Sigma-Aldrich, St. Louis, MO). Titrations were performed using the tube method by indirect antiglobulin test (IAT) against untreated RBCs and papain-treated RBCs. All procedures were performed according to the protocols in the *AABB Technical Manual*.[2]

Molecular Study

The molecular study was performed following the protocol of Gassner et al.[3] The DNA was extracted from EDTA-anticoagulated whole blood obtained from both parents and the newborn, using the QIAcube equipment (QIAGEN, Hilden, Germany). The *RHD/RHCE* testing was performed by polymerase chain reaction (PCR) using sequence-specific primers (PCR-SSP; Eurofins Genomics, Ebersberg, Germany) (Table 1).

In each PCR, human growth hormone was used as a positive amplification control. The detection concentration of the primers was 0.2 µmol/L, with the exception of PCRs 7 and 11, in which 0.1 µmol/L was used, respectively. The concentration of the control primers was 0.05 µmol/L (0.08 µmol/L in PCRs 9, 10, and 12). The amplification reaction was performed with a final volume of 25 µL, containing Colorless Gotaq Flexi Buffer 5× (Promega, Madison, WI) and a DNA concentration of 50 pmol/L. $MgCl_2$ concentrations were different between the different mixtures: 0.9 mmol/L (PCR 10), 1.1 mmol/L (PCR 5), 1.2 mmol/L (PCRs 1 and 4), 1.35 mmol/L (PCR 12), and 1.5 mmol/L (PCRs 2, 3, 6, 9, and 11). To facilitate the study, all mixtures were subjected to the same amplification conditions in the thermal cycler (Quanta BiotechQ Cycler II). The conditions were an initial denaturation step of 120 seconds at 94°C, 10 incubation cycles for 10 seconds at 94°C and 60 seconds at 65°C; and 20 incubation cycles for 30 seconds at 94°C, 60 seconds at 61°C, and 30 seconds at 72°C.

The amplification was read by capillary electrophoresis in QIAxcel Advance equipment (QIAGEN) with the use of the QIAxcel DNA Fast Analysis Kit (QIAGEN). The interpretation of the results was made through the presence of the amplification of the specific band and with the use of Helmberg-Score (Olerup) software.

Results

RBC typings on the three samples were as follows: mother: group A, D+C–c+E+e–; K–; father: group O, D+C+c+E+e+; K–; and baby: A, D+C+c+E+e+; K–. DATs performed on the infant's RBCs with polyspecific AHG and monospecific anti-IgG were positive (2+ and 3+, respectively).

The maternal plasma was positive by the IAT with antibody detection RBCs. Anti-e was identified, reacting by the IAT with untreated and papain-treated RBCs (Table 2). Since anti-C can be present with anti-e, anti-C exclusion was verified by confirming the results on panel cells 1, 3, 5, and 7 in repeat testing. Titer results were 512 and 1024 using untreated and papain-treated RBCs, respectively. These results were supported by the molecular testing that showed the absence of the corresponding primer amplification product for e in the mother but showed its presence in the father and the newborn. (Fig. 1) Based on the serologic and molecular testing and on the phenotype and population frequencies, the haplotypes for the mother, father, and newborn were predicted to be DcE/dcE, DCE/dce, and DcE/dce, respectively. The investigation was concluded as HDFN due to anti-e.

Discussion

HDFN due to antibodies in the Rh system (HDFN-Rh) is usually severe, particularly if due to anti-D, which historically has an incidence of 18 percent in the pregnancies of D– women. With the introduction of Rh immune globulin (RhIG) immunoprophylaxis, the incidence of HDFN due to anti-D dramatically decreased to ~1 percent. In Cuba, its incidence is <1 percent. The remaining cases of RhD-HDFN occur because of sensitization during pregnancy; no administration of RhIG after delivery of a D+ child, an abortion, or other immunizing event (poorly matched transfusions); and administration of an insufficient dose of RhIG to cover a large antigenic stimulus.[4] Maternal alloimmunization against other blood group antigens is very rare.[5]

While antibodies to C, c, E, and e have been implicated as a cause of HDFN, anti-e has been noted to be only weakly antigenic and not a significant cause of severe isoimmunization and hemolytic disease.[6,7]

The e antigen has a prevalence of 98 percent in the white and black populations and 96 percent in the Asian population. In Cuba, the prevalence of e is >98 percent.[8] Because few individuals can produce alloanti-e, the occurrence of HDFN by immunization against this antigen alone is uncommon.[9] The literature contains several case reports.[6,10,11] In 2012, Karagol et al.[12] studied 106 HDFN cases in Turkey caused by antibodies against K, C, c, E, and e. HDFN due to anti-e was reported in 5 (4.7%) of the 106 cases.[12] Our facility is the national reference laboratory for HDFN investigation; there are no cases of HDFN due to anti-e in our historical archives. This case constitutes the first report.

