Review Article

Indian J Med Res 146, September 2017, pp 305-315
DOI: 10.4103/ijmr.IJMR_914_16

Evolution of technology for molecular genotyping in blood group systems

Ajit Gorakshakar[1], Harita Gogri[1] & Kanjaksha Ghosh[2]

[1]*Department of Transfusion Medicine, ICMR- National Institute of Immunohaematology, Mumbai &*
[2]*Surat Raktadan Kendra & Research Centre, Surat, India*

Received June 10, 2016

The molecular basis of the blood group antigens was identified first in the 1980s and 1990s. Since then the importance of molecular biology in transfusion medicine has been described extensively by several investigators. Molecular genotyping of blood group antigens is one of the important aspects and is successfully making its way into transfusion medicine. Low-, medium- and high-throughput techniques have been developed for this purpose. Depending on the requirement of the centre like screening for high- or low-prevalence antigens where antisera are not available, correct typing of multiple transfused patients, screening for antigen-negative donor units to reduce the rate of alloimmunization, *etc*. a suitable technique can be selected. The present review discusses the evolution of different techniques to detect molecular genotypes of blood group systems and how these approaches can be used in transfusion medicine where haemagglutination is of limited value. Currently, this technology is being used in only a few blood banks in India. Hence, there is a need for understanding this technology with all its variations.

Key words ABO alleles - India - microarrays - molecular genotyping - red blood cell antigens - single nucleotide polymorphisms

Introduction

Since the time Karl Landsteiner discovered ABO blood groups, agglutination was the method of testing for detecting the presence of blood group antigens and antibodies. Apart from this, adsorption-elution, serum inhibition and anti-human globulin test are some other techniques routinely used in transfusion medicine. The first major breakthrough for blood group genotyping at molecular level occurred when *GYPA*, the gene for the MN blood group system was cloned in 1986[1]. This was followed by cloning of the genes for ABO and Rh blood group systems in 1990 and 1992 respectively[2,3]. Subsequently, the genes for the other blood group systems were also cloned. After studying these genes carefully, it was observed that single nucleotide polymorphism (SNP) is the main cause of variation in these genes. One or more SNPs in particular blood group system can help to identify specific alleles of that system. Apart from this, various other causes responsible for variation seen in different blood group alleles at the molecular level include deletion of a gene or an exon or a nucleotide(s) (*e.g*. whole gene deletion seen in case of Rh system, point deletion(s) in case of ABO, Kell, Duffy, Dombrock blood group systems), sequence duplication plus a nonsense mutation (*e.g*. inactive *RHD* gene), formation of hybrid genes

(*e.g*. MNS, Rh, ABO and Ch/Rg blood group systems), duplication of an exon (*e.g.* Gerbich blood group system), *etc (https://www.ncbi.nlm.nih.gov/projects/gv/mhc/xslcgi.cgi?cmd=bgmut/systems*).

In the ABO blood group system, initially, the DNA sequence of 'A' group specific transferase was partially sequenced from human lung tissue[4]. Subsequently, various A, B and O alleles were cloned and sequenced, and sequence variations among them were identified[5-8]. These variations were based on the presence of different SNPs as well as due to insertion or deletion of single nucleotides. This prompted the scientists to develop molecular techniques to identify these alterations to characterize various alleles. The main advantages of these techniques were: (*i*) small amount of DNA was required, and (*ii*) an individual's genotype could be determined without doing laborious and time-consuming family investigations or without detecting blood group specific molecules on the surface of red blood cells (RBCs).

Today, 35 blood group systems comprising of more than 300 specific antigens are known [International Society of Blood Transfusion (ISBT), *http://www.isbtweb.org*]. At molecular level, more than 1200 alleles have been identified while 50 genes are involved in the expression of blood group antigens[9]. Several review articles on blood group genotyping covering various aspects have been published so far[10-12]. In the ABO blood group system, several alleles encoding each antigen are identifiable and this has tremendous application in forensic science, chimerism, *etc*. Similarly, such heterogeneity may also throw some light on hitherto unexplainable or partially explainable phenomenon of ABO isoimmunization. For antigens from other blood group systems, this technology helps to identify several alleles belonging to these systems and type in alloimmunized patients[11].

In the present review, initial development of DNA-based technology for the detection of molecular genotypes has been discussed in the context of the ABO blood group system along with the evolution of these technologies from low to medium to high throughput for genotyping of other blood group antigens as well. This field is currently at the crossroads, bringing in new perspectives and techniques to replace a century-old practice of haemagglutination-based cross-matching in transfusion medicine.

Structure of *ABO* gene and alleles

The *ABO* gene is located on 'q' arm of chromosome 9 (9q34). It encodes a glycosyltransferase which catalyses the addition of a monosaccharide onto a carbohydrate sequence expressing the H antigen. N-Acetyl-galactosamine is a specific sugar molecule responsible for expression of 'A group' whereas D-galactose is a specific sugar molecule responsible for 'B group' expression. The entire locus spans over 18 kb and consists of seven exons[13]. Fig. 1 gives a schematic representation of *ABO* gene and Table I depicts the sizes (bp) of exons and introns. Exons 6 and 7 of the gene encode for 77 per cent of the full coding region of the glycosyltransferase and 91 per cent of the catalytically active soluble transferase protein[13]. Therefore, initially, techniques were developed to identify ABO alleles by screening SNPs from exons 6 and 7 of the gene.

Fig. 1. Schematic diagram of the *ABO* gene. The numbers indicate positions of exons. UTR, untranslated region.

