الأنماط الجينية للعامل الريزيسي في قطاع غزة - فلسطين

المتخص

يعتبر العامل الريزيسي من أهم وأكثر الفصائل الدموية تعقيدًا، حيث يحتوي هذا النظام على عدد كبير من الانتصاعات التي هي عبارة عن مواد بروتينية تخلق بواسطة رجوع من المورثات الجينية المتداخلة، الموجودة على الكروموسوم رقم 1. انها المورثات يقدم RHC والـ RHD والتي يعتبر الأساس في هذا النظام والية تعزى فصيلة الدم السارية والموجبة.

RHD Gene Polymorphism among Palestinians of Gaza Strip.
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Rhd أورثى وأمنوًا، ولا أورثى طبية بالغة في مجالات تقلل الدم وهم ذو قدرة عالية بعد Rhd على إحداث رد فعل مناعي لتكوين أجسام مضادة وتركيز عال، والتي تسبب السبب الرئيسي في مرضى تقلل الدم عند الأطفال حديثي الولادة، والكثير من حالات عدم التطرق أثناء عملية تقل الدم. الفصيلة السارية للدم عادة ما تكون من علاج أو إزالة كنتيجة لخلال في المورثة، والتي تنتج اما عن طفرة أحادية في المورثة أو حدوث عملية تهجين بين كلًا المورثتين RHD و RHC في نظام Rh و في Anti-D لذا الفرصة على حث جهاز المناعة لتكوين أجسام مضادة. نتائج والتي يمكن أن تسبب تقلل الدم عند الأطفال حديثي الولادة.

Rhd أورثى وأمنوًا، ولا أورثى طبية بالغة DNB و Dvi ويطلق عليها Partial D وهم ذو قدرة عالية بعد Rhd على إحداث رد فعل مناعي لتكوين أجسام مضادة وتركيز عال، والتي تسبب السبب الرئيسي في مرضى تقلل الدم عند الأطفال حديثي الولادة.

PCR-SSP.

الكلمات المفتاحية: النظام الريزيسي، العامل الريزيسي D والمورثة الهجينة.
Abstract

Rh system is one of the highly complex blood group systems with many serologically defined Rh antigens. These antigens are expressed by proteins encoded by a pair of highly homologous genes located on chromosome 1. RHCE gene encodes the CcEe antigens, while the RHD encodes the D antigen. RhD is the most important, immunogenic and polymorphic Rh antigen from the clinical aspects (comprising at least 30 epitopes), as it plays a key role in transfusion medicine. Anti-D antibodies remain the leading cause of the hemolytic disease of the newborn (HDN), and antigen D compatible transfusion is a standard practice in transfusion therapy. Partial D lacks one or more D epitopes, and a partial D individual may be immunized on exposure to a normal D positive during blood transfusion or pregnancy. The DVI and DNB variants are the most frequent partial Ds that lack some D epitopes, DVI is usually typed as D negative while DNB is typed as D positive.

We have examined 102 genomic DNA samples collected from blood donors expressing D positive (79 samples) and negative phenotypes (23 samples), to detect DVI and DNB variants, and to investigate the molecular basis of Rh negative phenotype. To verify the DVI variant; simplex PCR was used to detect the presence or absence of RHD exon 10/intron 4, while PCR-SSP was used to detect the DNB variant. Three DVI and three DNB samples were detected. The PCR results indicated a deletion of RHD gene in D negative specimens.

The results show that the frequency of the DVI phenotype in Palestinians is greater than expected and routine screening for this phenotype should become mandatory for equivocal weak D blood samples.

Key words: RHD gene, DVI, DNB, Partial D.

Introduction:

The Rh system is one of the most and highly complex blood group systems known in humans, with many serologically different Rh antigens. These antigens are expressed as part of a protein complex on the red blood cell (RBC) membrane. The expression is confined to the erythroid cell line, and therefore Rh antigens are only expressed on RBCs. The composition of the complex is unknown, but it is supposed to be a tetramer, consisting of two molecules of Rh associated glycoprotein (RhAG) and two molecules of Rh proteins. The Rh proteins may be RhD (carrying the D antigen) or RhCE (carrying the C or c antigen and the E or e antigen). It is unknown whether both RhCE and RhD can be in a single complex, but in D negative individuals the complex would only contain RhCE [1,2].

