Detection of RhD_{el} in RhD-negative persons in clinical laboratory

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The Rhesus (Rh) blood group is the most polymorphic human blood group system, and it is clinically significant in transfusion medicine. About 15% of Caucasoid people are RhD-negative, whereas in the Asian population, the RhD-negative blood type only occurs in 0.1% to 0.5%. However, approximately 30% of apparently RhD-negative Taiwanese people actually were RhD_{el}. Traditionally, we verify RhD_{el} by a serologically adsorption-elution procedure with polyclonal anti-D. In our recent report, RhC phenotype is highly associated with RhD_{el}, and *RHD*1227A is a useful genetic marker for RhD_{el}. For setting up a rapid protocol to detect RhD_{el} in clinical laboratory, a total number of 395 Taiwanese serological RhD-negative blood samples, those with RhC (+) phenotypes as selected by serological tests, were further screened by adsorption/elution tests and *RHD*1227A allele by specific sequence primer-polymerase chain reaction (SSP-PCR) for RhD_{el}. Among 395 blood samples collected from RhD-negative subjects, the incidence of RhC (+) was 43% (171/395). One hundred and twenty six of the 171 RhC (+) samples were positive for both adsorption/elution for RhD detection and SSP-PCR assay for *RHD*1227A. The sensitivity and specificity were 96.9% and 97.5%, respectively, for *RHD*1227A detection as compared with the traditional adsorption/elution test. Our results also indicated that *RHD*1227A was highly linked to Ce haplotypes (95.2%). In conclusion, combined RhC (+) phenotyping and *RHD*1227A analysis can be a simple and accurate laboratory screening protocol for RhD_{el} detection in RhD-negative population. (J Lab Clin Med 2005;146:321–5)

Abbreviations: Rh = rhesus; SSP-PCR = specific sequence primer-polymerase chain reaction; HDN = hemolytic disease of newborn

Rh antigens are acylated red cell transmembrane proteins with a molecular weight of 30–32 kDa and encoded by two highly homologous genes *RHD* and *RHCE*. Both genes are located on chromosome 1p34-36 and about 30,000 bp apart. Although *RHD* appears as an ancestral duplicate of *RHCE*, which occurs in four allelic forms ce, cE, Ce, or CE.[1] They have opposite orientation and are highly homologous retaining more than 90% identity.[2,3] The Rh blood group is the most polymorphic human blood group system, but with high clinically significance in transfusion medicine. Persons are clinically classified as Rh-positive and Rh-negative depending on the presence or absence of the D antigen on the red cell surface.

The RhD-negative trait could be generated by multiple genetic mechanisms, which have been shown to be ethnic group-dependent. About 15% of Caucasoid people are RhD-negative; most are associated with the

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deletion of *RHD* gene between the upstream and downstream *Rheus* boxes.[4,5] However, the RhD-negative blood type only occurs in 0.1% to 0.5% of the Asian population.[6–9] In contrast to Caucasoid persons, nearly 30% of the Chinese RhD-negative were RhD_{el}, a rare variant of the Rh system with intact *RHD* gene, but the D antigen is detectable only by adsorption and elution tests.[7,8] In recent years, the effort to molecular catalog Rh genetic variations has continued to impact on transfusion medicine. Based on our previous molecular studies, *RHD*1227A was an important genetic marker in RhD_{el} persons.[9–12]

A considerable proportion of seemingly RhD-negative samples in Asian are carrying the intact *RHD* gene presented RhD_{el} phenotype.[8–12] The classic adsorption/elution method for RhD_{el} typing is difficult and impractical for use in all transfused patients. Therefore, the development of a simple laboratory protocol for correct detection of RhD_{el} is very important especially in Taiwan and some other countries that have a high prevalence of RhD_{el}. This rationale prompted us to investigate the performance of combined RhC phenotyping by serological testing and genotyping identification of *RHD*1227A by SSP-PCR in distinguishing RhD_{el} and true D negative in 395 RhD-negative samples, which allowed us to set up a protocol for RhD_{el} identification feasible in a routine laboratory.

MATERIALS AND METHODS

Serological Rh phenotyping. A total of 395 RhD-negative unrelated blood donors were kindly provided by Tainan and Kaohsiung Blood Centers, Chinese Blood Service Foundation. The above apparently RhD-negative blood samples were serotyped for RhC/c and RhE/e antigens by monoclonal anti-C, anti-c, anti-E, and anti-e antibodies (Gamma Biologicals, Houston, Texas). RhD-negative samples with RhC (+) were further screened by adsorption and elution tests with polyclonal anti-D (Dominion Biologicals, Dartmouth, Canada), and *RHD*1227A polymorphism by SSP-PCR.

