Allele-specific oligonucleotide polymerase chain reaction for the determination of Rh C/c and Rh E/e antigens in thalassaemic patients

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Background. Thalassaemia is a genetic disease in which there is a relative or complete lack of alpha or beta globin chains. Patients with moderate to severe forms of thalassaemia need transfusions from the early years of life. Antibody production against blood group antigens may cause many problems in preparing compatible blood units for transfusion. The identification of definite blood group phenotypes by the haemagglutination method can be difficult because of the mixed population of red blood cells from the donor and recipient.

Materials and methods. Forty multiply transfused thalassaemic patients and ten healthy controls with no history of blood transfusion were enrolled in this study. Allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) and haemagglutination methods were used to determine the presence of Rhesus (Rh) C, c, E and e antigens.

Results. In this study four primer sets were used for ASO-PCR amplification of RhC/c and RhE/e. Although PCR assays for RhC/c and RHE/e genotyping have been described previously, in this study we used a new condition for PCR by decreasing the annealing temperature from 63 ℃ to 58 ℃ in order to amplify all four genes in the same condition. In order to evaluate this single run molecular method, we used the haemagglutination test as the standard method and compared the results from the two methods. We found discrepancies between phenotype and genotype results among patients with beta thalassaemia, but complete agreement between phenotype and genotype in the control group.

Conclusions. The advantage of this new ASO-PCR method compared to a restriction fragment length polymorphism (RFLP) PCR method is that with the former all four genes can be amplified at the same time by PCR, and electrophoresis can be performed immediately to determine individual antigen profiles. The simplicity of the ASO-PCR method makes it suitable for routine use in medical centres and it is also cheaper than RFLP-PCR. Furthermore, as shown by previous studies, the results of haemagglutination and PCR tests often differ because the existence of donor red blood cells in the patient's circulation can interfere with the interpretation of the haemagglutination test.

Key words: Rh typing, polymerase chain reaction, thalassaemia, allele-specific oligonucleotide polymerase chain reaction, ASO-PCR.

Introduction

Thalassaemia is a genetic disease in which there is a relative or complete lack of alpha or beta globin chains. Patients with moderate to severe forms of thalassaemia need transfusions from the early years of life. Regular blood transfusion is necessary for the satisfactory growth and development of thalassaemic patients, to improve their living conditions during

childhood and to sustain a good quality of life during adulthood[1]. Repeated blood transfusions are, however, associated with a risk of alloimmunisation and the incidence of this complication in multiply transfused patients is about 30%[2,3]. Alloimmunisation may cause many problems with regards to long-term management and transfusion. It can lead to haemolytic transfusion reactions as well as create difficulty and delays in finding compatible blood units[4,5].

The Rhesus (Rh) blood group system has the highest prevalence of polymorphisms among human blood group systems and is clinically significant in transfusion medicine. The genes coding for the Rh antigens are located on the short arm of chromosome 1 (1p34-p36) and consist of two adjacent gene structures, RHCcEe and RHD. The Rh blood group system comprises more than 50 different antigens with very high polymorphism. Proteins C, c, E and e are detected as immunological isoforms[2,6]. Nowadays only compatibility for RhD is tested serologically on blood units before transfusion. Antigen compatibility for the other systems, such as C, c, E and e, is not tested routinely until the patient shows alloantibodies against them. The production of alloantibodies against these antigens will, however, cause problems in preparing compatible blood units for transfusion. In multiply transfused patients, donors' red blood cells circulate in the recipients' vessels for a few weeks and a precise diagnosis of blood group phenotypes is rendered difficult by the presence of the mixed population of donors' and recipients' red blood cells, especially when patients' pre-transfusion blood samples are not available[8]. Since anti-RhC/c and anti-RhE/e may also cause transfusion reactions, their identification in multiply transfused patients, such as those with thalassaemia, is important[9].

Materials and methods

Forty multitransfused thalassaemic patients and ten healthy controls with no history of transfusion were enrolled in this study. Peripheral blood samples (4 mL) were drawn into test-tubes containing EDTA. The Rh phenotypes were determined by the haemagglutination method (CE-Immunodiagnostika, Germany).