A101 is considered as a reference allele. B101 allele differs from A101 allele in seven positions. Four of these changes [nucleotide (nt) 526, 703, 796, 803] result in amino acid substitution. There are two common A alleles responsible for A_1 group; one is A101 reference allele and the other one is A102. The latter has single nucleotide substitution at position 467. The rest of the sequence is same as the A101. The A201 allele has two alterations as compared to the A101: a single base substitution (nt467) and a single base deletion (nt1061). The O alleles are divided into two categories: deletional and non-deletional. The O01 is the most common type of deletional allele, and it differs from the A101 allele by a single nucleotide deletion at nt261. The O^1 variant *i.e.* O02 allele also from the deletional category shows nt261 deletion

Table I. Sizes of the exons and introns of the *ABO* gene

Exons		Introns	
No	Size (bp)	No	Size (bp)
1	28	1	12, 982
2	70	2	724
3	57	3	1451
4	48	4	1686
5	36	5	554
6	135	6	1052
7	688		

Source: Ref 14

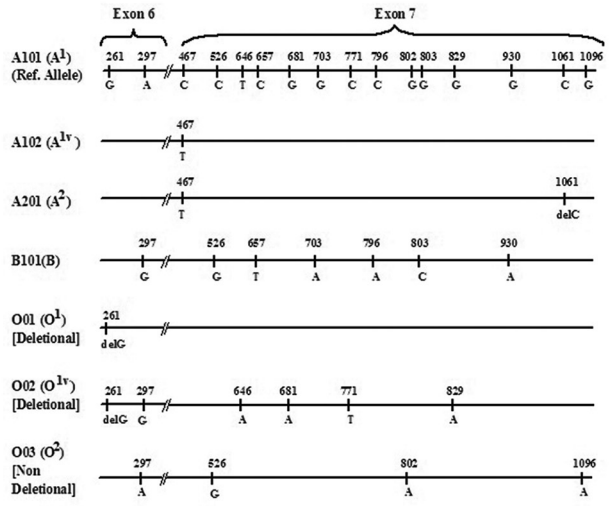

Fig. 2. Single nucleotide polymorphisms in exons 6 and 7 of the *ABO* gene describing the seven common ABO alleles. Allele names described in parentheses are as per old nomenclature.

and nine single base substitutions as compared to A101 allele. The O03 allele is an allele from non-deletional category and shows four single nucleotide substitutions when compared with A101 reference allele. The detailed SNP positions occurring in the seven common ABO alleles are shown in Fig. 2.

Low-throughput techniques

***ABO genotyping*:** Techniques were first developed to discriminate between O and non-O groups using Kpn I/Bst EII enzymes to determine the presence or absence of G at nucleotide position 261 specific to the O allele[2]. Polymerase chain reaction (PCR) followed by allele-specific restriction fragment length polymorphism (RFLP) using BssHII/NarI and HpaII/AluI restriction enzyme pairs were used to distinguish between A and O alleles from B alleles. Initially, 14 individuals of different blood groups were analyzed by Southern Blot technique and the results were compared with those of PCR-RFLP technique. This allowed the homo/heterozygous detection of SNP at this position[2].

Later on, techniques were developed to identify common ABO alleles by characterizing minimum number of SNPs. For example, normal A and B allele polymorphisms are present at seven positions. Of these, four sites namely, 526,703,796 and 803 are crucial. Hence, techniques were developed to identify A and B alleles by analyzing these four sites. G261 deletion for detection of O allele and G703A substitution specific for B allele were taken into account by some researchers for differentiating these alleles[15,16]. A similar approach using C526G polymorphism instead of G703A to detect B alleles was used by Stroncek *et al*[17]. However, they found an anomalous A allele which showed all polymorphisms as a normal A allele except for C526G polymorphism. This clearly showed that more than one polymorphism were required to differentiate between A and B alleles. In 1996, PCR-RFLP was developed to detect polymorphisms at four sites, namely, 261, 526, 703 and 796[18]. This helped to identify A, B and O alleles. The use of NarI which has a cleavage site at nt 526 helped them to differentiate between O01 and O03 alleles. A clinically applicable and simple genotype screening technique based on previously undescribed HpaII site in 3' untranslated region of the ABO gene was developed[19]. The polymorphism G1096A was found in A101 and O01 alleles but not in B101 and O03 alleles. This polymorphism abolishes the HpaII site and is valuable marker in identification of ABO alleles. The same enzyme was found to be useful in identifying polymorphisms associated with alleles such as A201 (C467T), B101 (G703A) and O03 (G1096A). Direct sequencing of the PCR amplified fragment for ABO genotyping was then developed in 1997[20].

The second approach which was simultaneously developed was PCR using allele-specific primers (PCR-ASP). The main advantage of this method was that it did not require post-amplification treatment with restriction enzymes thus reducing the processing time. However, in case of ABO blood group system, more than one SNP is required to be identified to characterize the alleles. Hence, more than one set of primers are required. Initially, ASP incorporated with P^{32} were developed to characterize common ABO alleles[21]. However, due to radioactivity extra care was required while using this technique. PCR-sequence specific oligonucleotide method developed in 1996 was based on specific sequence of the allele using an oligonucleotide probe hybridization reaction[22]. To detect three alleles (O01, O02 and O03) within O group, ten probes were developed. Alternatively, other researchers developed methods that required seven or eight PCR reactions to identify the common ABO alleles[23,24]. Later on, the reactions were multiplexed, and thus, only two PCR reactions were required to identify the common ABO alleles[25]. One of the drawbacks of this approach was that homo- or heterozygosity at each SNP could not be detected.

Initially, all the SNPs covered by ASPs to identify common alleles were from exons 6 and 7 of the *ABO*

gene. Later on, primers were designed to screen all exons, two regulatory regions and introns except intron 1[26]. This study revealed several unknown polymorphisms in coding as well as non-coding regions. Based on this, and other similar studies, various ABO alleles were reanalyzed and renamed, and database of these alleles has been developed (dbRBC, *www.ncbi.nlm.nih.gov/projects/gv/mhc/xslcgi.cgi?cmd=bgmut/systems*).
Some investigators developed another approach which involved combined use of PCR-RFLP and PCR-ASP techniques to detect hybrid alleles as well as weaker variants of A and B[27,28].