The significance of Rh system is related to the facts that: 1) The Rh antigens are highly immunogenic and of great importance for transfusion medicine; 2) The complexity of its antigens, which stems from the highly polymorphic genes that encode them; 3) The great differences among races in the frequencies of the alleles of the RH gene complex. According to those facts, the Rh system remains the most
polymorphic and immunogenic blood group system known in humans [1].

The D antigen is the most important Rh antigen consisting of at least 30 epitopes. Partial D phenotypes occur when there is absence of one or more of these epitopes. The DVI phenotype is the most common partial D phenotype in Europeans, lacking most D antigen epitopes. DVI mothers may become immunized by transfusion with D positive blood (if typed as D positive using polyclonal typing reagents) or by fetuses which have all of the D epitopes. This situation can give rise to HDN [3].

DNB is another partial D that presents a normal D in routine typing, indicating that DNB carriers are generally able to produce strong allo-anti-D [4].

Despite the importance of the Rh antigens in blood transfusion and HDN, the function of its proteins is speculative, and may involve transporting ammonium ions across the RBC membrane and maintaining the integrity of the RBC membrane. Substitutions of amino acids that are located in Rh transmembrane segments, may affect the function of the Rh protein [1]. New evidence, however, suggest that the RhAG possesses a channel for passage of neutral gases, and that D and CE polypeptides are unlikely to have a transport function [5].

Hitherto, no previous studies were conducted in the field of blood transfusion medicine and its adverse reactions in Gaza Strip, Palestine. RhD typing discrepancies of blood donors, patients, mothers and babies which complicate the transfusion process are not uncommon. To detect and characterize the more frequent partial Ds namely, DVI and DNB, and to identify the molecular basis of Rh negative phenotype, PCR-SSP and monoplex PCR were used to screen 102 positive and negative RhD blood donors’ specimens.

Materials and Methods:

Study design:

This descriptive study was designed to detect the D variants that are frequent in other ethnic groups (namely; DVI and DNB) and to determine the molecular basis of RhD negative phenotype among a group of blood donors residing in Gaza Strip.

The detection of DVI variant is based on exploiting the differences between RHD and RHCE genes, where, RHCE has a larger intron 4, whereas the RHD gene has a larger exon 10. DVI phenotype is characterized by absence of RHD intron 4. Thus, the presence of RHD exon 10 with RHCE intron 4 along with absence of RHD intron 4 is a strong indicator for the existence of the RH D/CE/D hybrid allele and consequently the presence of DVI phenotype [6].

Amplification of both RHD exon 10/ intron 4 is also used for investigating the molecular basis that generates the Rh negative phenotype. To define the DNB variant, PCR-SSP was used to detect the point mutation in RHD exon 7 that causes G355S substitution [4,7].

Blood samples:

The D negative and D positive blood samples used in this study were collected at random from 102 unrelated Palestinian blood donors: 79 RhD positive and 23 RhD negative. Ninety blood donor samples were obtained from Al Shifa hospital Central Blood Bank, and 12 blood donor samples were obtained from the Central Blood Bank Society.
Ethical Considerations:
The local ethics committee (Helsinki Committee) approved the study protocol.

Serological RhD typing:
Phenotyping of the RhD for all samples was performed in blood bank centers using commercial monoclonal anti-D (LORNE Laboratories, UK). Blood samples were serologically typed for RhD by the method of direct hemagglutination test according to the manufacturer protocol.

Molecular analysis:

DNA Extraction:
DNA was extracted from blood samples using Wizard® Genomic DNA purification Kit (Promega Corporation, Madison, USA). Extraction was done following the manufacturer instructions.

DVI Detection by PCR:
All D positive and D negative genomic DNAs were examined for DVI. The method is based on analyzing two RHD regions by using two separate monoplex PCR assays.Briefly, the assay involved the amplification of RHD exon 10/ intron 4, and RHCE intron 4, the latter was used as an internal control. The first region that was subjected for PCR investigation was the RH intron 4. Specific primers; 4-F (CGATACCCAGTTTGTCTGCC) and 4-R (AGAACATCCACAAGAGGG) were used to distinguish and identify intron 4 of both RHD and RHCE. The two RHD and RHCE intron 4 PCR fragments were obtained from the same primer pair, when the primers paired with the RHD intron 4, it amplified a product of 478 bp, whereas, when paired with the RHCE intron 4, a product of 1126 bp was produced. Each PCR run was performed with necessary negative controls [8].