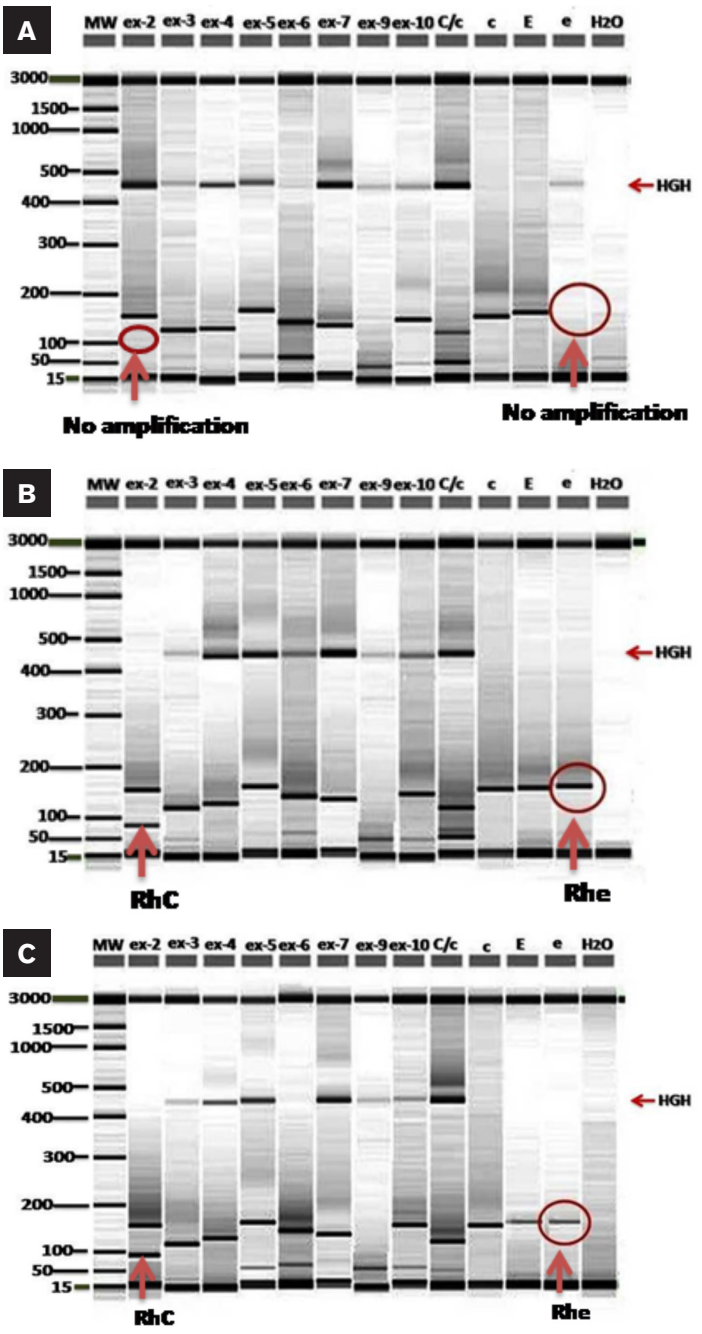

Fig. 1 Amplification products corresponding to the Rh genotype of mother (A), father (B), and baby (C). The control of the amplification used, human growth hormone (HGH), is indicated with the arrow. The presence or absence of amplification for RhC (137 bp) and Rhe (158 bp) are indicated by the circled results of each tested sample. (Because both parents and the baby are D+, the D antigen zygosity is not present.)

Despite the few reports, there are differences between the cases relative to the degree of severity and the presentation of the disease. For example, Hanzlick and Senhauser[13] reported a subclinical HDFN due to anti-e in 1979. Our assessment

of severity is based on the criteria of the Subcommittee on Hyperbilirubinemia of the American Academy of Pediatrics, which assigns a high risk of kernicterus in term neonates when total bilirubin levels are ≥137 μmol/L at 24 hours, ≥222 μmol/L at 48 hours, and ≥290 μmol/L at 84 hours of life.[14]

The most clinically significant forms of HDFN are caused by maternal blood group alloantibodies of IgG1 and IgG3 subclasses, which cause hemolysis more effectively than other IgG subclasses. IgG1 and IgG3 are transported across the placenta by the Fc receptor beginning in the second trimester. Once in the fetal circulation, the antibody binds to antigen-positive fetal RBCs that are then cleared by the fetal spleen. Free hemoglobin is metabolized into bilirubin that is conjugated by the maternal liver.[15]

At birth, the connection to the maternal circulation is severed, causing the risk of neonatal hyperbilirubinemia to increase significantly because of the immature development of the metabolic pathway to break down bilirubin in the neonatal liver. Although most jaundice in newborns is benign, management of hyperbilirubinemia is critical in the neonatal period because of the risk for bilirubin-induced encephalopathy.[14] Affected infants may need phototherapy to oxidize unconjugated bilirubin to allow for urinary excretion. For neonates diagnosed with HDFN, close observation of bilirubin and hemoglobin levels is warranted to determine whether exchange transfusion is needed to remove bilirubin and maternal antibody and/or if transfusions are indicated to support oxygen-carrying capacity to the tissues. Administration of IVIG to the newborn has been used to reduce the need for exchange transfusions and phototherapy, but it does not affect the need for top-off transfusions. A Cochrane review by Rath et al.[16] suggests that more study is needed to determine the best use of IVIG in this setting. When bilirubin reaches critical levels, exchange transfusion is indicated. Blood product selection is similar to that for intrauterine transfusions; however, because the infant's whole blood is being removed, the RBC unit is usually mixed with a plasma unit to create reconstituted whole blood. After a two-volume exchange transfusion, ~90 percent of the RBCs have been replaced and 50 percent of the bilirubin has been removed.[14]