Due to extremely heterogeneous nature of *ABO* gene and the possibility of identifying new alleles based on the SNPs that were not detected earlier, some researchers tried to use mutation scanning techniques such as denaturing gradient gel electrophoresis (DGGE) or single strand conformation polymorphism (SSCP) to detect ABO alleles. Johnson and Hopkinson[29] identified four different O alleles and two B alleles by amplifying 250 bp fragment of *ABO* gene and by running DGGE for 19 h at 61°C. Akane *et al*[30] could identify four common ABO alleles by SSCP analysis of a single PCR product covering exon 6 of the *ABO* gene. Ogasawara *et al*[31] further developed this technique and analyzed four PCR products amplified from exons 6 and 7. Thirteen different alleles (common as well as rare) were identified. This approach was further developed by multiplexing three PCRs in a single tube and analyzing the three amplified products by SSCP in a single lane[32]. This could identify polymorphisms at nine positions in exons 6 and 7 (nt 261, 297, 467, 526, 646, 657, 681, 1059 and 1096). Based on these SNPs, seven common ABO alleles could be differentiated using a 'single tube-single lane format'. Initially, a catalogue of various patterns corresponding to different ABO genotypes has to be prepared. For this, genotypes of the samples should be determined by PCR-RFLP technique. The same samples are then analyzed by SSCP to develop a catalogue. This method can also identify new alleles based on unknown SNPs in the three amplified fragments. This technology has been used to identify common ABO alleles in the Indian population[33].

To genotype three major alleles (A^1, B and O^1) of the ABO blood group system simultaneously, inverse PCR technique was developed[34]. In this technique, sequence (about 1.7 kb) from exons 6 and 7 of each allele was amplified, both the termini of the fragment were then ligated and allele typing was performed by the inverse PCR-RFLP and inverse PCR ASP techniques. In a modified assay, labelled primers were used and the alleles were identified by measuring the excess radioactivity present in the amplified reaction mixture[35].

A multiplexed single base primer extension reaction which allows the simultaneous determination of six SNPs (nt 261, 297, 681, 703, 802, 803) has also been described to detect common ABO genotypes[36-38]. The evolution of the molecular genotyping techniques for characterization of ABO alleles is illustrated in Table II.

The technology was developed to extract DNA from various tissues and characterize the ABO alleles. This was a very sensitive technique as ABO genotype was determined using only 0.1 ng of genomic DNA. In addition, the ABO genotype could also be detected from the tissues obtained from bones, muscles, teeth, nails, semen contaminated vaginal fluid, *etc*. where the conventional serological technique could not be used. Lee *et al*[39] developed ABO genotyping technique using four reactions of allele-specific multiplex PCRs to detect five common ABO alleles. In this, whole blood without extracting DNA was used. Here, 'AnyDirect' PCR reaction buffer was used. It conserved the enzyme activity of DNA polymerase for effective use in direct PCR from whole blood which contained PCR inhibitors[40]. The specificity and sensitivity of the novel buffer used in this reaction was good. This is a rapid and convenient technique and has many applications in forensic medicine. ABO genotyping using fresh blood, hair, body fluids, *etc*. without extracting DNA has also been described[41] where, a fast PCR instrument and optimized Taq polymerase were used. The amplified products were analysed by GeneScan programme after capillary electrophoresis. For amplifications, ASP was used. This technique saved lot of time[41].

A kit based on PCR-SSP technique has been developed for detection of common ABO alleles as well as for some weaker variants such as A_3, A_x, B_3 and B_x[42]. The kit also contains ASP to detect common alleles of Kell, Kidd and Duffy blood group systems.

***Genotyping of Rh and other minor blood group systems*:** After ABO, Rh is the second clinically important blood group system. It is encoded by two genes, namely, *RHD* and *RHCE*, which are closely linked and highly homologous and located on chromosome 1, exact location being 1p36.1. Both these genes are inherited together. The *RHD* gene

Table II. Evolution of different techniques for molecular genotyping with reference to ABO blood system

Year	Author	Method	Nucleotide position analyzed	No of PCR reactions	Reference No.
1990	Yamamoto *et al*	PCR-RFLP	261, 526, 703, 796	4	2
1992	Uggozoli and Wallace	PCR-ASP using P^{32} labelled primers	261, 526, 703	1	21
1992	Lee and Chang	PCR-RFLP	258, 700	2	15
1992	Johnson and Hopkinson	PCR-DGGE	261, 297	1	29
1993	O' Keefe & Dobrovic	PCR-RFLP	261, 703	1	16
1995	Stroncek *et al*	PCR-RFLP	261, 526	2	17
1995	Olsson and Chester	PCR RFLP	261, 467, 703, 1096	1 (multiplex)	19
1996	Mifsud *et al*	PCR-RFLP	261, 526, 703, 796	4	18
1996	Gassner *et al*	PCR-ASP	261, 802, 803, 1059	2	23
1996	Mifsud *et al*	PCR-SSO	261, 297, 526, 802	2	22
1996	Akane *et al*	PCR_SSCP	-	1	30
1996	Ogasawara *et al*	PCR-SSCP	261, 297, 467, 526, 703, 796, 802, 1061	4	31
1997	Nata *et al*	Direct sequencing	All positions encompassing the amplified product		20
1997	Procter *et al*	PCR-ASP	261, 467, 796, 802, 1061	7	24
1998	Pearson & Hessner	PCR-ASP	261, 796, 802, 1061	2	25
2000	Yip	PCR-SSCP	261, 297, 467, 526, 646, 657, 681, 1059 and 1096	4	32
2000	Kobayashi and Akane	IP-RFLP, AISP	261, 796, 803	2	34
2001	Watanabe *et al*	CASPA	261, 796, 803, 1065	1	35
2003	Seltsam *et al*	PCR-SSP	52 known polymorphisms and remaining rare	102	26
2004	Doi *et al*	Multiplex single-base primer extension reaction	261, 297, 681, 703, 802, 803	1	38