The second target region that was subjected to PCR was RHD exon 10. The specific primers; 10-F (TTGGATTTTAAGCAAAGCATCC) and exon 10-R (ATTCTCCTCAAAGAGTGCGAG) were applied to amplify and detect a sequence located between RHD specific 3’ untranslated region (UTR) and RHD exon 10, which produced a product of 185 bp [8].

The two separate PCRs were performed in similar conditions and carried out in 0.2 ml PCR microfuge tubes with a final volume of 20μl, containing, 2 μl of 2.0 μmol of each specific primer, 10 μl of PCR Mater Mix (Promega Madison, USA), 2.0 μl of template genomic DNA (100-200 ng), 4.0 μl of nuclease free ultra-pure water. The following profile was used for both RHD exon 10 and RHD/RHCE intron 4 PCR assays. After an initial denaturation for 10 minutes at 94°C, the samples were subjected to 30 cycles of PCR in a DNA thermal cycler (Eppendorf, Germany). Each cycle consisted of: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes, followed by a final extension at 72°C for 5 minutes. Equal volumes of the RH intron 4 and RHD exon 10 PCR products were mixed and analyzed by electrophoresis on 1.5 % agarose gels containing 10 μg/ml ethidium bromide.

PCR-SSP for DNB Detection:
A PCR with sequence specific priming (PCR-SSP) was used to detect or to confirm the 1063G→A substitution in the RHD exon 7 (DNB allele). DNB specific sense primer re77 (TCTCCACAGCTCCATCATGGG) and antisense primer DNBb (CAGTGACCCAC ATGCCATTACT) at a concentration of 2.0 μmol were
used to detect the specific mutation in \textit{RHD} exon-7. PCR should amplify a fragment with 118 bp product across \textit{RHD} exon 7 \cite{4,7}. Human growth hormone (HGH) gene was used as a positive control where a 434 bp fragment was co-amplified using the primers (hgh-F) (tgccctcacaacacctcctta) (intron/intron) and (hgh-R) (ccactccaggttcttggtgctc). Amplification was carried out in 0.2 ml PCR microfuge tube with a final volume of 20 μl, each containing 2 μl of 2.0 μmol DNB primers (DNBrec77, DNBb), 0.5 μl of 2.0 μmol HGH primers (hgh-F, hgh-R), 10 μl of PCR Mater Mix (Promega Madison, USA), 1.5 μl of template DNA (100-200ng), 3.5 μl of nuclease free ultra-pure water. Each PCR run was performed with a negative control without a DNA template.

Thirty two PCR cycles were performed on the DNA consisting of an initial denaturation of 2 min at 94°C, followed by ten cycles of 10 seconds denaturation at 94°C, 1 min annealing/extension at 65°C, and 30 sec at 72°C followed by 22 cycles of 30 sec denaturation at 94°C, 1 min annealing at 61°C, 30 sec extension at 72°C and a final extension of 4 min at 72°C. The PCR products were then analyzed by electrophoresis as described above.

**Statistical Analysis:**
z test for two proportions, at 95% confidence level, was applied to test for significant difference between the Dvi frequency observed in this study and that reported for Caucasians.

**Results:**

**Immunohematology:**
All the 102 donor blood samples were serotyped for RhD using commercial MoAb. Seventy nine samples were D positive and 23 were D negative.

**Molecular screening for Rh D\textsuperscript{VI}:**
Detection of the D\textsuperscript{VI} alleles depends on the absence of \textit{RHD} intron 4 with concomitant presence of \textit{RHD} exon 10. The D\textsuperscript{VI} phenotype was verified by using two independent PCRs: one PCR coamplifies introns 4 of the \textit{RHD} and \textit{RHCE} genes, and produces two products, a 478 bp corresponding to \textit{RHD} intron 4 and a 1126 bp from \textit{RHCE} intron 4 (was also used as an internal control) from D positive genomes (two bands were detected). The D\textsuperscript{VI} which is characterized by absence of \textit{RHD} intron 4 (should yield only one 1126 bp fragment) and the same applies for RhD negative specimens. The second PCR primer pair is specific for exon 10 of the \textit{RHD} gene only, and amplifies a product of 185 bp in D positive and D\textsuperscript{VI} but not in D negative samples.

