RHESUS D GENOTYPING USING POLYMERASE CHAIN REACTION

To the Editor:

The Rhesus blood group antigens are clinically important because of their highly immunogenic nature. Specifically, they are central in the pathogenesis of Rh hemolytic disease of the newborn (HDN) and some autoimmune hemolytic anemias. Furthermore, in blood transfusion it is important to avoid immunization of Rh-negative recipients, particularly women, with Rh-positive blood and to avoid transfusion of immunized patients with Rh-incompatible blood products. There are five most commonly typed Rh antigens: C/c, E/e, and the D antigen which is the most immunogenic, defining an individual as Rh-positive or -negative. Previously RhD was thought to have an alternative allelic antigen (d); however, Southern analysis has since shown that RhD phenotypes result from the absence of RhD genes that code for the D antigen. That is, RhD-positive individuals have either one or two RhD genes per cell and RhD-negative individuals have no RhD genes at all.

RhD typing was initially performed by agglutination with human polyclonal anti-D sera but has recently progressed to agglutination with IgM and/or blends of IgM and IgG anti-D monoclonal antibodies (MoAbs). However, even these MoAbs may not detect some weak RhD antigens (D*) and RhD variants. Additionally, these serological techniques only allow a probable RhD genotype (one or two D genes) to be assigned based on Rh phenotype and available population statistical data. Often, unambiguous RhD genotypic information is required such as in the case of prenatal counseling of Rh-negative mothers previously immunized with an Rh-positive child.

Using published RhD and RhCcEe genes sequence data, a reliable, single-tube RhD PCR method has been designed enabling not only detection of the RhD gene but also a direct measure of D gene zygosity for a particular individual. Furthermore, this technique can be used to type various samples such as hair follicles, totally avoiding the need for red blood cells.

The published Rh D and CcEe gene sequences differ by only 44 bases. Using sequence differences in exon 7, a forward primer (gattacgaattcGTAACCGAGTGCTGGGGATT) was designed common to both genes, starting at nucleotide position 947, and reverse primers (taccagattaatcATGCCATTGCGGCTC and gattacgaattiCATTGCCGGCTCAGACA), specific for D starting at nucleotide 1058 and CcEe starting at nucleotide 1053, respectively. These PCR primers give 155-bp and 146-bp fragments for the D and CcEe genes allowing these products to be distinguished by agarose gel electrophoresis.

The PCR reaction consisted of 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0 at 25°C), 1% Triton X-100, 2 mmol/L MgCl2, 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP in a volume of 50 μL H2O containing 80 ng forward primer, 120 ng of each reverse primer, 2.5 U Taq polymerase (Promega, Madison, WI) and 50 ng human genomic DNA. It is important that the common forward primer be in reaction limiting amounts for a quantitative result. Standard polymerase chain reaction (PCR) conditions were one cycle of 94°C for 3 minutes, 56°C for 2 minutes, 72°C for 2 minutes, and 35 cycles of 95°C for 30 seconds, 65°C for 1 minute, and a final extension of 72°C for 3 minutes.

The PCR results in Fig 1 show either one PCR product (Rh neg) or two PCR products (Rh pos). The CcEe gene product is the lower band (146 bp) and the D gene product is the upper band (155 bp). The number of copies of the D gene can be determined by comparing the intensity of the two bands. The CcEe gene that has two gene copies per cell acts as an internal PCR control (Rh neg; lane 1).

Therefore, a half intensity of the D band compared with CcEe band predicts a single D gene, a heterozygous carrier of D (lane 2). Likewise, an equal or greater intensity of D band compared with CcEe band (lane 3) indicates two D gene copies, a homozygous carrier for D. In practice, the D band intensity of a homozygote consistently appears slightly stronger than the CcEe band probably because of better priming of the D gene-specific primer. Results were also obtained using whole blood (lane 4) and hair follicles (lane 5).

There are a number of advantages with RhD genotyping by this PCR method. Firstly, PCR using DNA enables the RhD genotype of an individual to be unambiguously known. Prenatal counseling often involves prediction of fetal Rh type and a precise RhD genotype would allow accurate predictions of fetal RhD zygosity to be made and advised. Other applications of RhD PCR genotyping exist in Rh paternity testing and in family and genetic studies using Rh as an inheritance marker.

Secondly, unlike serology, the RhD PCR method does not require red blood cells or large quantities of human tissue. In the case of fetal Rh testing this could lead to safer testing, avoiding the need for percutaneous umbilical blood sampling (PUBS) and the attendant risks of immunization. RhD PCR typing of a fetus would even be possible using any fetal cell containing DNA such as chorionic villi biopsy or even fetal-derived white blood cells circulating peripherally in the pregnant mother. Another application exists in forensic investigations where materials such as hair, teeth, seminal fluid, or dried blood could potentially be used, virtually independent of age, quantity, and quality.

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REFERENCES
Rhesus D genotyping using polymerase chain reaction [letter] [see comments]

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