RFLP, restriction fragment length polymorphism; DGGE, denaturing gradient gel electrophoresis; SSO, sequence specific oligonucleotide; SSCP, single strand conformational polymorphism; ASP, allele specific PCR; IP-RFLP, inverse PCR- restriction fragment length polymorphism; AISP, allele specific inverse PCR; CASPA, consumed allele-specific primer analysis

is responsible for the expression of the D antigen. Multiple genetic events may be responsible for the absence of D antigen on RBCs. Among Caucasians, D negativity is associated with deletion of *RHD* gene between upstream and downstream of Rhesus boxes, while D negativity with intact *RHD* gene has been observed in many other populations[43-45]. Shao *et al*[46] studied 76 RhD-negative cases and 26 Del cases from China and found as many as five alterations responsible for affecting the expression of D antigen on RBCs. At phenotypic level, 54 antigens have been detected while at molecular level 493 alleles have been illustrated[47]. Thus, Rh is a very complex system and to develop a strategy like PCR-SSP for detecting various Rh alleles in a particular population group or geographical area, knowledge about the profile of alterations in the RH gene in that particular group or region is essential. Very few studies have reported alterations in RHD at molecular level[46,48,49]. As it is very difficult to design a generalized technique to detect Rh alleles in different populations, only a few investigators have included detection of RH alleles in genotyping platforms[50-52].

Subsequently, molecular basis of other blood group systems was investigated. MNS (46 antigens), Diego (22 antigens) and Kell (35 antigens) are some of the blood group systems where many antigens are recognized phenotypically, and 59, 91 and 92 alleles have been identified among these blood group systems, respectively. In majority of the cases, alleles have been identified on the basis of SNPs. Hence, PCR-SSP technique was developed to detect these alleles. Olsson

et al[53] developed this technique to detect various alleles of Duffy blood group system, while Hessner *et al*[54] designed sequence-specific primers to detect alleles of Kidd blood group system. Yan *et al*[55] used this technique to identify alleles of eight blood group systems (ABO, Rh, MNS, Kidd, Duffy, Cartwright, Scianna and Colton) among Chinese; while Touinssi *et al*[56] developed this approach to screen French Basques to detect the alleles of six blood group systems (Kell, Kidd, MNS, Dombrock, Colton and Cartwright).

Medium-throughput techniques

Assays with medium throughput include real-time PCR, Sanger DNA sequencing and Pyrosequencing. In real-time PCR, amplified DNA is detected as the reaction progresses[57]. Three methods are used for the detection of the products[58] (*i*) Non-specific fluorescent dyes like SYBR green which intercalates with any double-stranded DNA; (*ii*) TaqMan probes; and (*iii*) Hybridization probe protocol involving fluorescence resonance energy transfer (FRET). Sanger DNA sequencing involves the principle of termination of growing DNA chain after inclusion of dideoxynucleotide triphosphates with fluorochrome labelled bases. SNPs can be identified after reading the sequence of the gene. In pyrosequencing, a pyrophosphate molecule is detected on nucleotide incorporation. Pyrosequencing technique has been used to detect alleles of Kell, Kidd and Duffy blood group systems[59].

High-throughput techniques

Assays with high throughput involve the use of microarray technology. Majority of the alleles of various blood group systems can be identified by detecting one or two SNPs. Microarray technology can identify large number of SNPs at the same time from genomic DNA. This technology is generally used to detect the extended genotype of a donor. The Blood Chip (Progenika Biopharma, Spain), HEA BeadChip (IMMUCOR, USA), GenomeLab (Beckman Coulter USA), Progenika IDcore+ (Progenika Biopharma, Spain) and The Bead Chip (Bioarray Solutions, USA) are some of the microarray platforms available for molecular genotyping. Glass slides or beads or microtitre plates are used to attach SNP specific DNA probes. The 'on-chip' test is based on the hybridization of targets which were amplified earlier by multiplex PCR followed by a detection step allowing the simultaneous identification of many SNPs involved in detection of various alleles of different blood group systems which in turn help to determine the extended genotype.

Three studies describing molecular genotyping of various blood group systems and platelet antigens using SNP platforms specific for blood group genotyping were published simultaneously in 2005[50,60,61]. All the three groups used multiplexed PCR based assays with visual endpoints. Hashmi *et al*[60] identified 18 SNPs describing 36 alleles of more than 11 blood group systems. Denomme and Van Oene[50] screened 372 samples for 12 SNPs detecting several blood group and platelet antigens. Beiboer *et al*[61] detected various platelet antigens in 92 blood donors. Subsequently, several investigators reported[52,62-67] their results of molecular genotyping using various platforms of microarray technology, the details of which is summarized in Fig. 3.

Fluidic microarray system (Luminex xMAP) is a microsphere-based technology used for blood group genotyping. In this method, microspheres are dyed with two spectrally distinct fluorochromes. Using precise amounts of each of these fluorochromes, an array is created. The system detects PCR amplified targets involving various SNPs by direct hybridization to microspheres which are coupled to allele (SNP) specific oligonucleotides. Using this technology, Karpasitou *et al*[64] analysed alleles of seven different blood group systems. Later on, this technique was validated by the same group using biotinylated PCR product. This method involves two multiplex PCRs for screening of 16 antigens of Kell, S, Duffy, Kidd, Lutheran and Colton blood group systems[68].