Adsorption/elution test for RhD. Adsorption/elution tests were performed on 171 RhC (+) samples. Red blood cells were incubated with an equal part anti-D (Dominion Biologicals) at 37°C for 1 hour. The cells were then washed thoroughly. An eluate was prepared by heat elution technique. The eluates and last-washed supernatants were used for indirect antiglobulin tests (IAT) against an in-house panel made up of RhD-positive and RhD-negative red cells.

***RHD*1227 analysis.** Genomic DNA was extracted from buffy coat fraction by a commercial kit (Blood & Tissue Genomic DNA Extraction Miniprep kit; Viogene, Hsichih, Taiwan). *RHD*1227 polymorphism in samples with RhC (+) was determined by SSP-PCR. Forward primer for *RHD*1227A allele was 5′-GATGACCAAGTTTTCTGGAAA-3′ and for *RHD*1227G allele was 5′-GATGACCAAGTTTTCTGGAAG-3′, respectively. The reverse primer for both *RHD*1227A and *RHD*1227G, 5′-GTTCTGTCACCCGCATGTCAG-3′, was used to amplify a 348-bp product.[10] Another pair of nucleotides (forward primer: 5′-GCCTTCCCAACCATTCCCTTA-3′, reverse primer: 5′-TAGACGTTGCTGTCAGAGGC-3′) were included as an internal control to generate a 629-bp PCR fragment from the growth hormone gene. The PCR reactions were performed at a total volume of 10 μl, each containing 1 μl of genomic DNA, 0.5 U DNA polymerase (Supertherm Gold, Sevenoaks, England), 200-μM dNTPs, primers, and 2.5-mM $MgCl_2$ in a buffer provided by the manufacturer. Forty cycles were programmed on a thermocycler (PE 9600 GeneAmp PCR system; Applied Biosystems, Foster City, Calif) as follows: denaturation at 94°C for 5 min, then 35 cycles of 30 seconds at 94°C, 40 seconds at 68°C and 30 seconds at 72°C. PCR products were visualized in a 2% agarose gel.

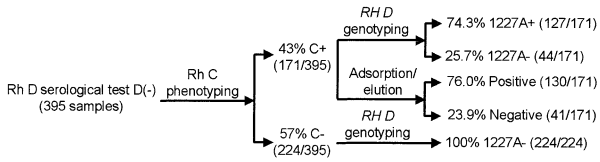

Fig 1. Combined RhC phenotyping and *RHD*1227A genotyping in 395 RhD-negative subjects.

***RHD* zygosity determination.** Allele-specific primers were designed to directly amplify the hybrid *Rheus* box[13] (forward primer: 5′-TGAGCCTATAAAATCCAAAGCAAGTTAG-3′; reverse primer: 3′CCTTTTTTTGTTTGTTTTTGGCGGTGC 5′). An internal control of the 629-bp PCR fragment from the growth hormone gene was included in the amplification reaction. Thermal profile started with a first denaturation step of 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, annealing at 67°C for 1 min, and extension at 72°C for 2 min, with a final step of 72°C for 5 min. The PCR products were visualized on 1% agarose gels with ethidium bromide staining. Consequently, the primers selectively amplify a 2778-bp segment of the hybrid *Rheus* box that can accurately detect *RHD* gene deletion.

RESULTS

The incidence of RhC (+) of the apparent RhD-negative persons was 43% (171/395). The incidences of RhD_{el} in RhC (+) persons as determined by *RHD*1227A, and adsorption and elution test were 74.3% (127/171) and 76.0% (130/171), respectively (Fig 1). The comparative results of these 2 tests are shown in Table I, 126 of the130 RhD adsorption/elution-positive (RhD_{el}) samples were also positive for *RHD*1227A by SSP-PCR analysis (sensitivity 96.9%), and 40 out of 41 RhD adsorption/elution-negative samples were negative (specificity 97.5%). Only 5 samples revealed an inconsistent result detected by these 2 methods. However, all 224 RhC (−) samples were negative in *RHD*1227A detection (Fig 1).

To understand the association of *RHD*1227A and *RHCE* loci, further analysis of the 126 RhD_{el} samples (with both *RHD*1227A and adsorption and elution pos-