Genomic DNA was isolated from whole blood using a commercial kit (KIAsorb purification kit). The primers that were used for RhC/c and Rh E/e genotyping are shown in Table I as described by Tanaka M. *et al.*[10]. Polymerase chain reaction was conducted using 100 ng of genomic DNA, 1 μM of each primer, 200 μM of each dNTP, 2.0 U Taq DNA polymerase, and 1.5 mM $MgCl_2$ in buffer, provided by the polymerase manufacturer, in a final volume of 50 μL. The amplification reaction were carried out in a thermal cycler (Gradient, Eppendorf). After initial denaturation at 94 ºC for 10 min, 30 three-step cycles at 94 ºC for 30 sec, 58 ºC for 45 sec and 72 ºC for 45 sec were performed, followed by 5 minutes of elongation at 72 ºC (Table II). PCR products were analysed by electrophoresis in 3% agarose.

Table I - PCR primers for RhC/c and RhE/e ASO-PCR.

Primer name	Sequence 5' to 3'	Product size	Allele specificity
TRH 1	CGCTGCCTGCCCCTCTGC	118	C
TRH 2	TTGATAGGATGCCACGAGCC		
TRH 3	CTTGGGCTTCCTCACCTCAAA	107	c
TRH 4	AAGCCGTCCAGCAGGATTGC		
TRH 5	TGGCCACGTGTCAACTCTC	143	E
TRH 7	CATGCTGATCTTCCTTTGGG		
TRH 6	TGGCCACGTGTCAACTCTG	143	e
TRH 7	CATGCTGATCTTCCTTTGGG		

Table II - PCR conditions for amplification.

Stage		Time	Temperature (℃)
		10 min	94
30 cycles	Denaturation	30 sec	94
	Annealing	45 sec	58
	Extension	45 sec	72
Final elongation		5 min	72

Results

In the present study four primer sets were used for allele-specific oligonucleotide (ASO)-PCR amplification of RhC/c and RhE/e in two genomic regions: exon 1 and exon 5. Primers TRH1/2 and TRH3/4 were used for RhC/c ASO-PCR and primers TRH5/7 and TRh6/7 were used for RhC/c ASO-PCR. Although PCR assays for RhC/c and RHE/e genotyping have been described previously, in this study we used a new condition for PCR by decreasing