Three patterns of PCR products were apparent when the mixture of the two PCR products was analyzed: 1) Detection of both \textit{RHD} intron 4/ exon10 regions. 2) Absence of both \textit{RHD} regions, 3) Presence of \textit{RHD} exon 10, but absence of \textit{RHD} intron 4. Of the 102 blood donors tested, 79 samples (77.5%) had both regions of \textit{RHD}, 20 samples (19.6%) lacked both \textit{RHD} regions and 3 samples (2.9%) had \textit{RHD} exon 10 only (Figure 1). \textit{RHCE} intron 4 was detected in all samples.
Screening D positive donor samples for RHD exon 10 and intron 4:
The first pattern was observed in RhD positive blood donors. When we screened the 79 serological RhD positive samples, all samples (100%) showed the presence of both introns 4 and exon 10 of RHD gene. Intron 4 of RHCE gene was always detected and indicated the success of PCR amplification (Figure 2).

RhD negative:
Of the 23 D negative blood specimens examined, twenty D negative (87%) lacked both RHD regions but retained the RHCE intron 4 band (the internal control). The specific primers could not recognize the presumably deleted sequences of RHD gene.

DVI variant:
The assay mentioned above was discordant in three D negative phenotypes. Of the 23 D negative samples 3 (13%) had RHD exon 10 only, suggestive for a DVI variant. See Figure 2 for representative results.

Statistical Analysis:
z test for two proportions (with a z value of 9.63) revealed a highly significant difference between Dvi frequency observed in this study (2.9%) and that reported for Caucasians (0.02%).
Molecular screening for DNB:
All the 79 D-positive blood samples were examined for DNB variant. The PCR-SSP amplified a product of 118 bp across *RHD* exon-7 in 3 of the 79 D-positive, and these were considered as DNB variants. The rest of the samples yield no product indicating absence of G355S substitution. The 434 bp product of human growth hormone gene (*HGH*) which served as an internal control was evident in all amplifications (Figure 3).

**Figure 3.** A photograph illustrating PCR-SSP results for detection of DNB allele. Samples # 10, 12 and 13 represent positive DNB results. Sample # 9 is negative for DNB. HGH represents the control 434 bp product of human growth hormone gene. S.M is the size marker.

Discussion:
The current study tested the correlation between the serological phenotype of Rh blood group and the molecular structure of the *RHD* gene and the relevant alleles.

In individuals expressing normal D phenotype a full concordance was observed between serological typing and presence of an intact *RHD* gene, where the two usually examined *RHD* regions (exon 10 and intron 4) were detected by PCR assay.

The frequency of D positive/negative is not yet estimated in the Palestinians residing in Gaza strip, but in Palestinians residing in Kuwait the D positive phenotype was reported as 92% [9]. In other ethnic groups like Caucasians, the D positive frequency was found to be about 82%-85%, in Africans 93%-97% and about 99% in the Far East populations [2,10,11].

The D negative phenotype is generally due to homozygosity of *RHD* gene deletion. Our results showed that, of the 23 serologically D negative donor samples, 20 D negative samples (87%) were completely concordant with both exon 10 and intron 4 bands absence pattern, which according to many authors indicates a gross deletion of the *RHD* gene [1,10,12]. PCR analysis for the rest of the *RHD* gene exons/introns and DNA sequencing are needed in order to support this conclusion.

The examination of *RHD* intron 4 and exon10 of negative samples by using the specific primers excluded the confusion of either *RHD* pseudogene (*RHD*²) or any other unexpected molecular variants with grossly intact *RHD* genes. This result supports the hypothesis that, the majority of D
negative genomes examined, appear to lack all portions of the RHD gene. In D negative African Americans and South African people of mixed race, 54% of African Americans and 81% of South African donors of mixed race were shown to have no RHD gene [13].

Other workers have described variant D negative English and Scottish individuals with an apparently intact RHD gene, and intact RHD transcripts. The molecular basis was explained by a point mutation in the RHD gene at codon 41 generating a premature stop codon. This pattern was also shown in Japan, where intact RHD gene was found in 27.7% of the D negative Japanese donors. Still, other investigators found a 37 bp insertion in RHD exon 4 introducing a premature stop codon at position 210. Two-thirds of D negative Africans were found to carry this mutant RHD allele [11,14].