Nanofluidic open array system has been used to genotype 32 SNPs for 42 blood group antigens in more than 40,000 donors[66]. The results were confirmed by phenotyping before release of blood unit. This helped them to get antigen-negative blood units. Hence, they abandoned the screening by serology. These results are encouraging as these help to shift to molecular genotyping from serological techniques, provided that the platform for molecular genotyping is well established.

Other techniques of high-throughput technology

***Mini sequencing or the SNaPshot assay*:** This method involves SNP analysis to detect the exact base and computer-assisted visualization of the specific alteration/polymorphism. Fluorescently labelled dideoxynucleotides are used with multiplex PCR product as a template. After the hybridization and extension steps, the fluorescent signals from the array are measured and the genotypes are determined by

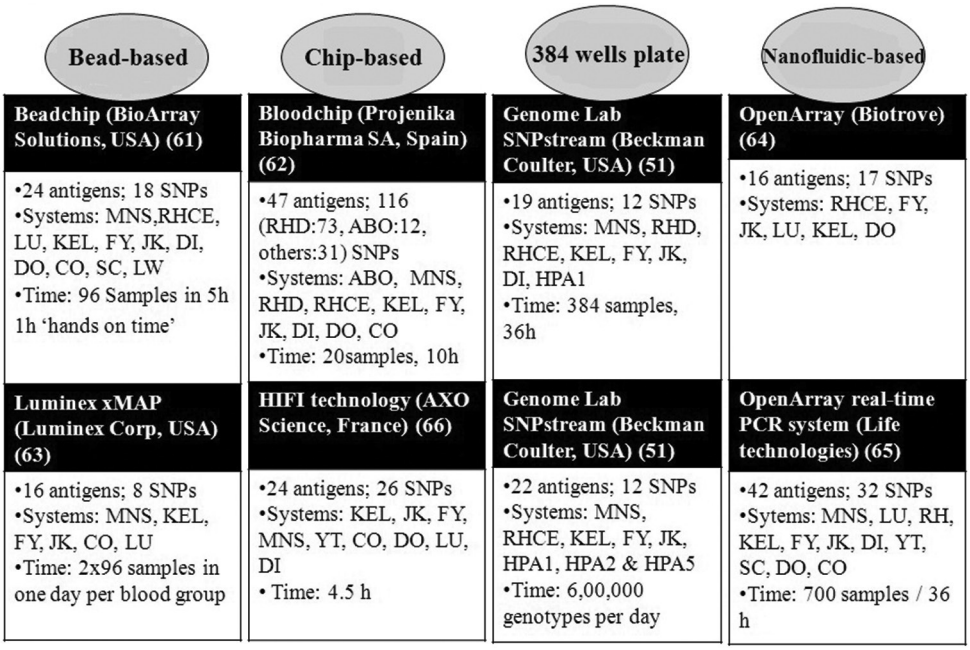

Fig. 3. Various platforms of microarray technology used for blood group genotyping. Numerals in parentheses denote reference number. LU, Lutheran blood group system; KEL, Kell blood group system; FY, Duffy blood group system; JK, Kidd blood group system; DI, Diego blood group system; YT, Cartwright blood group system; SC, Scianna blood group system; DO, Dombrock blood group system; CO, Colton blood group system; HPA, Human platelet antigens

cluster analysis. This technique has been successfully used to detect the most common alleles of the ABO blood group system[69]. The SNaPshot platform has been used to detect the alleles from 12 different blood group systems[70]. Using this platform, nine SNPs defining 16 blood group alleles from five blood group systems were simultaneously identified among blood donors from Brazil[71].

***Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS)*:** This technique can discriminate two DNA fragments which differ in a single nucleotide. The technique involves two parts: first, laser-induced desorption/ ionization of matrix molecules and second, separation and analysis of these molecules on the basis of their intrinsic physical properties. It is a quantitative as well as qualitative technique and can analyse several SNPs in a single reaction which takes about eight hours. This technique has been used to detect some alleles of Kell blood group system[72]. Two groups from Switzerland also used this system to identify several blood group alleles among Swiss blood donors[73,74].

***Limitations of molecular blood group genotyping*:** Low-throughput techniques such as PCR-SSP and PCR-RFLP were initially developed to detect various alleles of ABO blood group system. However, many SNPs need to be identified to characterize a particular ABO allele. Furthermore, considering the pace of discovery of new alleles in ABO as well as other blood group systems, it became difficult to develop a low-throughput method to detect all the alleles. These techniques require post-PCR analysis and may give false- or false-negative reactions with certain hybrid alleles. There is a preferential amplification of only one allele when present in heterozygous condition.

Medium-throughput techniques such as real-time SNP assay and DNA sequencing are time-consuming and complex techniques. Further, in DNA sequencing, large data are generated which are difficult to store and analysis of the data requires bioinformatic tools. Second, the sample to results takes days to weeks which later on decreased due to automation and optimization of workflow[74].

High-throughput donor typing techniques vary considerably in methodology, antigen selection, throughput and cost. Even though several techniques are reported, only a few report this as an ongoing activity and provide data on large number of samples[51,66,75]. Although these are highly efficient for testing a large number of samples for multiple blood group alleles simultaneously; but these may be suitable for screening only some populations. The new alleles identified

cannot be detected and have to be incorporated into the testing platform. It has then to be validated again before routine use. These technologies are expensive and one has to consider the cost. As per the requirement of the centre, the platform with number of antigens to be screened can be designed (*e.g.* clinically important antigens, minor antigens, rare alleles, null types) which can also take into account cost per sample.

***Molecular genotyping in India*:** Only one study has described molecular ABO genotyping in Indian population[33]. In this study, molecular genotyping was done by PCR-RFLP and PCR-SSCP techniques. Totally, 13 common and rare alleles belonging to the ABO blood group were identified. Considering the heterogeneity of Indian population many more alleles are likely to be detected among various population groups.