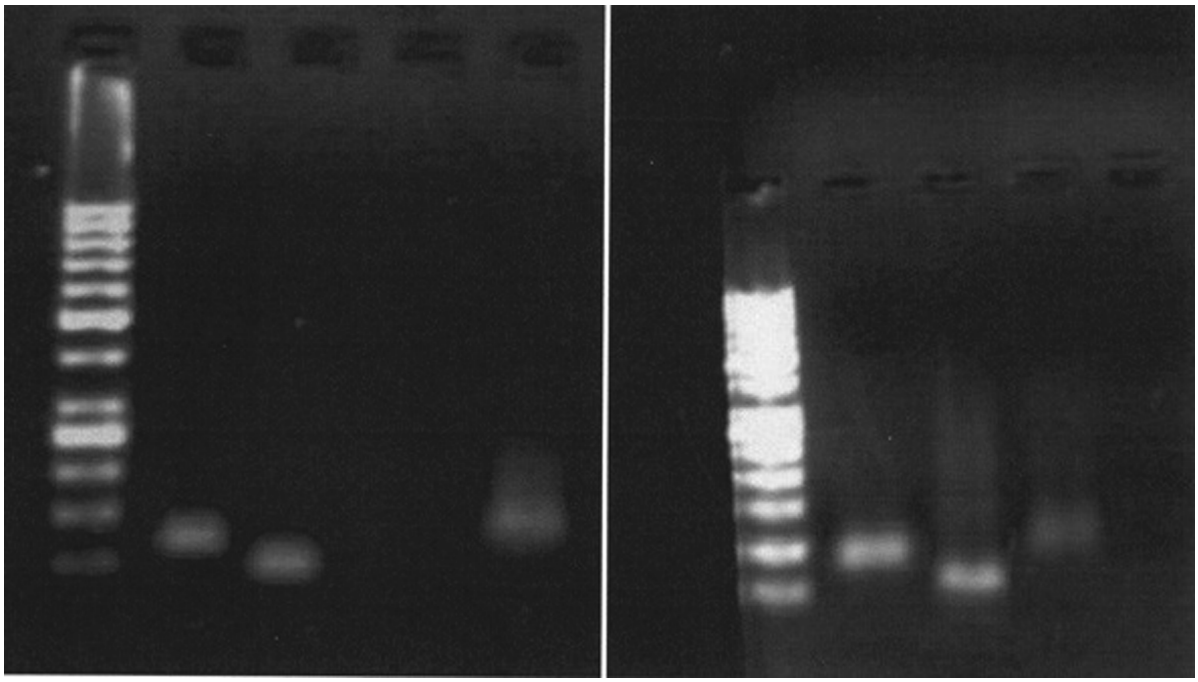

Figure 1 - RhC/c and RhE/e genotyping by PCR for two patients. Left: a patient typed RhCcee; right: a patient with RhCcEE.

the annealing temperature from 63 ℃ to 58 ℃ in order to amplify all four genes in the same condition (Figure 1). We were, therefore, able to amplify all four genes in each sample in a single run. A 118 bp fragment was amplified with primers TRH1/2 for the RhC allele in RhC-positive samples (CC or Cc). A 107 bp product was amplified with primers TRH3/4 for the Rhc allele in Rhc samples. A 143 bp product was amplified with primer sets TRH5/7 for the RhE allele in RhE-positive samples.

Finally another 143 bp product was amplified with primer set TRH6/7 from DNA samples containing the Rhe allele. In order to evaluate this single run molecular method, we used the haemagglutination test as the standard method and the results were compared (Table III). There were some discrepancies between phenotype and genotype in patients with beta thalassaemia, but complete agreement between phenotype and genotype was observed in the control group.

Discussion

The incidence of alloimmunisation in transfusion-dependent patients is higher than that in other groups of patients. The reported incidence of alloimmunisation in multiply transfused patients varies from 8% to 76%, generally tending to increase with transfusion load and age[11-15]. As shown in previous studies, serological methods are not suitable for the correct determination of blood groups in multiply transfused patients. However, over the last decades the molecular bases of almost all of the clinically significant polymorphisms have been elucidated and it is now possible to determine blood group genotypes.

The development of high-throughput molecular methods means that it may soon be feasible to test large numbers of blood donors for all clinically important blood group polymorphisms[16-20].

In this study we attempted to define a feasible, reliable and low-cost molecular method for Rh genotyping that could be used in clinical laboratories in order to decrease alloimmunisation reactions in multitransfused patients. Many polymorphisms of blood groups are due to point mutations[10,11]. Between the RhC and Rhc alleles, a single nucleotide substitution at position 48 in exon 1 and five base changes at positions 150, 178, 201, 203 and 307 in

Table III - Random sampling study of RHCE genotyping by ASO-PCR and phenotyping by the haemagglutination test.