The high prevalence of e in the Cuban population prevented the treatment of the high total bilirubin levels by exchange transfusion in accordance with current clinical practice guidelines.[14] A total of 135 units of RBC concentrates were tested and only 1 unit (0.7%) was e–.

In contrast to RhD- and ABO-HDFN, no critical antibody titer levels were determined for the monitoring of HDFN due to other blood group antibodies. However, in our report, we found high levels of maternal anti-e similar to those induced in RhD-HDFN, which resulted in significant anemia and hyperbilirubinemia. In the obstetric history of the mother, there is no reference to the performance of the IAT for the detection of antibodies against fetal erythrocytes, which limited the ability to detect the disease during the fetal period. Maternal alloimmunization was likely triggered by prior blood transfusions or by fetomaternal hemorrhage in a previous pregnancy. Antigenic stimulation was reinforced during the current pregnancy and is likely the cause of the high antibody titers observed.

The RBC antibody titer, or concentration, helps with further stratification, although the relatively subjective nature of these assays should always be kept in mind.[17] Traditionally, serial antibody titers are used to detect ongoing sensitization, with arbitrary thresholds of increasing antibody strength used to indicate ongoing and increasing immune stimulation, presumably due to the presence of the specific fetal RBC antigen. If there was a previously affected pregnancy, trending of the titer will not be a reliable measure of increasing sensitization. In addition, transfusion laboratories establish critical antibody titers at which the antibody concentration has reached a level that may lead to significant fetal anemia (titers of 16–32 are commonly used).[18]

Very few cases of HDFN due to anti-e are reported in the literature, and there are fewer cases in which other antibodies are also present.[19] Because the mother also lacks C, HDFN could be produced by anti-C that was present but masked by the anti-e. This possibility was addressed by the repeat testing to exclude anti-C performed during antibody identification studies.

The major limitation in our interpretation lies in the inability to confirm that anti-e caused the HDFN by eluting the antibody from the RBCs of the neonate. Umbilical blood samples were not collected for this study because the clinical manifestations in the newborn did not appear until 8 hours after birth, and the peripheral blood sample from the newborn sent to our laboratory was not large enough to perform the elution.

Although molecular studies are used to predict the risk of HDFN in the fetal period, this is not a test of choice for the postnatal diagnosis of HDFN. Given the infrequency of e within the Cuban population, we used molecular testing to corroborate the mother's RBCs were e– and to help us in making the diagnosis.

Currently, more than 100 RBC antigen/antibody pairs are recognized as potentially causing HDFN when incompatibility between mother's antibody and fetal RBCs exists. In most

countries that have prenatal diagnosis programs for HDFN, only those cases in which the mother's RBCs type as D– are monitored for maternal antibody formation. This finding means that HDFN caused by non-D blood group incompatibilities is not diagnosed in time or is overlooked, so these programs must include maternal antibody detection testing in all pregnancies.

At the same time, other considerations in the newborn need to be investigated when acute severe jaundice presents within the first 2 days and when there is an acute severe hemolysis as in this index newborn. Phototherapy and exchange transfusion remain the standard treatment modalities in newborns with severe jaundice. Considering similar pathophysiology to D immunization, however, early IVIG may prevent acute and severe onset of jaundice.

Since HDFN was first recognized, it has been the subject of concern for obstetricians, neonatologists, and hemotherapists. Currently, there is an arsenal of diagnostic and therapeutic methods that allow prevention, early diagnosis, and effective treatment; therefore, whenever the possibility of this disease is suspected, it is necessary to determine the antibody involved and its clinical importance to help reduce the incidence of morbidity and mortality.

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Gilberto Soler-Noda, MS (corresponding author), Auxiliary Professor, Auxiliary Investigator, Laboratory of Immunohematology, National Institute of Hematology and Immunology, La Habana, Habana, Cuba, CP 10400, gsolern@infomed.sld.cu; Yisenia Romero-Díaz, BS, Aggregate Investigator, Laboratory of Immunohematology, National Institute of Hematology and Immunology, La Habana, Habana, Cuba; Lisette Orbeal-Aldama, Specialist of Immunohematology, Laboratory of Immunohematology, National Institute of Hematology and Immunology, La Habana, Habana, Cuba; and Suharmi Aquino-Rojas, BS, Specialist in Medicine Transfusion, Laboratory of Immunohematology, National Institute of Hematology and Immunology, La Habana, Habana, Cuba.