Identification of rare donors is a critical factor in establishing a rare donor registry[76]. It can be done by performing mass screening of donors for clinically important blood group antigens by serological testing or by using gel technology. However, serological typing of large cohorts of donors is labour intensive and expensive exercise and many a times hampered by the lack of reliable antisera. To overcome this, genotyping of various blood group systems will be an important aspect.

There are many reports where healthy blood donors from different cities in India have been screened to look for the frequency of various blood group antigens[77-82]. Similarly, many studies report the prevalence of alloimmunization against various antigens from different parts of India[83-90]. Recently, in a screening among 1221 donors from Mumbai, 261 donors who lacked a combination of clinically important common antigens, were detected[91]. All these studies have used serological techniques. At National Institute of Immunohaematology, Mumbai, India, using a PCR-SSP technique, a study has been initiated to detect antigens of Kell, Kidd, Duffy, MNS, Indian, Diego blood group systems among donors[92,93]. Also RhD typing of the foetus of a Rh-negative mother using non-invasive DNA-based technique has been established[94].

Conclusion

Molecular genotyping is set to play an important role in the routine blood banks in the future. Whether it will replace serology completely or not needs to be seen. ABO genotyping will be useful in ABO-incompatible bone marrow transplant cases and in case of identifying weaker subtypes. However, complex genotyping strategies are required to identify correct ABO or Rh alleles. So serology will still be required to type for these systems. Molecular genotyping will help to type donors for a wider spectrum of minor blood group antigens and also genotype blood group antigens of multiply transfused patients such as sickle cell anaemia or β-thalassemia or patients having positive direct antiglobulin test. Providing a donor's blood to the patient after studying extended antigen profile will help in preventing alloimmunization. Alloimmunized transfusion recipients will also be benefited if the donor's blood is electronically cross matched using the extended array of SNPs. This technology can be used to screen for uncommon or rare antigens or to look for the absence of high-frequency antigens or to detect the antigens where specific antisera are not available. Molecular genotyping will also play an important role in non-invasive prenatal RhD typing of foetus of RhD-negative pregnant woman. In short, molecular genotyping will make transfusion medicine more personalized and patient-oriented.

***Conflicts of Interest*:** None.

References

1. Siebert PD, Fukuda M. Molecular cloning of a human glycophorin B cDNA: Nucleotide sequence and genomic relationship to glycophorin A. *Proc Natl Acad Sci U S A* 1987; *84* : 6735-9.
2. Yamamoto F, Clausen H, White T, Marken J, Hakomori S. Molecular genetic basis of the histo-blood group ABO system. *Nature* 1990; *345* : 229-33.
3. Le van Kim C, Mouro I, Chérif-Zahar B, Raynal V, Cherrier C, Cartron JP, *et al.* Molecular cloning and primary structure of the human blood group RhD polypeptide. *Proc Natl Acad Sci U S A* 1992; *89* : 10925-9.
4. Clausen H, White T, Takio K, Titani K, Stroud M, Holmes E, *et al.* Isolation to homogeneity and partial characterization of a histo-blood group A defined Fuc alpha 1-2Gal alpha 1-3-N-acetylgalactosaminyltransferase from human lung tissue. *J Biol Chem* 1990; *265* : 1139-45.
5. Yamamoto F, Marken J, Tsuji T, White T, Clausen H, Hakomori S. Cloning and characterization of DNA complementary to human UDP-GalNAc: Fuc alpha 1-2Gal alpha 1-3GalNAc transferase (histo-blood group A transferase) mRNA. *J Biol Chem* 1990; *265* : 1146-51.
6. Yamamoto F, McNeill PD, Hakomori S. Human histo-blood group A2 transferase coded by A2 allele, one of the A subtypes, is characterized by a single base deletion in the coding sequence, which results in an additional domain at the carboxyl terminal. *Biochem Biophys Res Commun* 1992; *187* : 366-74.
7. Yamamoto F, McNeill PD, Yamamoto M, Hakomori S, Harris T, Judd WJ, *et al.* Molecular genetic analysis of the

ABO blood group system: 1. Weak subgroups: A3 and B3 alleles. *Vox Sang* 1993; *64* : 116-9.