	Genotyping					Phenotyping				
	C	C	E	e		C	c	E	E	
C 1*	P	P	N	P	Ccee	P	P	N	P	Ccee
C 2	P	N	P	P	CCEe	P	N	P	P	CCEe
C 3	P	P	P	P	CcEe	P	P	P	P	CcEe
C 4	P	N	P	P	CCEe	P	N	P	P	CCEe
C 5	P	P	P	P	CcEe	P	P	P	P	CcEe
C 6	N	P	P	N	ccE	N	P	P	N	ccEE
C 7	P	N	P	P	CCEe	P	N	P	P	CCEe
C 8	P	P	P	P	CcEe	N	P	P	P	ccEe
C 9	P		P	N	CCEE	P	N	P	N	CCEE
C 10	P	P	P	N	CceEE	P	P	P	N	CcEE
T1**	P	P	P	P	CcEe	P	N	P	P	CCEe
T2	P	P	P	N	CcEE	P	P	P	P	CcEe
T3	P	P	P	P	CcEe	P	P	P	P	CcEe
T4	P	P	N	P	Ccee	P	P	P	N	CcEE
T5	P	N	P	P	CcEe	P	N	P	N	CCEE
T6	P	P	P	N	CcEE	N	P	N	P	Ccee
T7	P	P	P	P	CcEe	P	P	P	N	CcEE
T8	P	P	N	P	Ccee	P	P	P	P	CcEe
T9	P	P	P	P	CcEe	P	P	P	P	CcEe
T10	P	P	P	N	CcEE	P	P	P	P	CcEe
T11	P	N	P	N	CCEE	P	P	N	P	Ccee
T12	N	P	N	P	Ccee	P	N	P	P	CCEe
T13	P	P	P	N	CcEE	P	P	P	P	CcEe
T14	P	P	P	P	CcEe	N	P	P	P	ccEe
T15	P	P	P	P	CcEe	P	N	P	N	CCEE
T16	P	P	N	P	Ccee	P	P	N	P	Ccee
T17	P	P	P	P	CcEe	P	N	N	P	Ccee
T18	P	P	P	P	CcEe	P	N	P	P	CCEe
T19	P	P	N	P	Ccee	P	P	P	N	CcEE
T20	P	P	P	P	CcEe	P	N	P	P	CCEe
T21	P	P	N	P	Ccee	P	P	P	P	CcEe
T22	P	P	P	P	CcEe	P	N	P	N	CCEE
T23	P	N	P	N	CCEE	P	N	P	N	CCEE
T24	P	P	P	P	CcEe	P	P	P	P	CcEe
T25	P	P	N	P	Ccee	P	P	N	P	Ccee
T26	P	N	P	N	CCEE	P	P	P	P	CcEe
T27	P	P	N	P	Ccee	P	N	P	N	CcEe
T28	P	N	N	P	CCee	P	N	P	P	CCEe
T29	P	N	P	P	CCEe	N	P	P	P	ccEe
T30	P	N	P	N	CCEE	P	P	P	N	CcEE
T31	P	N	P	P	CCEe	P	P	P	P	CcEe
T32	P	P	P	P	CcEe	P	N	N	P	Ccee
T33	N	P	P	P	ccEe	P	P	P	N	CcEE
T34	P	P	P	N	CcEE	P	N	P	P	CCEe
T35	P	N	P	P	CCEe	P	P	P	P	CcEe
T36	P	P	P	P	CcEe	P	N	P	N	CCEE
T37	P	P	N	P	Ccee	P	N	P	P	CCEe
T38	P	N	P	P	CCEe	P	P	P	P	CcEe
T39	P	P	P	P	CcEe	P	P	P	P	CcEe
T40	N	P	P	P	ccEe	P	P	N	P	Ccee

*C1 to C10: control group; **T1 to T40: thalassaemic patients.

exon 2 of the RhCE gene have been detected. These six nucleotide substitutions lead to four amino acid changes. Among these amino acids, one at residue 103 is the outer leaflet of the plasma membrane and is, therefore, of importance. The polymorphism at nucleotide 307 leads to the Rhc phenotype and Rhe alleles differ by a single nucleotide substitution (C to G) at position 676 along exon 5 of the RHCE gene, which results in a proline to alanine substitution. Several PCR assays for the Rh alleles have been developed. Faas *et al.*, relying on the polymorphism at nucleotide 676, introduced an allele-specific primer

amplification (ASPA) method[21]. Tanaka *et al.* designed an ASO-PCR method to distinguish RhC and Rhc genes in separate runs[10]. A real-time PCR method has also been used for the detection of RhCE alleles by Legler *et al.*[16]. Castilho *et al.* found phenotype and genotype discrepancies in thalassaemia patients using a restriction fragment length polymorphism (RFLP)-PCR method[19]. Shayegan *et al.*, using RFLP-PCR on 44 thalassemic patients and 20 healthy controls, reported that most discrepancies between phenotype and genotype were seen in the Rh blood group[21].

In conclusion, in this study we introduced an ASO-PCR method for simultaneous genotyping of RhC/c and RhE/e alleles which could be performed in a single procedure within a short time. The advantage of this method compared to the RFLP-PCR method is that all four genes are amplified under the same conditions; it is also a fast and low-cost procedure, making it suitable for use in medical centres and by researchers.

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Received: 16 June 2010 - Revision accepted: 8 October 2010
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