DVI phenotype is the most common among Europeans and the most clinically significant partial D phenotype in transfusion medicine. It lacks most epitopes compared to any other D variant, with very low antigen density. Among the D variants, DVI is still considered as the major cause of allo-immunization with anti-D [2,3,15,16]. Of the 23 D negative blood donors investigated in this study, the PCR assay results of 3 samples (13%) proved the absence of RHD intron 4, and these were considered as DVI variant.

Many investigators confirmed that all RHD genes in individuals expressing the DVI phenotype lack intron 4 of the RHD gene. A hybrid RHD-RHCE-RHD gene, resulting from a probable gene conversion event in which the complete RHD gene exons 4, 5, and 6 are replaced by the corresponding exons of the RHCE gene is the most accepted mechanism for generating this DVI phenotype [2,3,11].

The negative phenotype of the DVI cannot be explained by the lack of the RhD epitopes alone, but may be also due to a reduced number of RhD proteins accessible on the RBCs' surface. The four known DVI types (I, II, III, IV) have the same clinical significance, because all types share RHCE exons 4 and 5 and their negative reaction patterns don't differ with polyclonal and most monoclonal antibodies and all four types can readily become allo-immunized when encountering conventional D [2,3,6]. The frequency of DVI variant differs among populations, the phenotype has a frequency ranging between 0.02% to 0.05% in Caucasians, about 0.02% in Germany, and 0.04% in the English people [15,16]. The results of our study show that the frequency of the DVI phenotype in Palestinian, and probably in Arab populations, may be greater than expected. Moreover, statistical analysis revealed that the observed frequency (2.9%) is significantly different from that reported for Caucasians, a result suggesting differences in the genetic composition of the two populations.

Several partial D antigens permit anti-D immunization in their carriers and DNB variant is one of these. In routine D typing, DNB samples are usually typed as positive RhD and are agglutinated by most anti-Ds, including almost all commercial anti-D typing reagents.

Although the DNB variant lacks 4 epitopes (epD6 and epD31 as well as part of epD18 and epD23), the three
samples observed in this study were serologically typed as D positive without noticeable weakening of the antigen D reaction. Of the 79 D-positive donors, 3 (3.8%) were positive with PCR-SSP. This finding confirms one of the mechanisms that generate the partial D phenotype, where a point mutation in the exofacial loop 6 characterizes the DNB features and classifies it as a partial D [4,12].

The application of molecular approaches in determining the genetic polymorphisms of RHD, their frequencies and distribution in the population have critical importance for the practical application of blood transfusion, for example, RHD PCR may allow the identification of D positive blood units missed by routine serology. In instances where DVI phenotype blood donors are identified as D negative by use of inappropriate D typing reagents, the recipient will be at risk of alloimmunization, so appropriate anti-D reagent must be used to type the DVI blood donor as a D positive. On the other hand, DVI recipient should be treated and typed as D negative, triggering D negative transfusions, which is the clinically favored management. Because the frequency of partial D is low, the D negative transfusion would not compromise the D negative blood supply. Similarly, when DVI phenotype mothers are identified as D positive by use of inappropriate D typing reagents (e.g. polyclonal anti-D), DVI mothers may become alloimmunized against their normal D positive infants where prophylactic anti-D is not administered [2,4,7,16]. In cases where alloimmunization has occurred prenatal detection of a fetus carrying a normal RHD gene can be achieved by PCR analysis of fetal DNA [17].

Today, DVI may be detected specifically by suitable combinations of monoclonal anti-D antibodies. This strategy became mandatory in Germany since 1996, and in the United Kingdom as well, where, D typing reagents are adopted to type DVI patients and mothers as D negative. All commercial monoclonal anti-Ds reagents that don't bind DVI can agglutinate DNB, therefore, a serologic strategy for detecting DNB would have to rely on a separate anti D that discriminates DNB from normal D. D negative transfusion strategy for DNB may be advantageous.

Conclusion:

The present study revealed that the D negative phenotype is common in our population and is largely due to complete deletion in the RHD gene. Two partial D variants namely DNB and DVI were detected and these should be considered in refining the transfusion strategy in our country. Further work is needed to unravel the allelic diversity of RHD gene in the Palestinian population and other Arab nations.

References:


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