8. Grunnet N, Steffensen R, Bennett EP, Clausen H. Evaluation of histo-blood group ABO genotyping in a Danish population: Frequency of a novel O allele defined as O2. *Vox Sang* 1994; *67* : 210-5.
9. Boyle J, Thorpe SJ, Hawkins JR, Lockie C, Fox B, Matejtschuk P, *et al.* International reference reagents to standardise blood group genotyping: Evaluation of candidate preparations in an international collaborative study. *Vox Sang* 2013; *104* : 144-52.
10. Daniels G. The molecular genetics of blood group polymorphism. *Transpl Immunol* 2005; *14* : 143-53.
11. Tournamille C. Molecular biology methods in immunohematology. *Transfus Clin Biol* 2013; *20* : 72-9.
12. Denomme GA. Molecular basis of blood group expression. *Transfus Apher Sci* 2011; *44* : 53-63.
13. Yip SP. Sequence variation at the human ABO locus. *Ann Hum Genet* 2002; *66* (Pt 1) : 1-27.
14. Seltsam A, Hallensleben M, Kollmann A, Blasczyk R. The nature of diversity and diversification at the ABO locus. *Blood* 2003; *102* : 3035-42.
15. Lee JC, Chang JG. ABO genotyping by polymerase chain reaction. *J Forensic Sci* 1992; *37* : 1269-75.
16. O'Keefe DS, Dobrovic A. A rapid and reliable PCR method for genotyping the ABO blood group. *Hum Mutat* 1993; *2* : 67-70.
17. Stroncek DF, Konz R, Clay ME, Houchins JP, McCullough J. Determination of ABO glycosyltransferase genotypes by use of polymerase chain reaction and restriction enzymes. *Transfusion* 1995; *35* : 231-40.
18. Mifsud NA, Haddad AP, Condon JA, Sparrow RL. ABO genotyping by polymerase chain reaction-restriction fragment length polymorphism. *Immunohematology* 1996; *12* : 143-8.
19. Olsson ML, Chester MA. A rapid and simple ABO genotype screening method using a novel B/O2 versus A/O2 discriminating nucleotide substitution at the ABO locus. *Vox Sang* 1995; *69* : 242-7.
20. Nata M, Kanetake J, Adachi N, Hashiyada M, Aoki Y, Sagisaka K. ABO genotyping by PCR-direct sequencing. *Nihon Hoigaku Zasshi* 1997; *51* : 1-5.
21. Ugozzoli L, Wallace RB. Application of an allele-specific polymerase chain reaction to the direct determination of ABO blood group genotypes. *Genomics* 1992; *12* : 670-4.
22. Mifsud NA, Haddad AP, Condon JA, Sparrow RL. ABO genotyping-identification of O1, O1*, and O2 alleles using the polymerase chain reaction-sequence specific oligonucleotide (PCR-SSO) technique. *Immunohematology* 1996; *12* : 149-53.
23. Gassner C, Schmarda A, Nussbaumer W, Schönitzer D. ABO glycosyltransferase genotyping by polymerase chain reaction using sequence-specific primers. *Blood* 1996; *88* : 1852-6.
24. Procter J, Crawford J, Bunce M, Welsh KI. A rapid molecular method (polymerase chain reaction with sequence-specific primers) to genotype for ABO blood group and secretor status and its potential for organ transplants. *Tissue Antigens* 1997; *50* : 475-83.
25. Pearson SL, Hessner MJ. A(1,2)BO(1,2) genotyping by multiplexed allele-specific PCR. *Br J Haematol* 1998; *100* : 229-34.
26. Seltsam A, Hallensleben M, Kollmann A, Burkhart J, Blasczyk R. Systematic analysis of the ABO gene diversity within exons 6 and 7 by PCR screening reveals new ABO alleles. *Transfusion* 2003; *43* : 428-39.
27. Hosseini-Maaf B, Hellberg A, Rodrigues MJ, Chester MA, Olsson ML. ABO exon and intron analysis in individuals with the AweakB phenotype reveals a novel O1v-A2 hybrid allele that causes four missense mutations in the A transferase. *BMC Genet* 2003; *4* : 17.
28. Olsson ML, Irshaid NM, Hosseini-Maaf B, Hellberg A, Moulds MK, Sareneva H, *et al.* Genomic analysis of clinical samples with serologic ABO blood grouping discrepancies: Identification of 15 novel A and B subgroup alleles. *Blood* 2001; *98* : 1585-93.
29. Johnson PH, Hopkinson DA. Detection of ABO blood group polymorphism by denaturing gradient gel electrophoresis. *Hum Mol Genet* 1992; *1* : 341-4.
30. Akane A, Yoshimura S, Yoshida M, Okii Y, Watabiki T, Matsubara K, *et al.* ABO genotyping following a single PCR amplification. *J Forensic Sci* 1996; *41* : 272-4.
31. Ogasawara K, Bannai M, Saitou N, Yabe R, Nakata K, Takenaka M, *et al.* Extensive polymorphism of ABO blood group gene: Three major lineages of the alleles for the common ABO phenotypes. *Hum Genet* 1996; *97* : 777-83.
32. Yip SP. Single-tube multiplex PCR-SSCP analysis distinguishes 7 common ABO alleles and readily identifies new alleles. *Blood* 2000; *95* : 1487-92.
33. Ray S, Gorakshakar AC, Vasantha K, Nadkarni A, Italia Y, Ghosh K. Molecular genotyping of ABO blood groups in some population groups from India. *Indian J Med Res* 2014; *139* : 105-11.
34. Kobayashi T, Akane A. ABO genotyping by inverse PCR technique. *Leg Med (Tokyo)* 2000; *2* : 15-20.
35. Watanabe G, Umetsu K, Yuasa I, Sato M, Sakabe M, Naito E, *et al.* A novel technique for detecting single nucleotide polymorphisms by analyzing consumed allele-specific primers. *Electrophoresis* 2001; *22* : 418-20.
36. Sasaki M, Shiono H. ABO genotyping of suspects from sperm DNA isolated from postcoital samples in sex crimes. *J Forensic Sci* 1996; *41* : 275-8.
37. Tun Z, Honda K, Nakatome M, Islam MN, Bai H, Ogura Y, *et al.* Rapid and clear detection of ABO genotypes by simultaneous PCR-RFLP method. *J Forensic Sci* 1996; *41* : 1027-30.
38. Doi Y, Yamamoto Y, Inagaki S, Shigeta Y, Miyaishi S, Ishizu H. A new method for ABO genotyping using a multiplex single-base primer extension reaction and its application to forensic casework samples. *Leg Med (Tokyo)* 2004; *6* : 213-23.

39. Lee SH, Park G, Yang YG, Lee SG, Kim SW. Rapid ABO genotyping using whole blood without DNA purification. *Korean J Lab Med* 2009; *29* : 231-7.

40. Yang YG, Kim JY, Song YH, Kim DS. A novel buffer system, AnyDirect, can improve polymerase chain reaction from whole blood without DNA isolation. *Clin Chim Acta* 2007; *380* : 112-7.

41. Lee HY, Park MJ, Kim NY, Yang WI, Shin KJ. Rapid direct PCR for ABO blood typing. *J Forensic Sci* 2011; *56* (Suppl 1) : S179-82.

42. Prager M, Scharberg EA, Wagner FF, Burkhart J, Seltsam A. ABO genotyping for diagnosis of unusual ABO blood groups: A comparative study in German blood donor centers. *Transfusion* 2007; *47* : SP305.

43. Wagner FF, Flegel WA. *RHD* gene deletion occurred in the Rhesus box. *Blood* 2000; *95* : 3662-8.

44. Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A, *et al.* The presence of an *RHD* pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the RhD-negative blood group phenotype. *Blood* 2000; *95* : 12-8.

45. Lan JC, Chen Q, Wu DL, Ding H, Pong DB, Zhao T. Genetic polymorphism of RhD-negative associated haplotypes in the Chinese. *J Hum Genet* 2000; *45* : 224-7.

46. Shao CP, Maas JH, Su YQ, Köhler M, Legler TJ. Molecular background of Rh D-positive, D-negative, D(el) and weak D phenotypes in Chinese. *Vox Sang* 2002; *83* : 156-61.

47. Patnaik SK, Helmberg W, Blumenfeld OO. BGMUT: NCBI dbRBC database of allelic variations of genes encoding antigens of blood group systems. *Nucleic Acids Res* 2012; *40* : D1023-9.

48. Kim JY, Kim SY, Kim CA, Yon GS, Park SS. Molecular characterization of D- Korean persons: Development of a diagnostic strategy. *Transfusion* 2005; *45* : 345-52.

49. Sun CF, Liu JP, Chen DP, Wang WT, Yang TT. Use of real time PCR for rapid detection of Del phenotype in Taiwan. *Ann Clin Lab Sci* 2008; *38* : 258-63.

50. Denomme GA, Van Oene M. High-throughput multiplex single-nucleotide polymorphism analysis for red cell and platelet antigen genotypes. *Transfusion* 2005; *45* : 660-6.

51. St-Louis M, Perreault J, Lavoie J, Émond J, St-Laurent J, Long A, *et al.* Genotyping of 21,000 blood donors in Quebec and RHD analysis. *Transfus Clin Biol* 2010; *17* : 242-8.

52. Perreault J, Lavoie J, Painchaud P, Côté M, Constanzo-Yanez J, Côté R, *et al.* Set-up and routine use of a database of 10,555 genotyped blood donors to facilitate the screening of compatible blood components for alloimmunized patients. *Vox Sang* 2009; *97* : 61-8.

53. Olsson ML, Hansson C, Avent ND, Akesson IE, Green CA, Daniels GL. A clinically applicable method for determining the three major alleles at the Duffy (FY) blood group locus using polymerase chain reaction with allele-specific primers. *Transfusion* 1998; *38* : 168-73.

54. Hessner MJ, Pircon RA, Johnson ST, Luhm RA. Prenatal genotyping of Jk(a) and Jk(b) of the human Kidd blood group system by allele-specific polymerase chain reaction. *Prenat Diagn* 1998; *18* : 1225-31.

55. Yan L, Zhu F, Fu Q, He J. ABO, Rh, MNS, Duffy, Kidd,Yt, Scianna, and Colton blood group systems in indigenous Chinese. *Immunohematology* 2005; *21* : 10-4.

56. Touinssi M, Chiaroni J, Degioanni A, Granier T, Dutour O, Bailly P, *et al.* DNA-based typing of Kell, Kidd, MNS, Dombrock, Colton, and Yt blood group systems in the French Basques. *Am J Hum Biol* 2008; *20* : 308-11.

57. Araújo F, Pereira C, Monteiro F, Henriques I, Meireles E, Lacerda P, *et al.* Blood group antigen profile predicted by molecular biology-use of real-time polymerase chain reaction to genotype important KEL, JK,RHD, and RHCE alleles. *Immunohematology* 2002; *18* : 59-64.

58. Monteiro F, Tavares G, Ferreira M, Amorim A, Bastos P, Rocha C, *et al*. Technologies involved in molecular blood group genotyping. *ISBT Sci Ser* 2011; *6* : 1-6.

59. van Dronen J, Beckers EA, Sintnocolaas K. Rapid genotyping of blood group systems using the pyrosequencing technique. *Vox Sang* 2002; *83* : 104-5.

60. Hashmi G, Shariff T, Seul M, Vissavajjhala P, Hue-Roye K, Charles-Pierre D, *et al.* A flexible array format for large-scale, rapid blood group DNA typing. *Transfusion* 2005; *45* : 680-8.

61. Beiboer SH, Wieringa-Jelsma T, Maaskant-Van Wijk PA, van der Schoot CE, van Zwieten R, Roos D, *et al.* Rapid genotyping of blood group antigens by multiplex polymerase chain reaction and DNA microarray hybridization. *Transfusion* 2005; *45* : 667-79.

62. Hashmi G, Shariff T, Zhang Y, Cristobal J, Chau C, Seul M, *et al.* Determination of 24 minor red blood cell antigens for more than 2000 blood donors by high-throughput DNA analysis. *Transfusion* 2007; *47* : 736-47.

63. Avent ND, Martinez A, Flegel WA, Olsson ML, Scott ML, Nogués N, *et al.* The BloodGen project: Toward mass-scale comprehensive genotyping of blood donors in the European Union and beyond. *Transfusion* 2007; *47* (1 Suppl) : 40S-6S.

64. Karpasitou K, Drago F, Crespiatico L, Paccapelo C, Truglio F, Frison S, *et al.* Blood group genotyping for Jk(a)/Jk(b), Fy(a)/Fy(b), S/s, K/k, Kp(a)/Kp(b), Js(a)/Js(b), Co(a)/Co(b), and Lu(a)/Lu(b) with microarray beads. *Transfusion* 2008; *48* : 505-12.

65. Hopp K, Weber K, Bellissimo D, Johnson ST, Pietz B. High-throughput red blood cell antigen genotyping using a nanofluidic real-time polymerase chain reaction platform. *Transfusion* 2010; *50* : 40-6.

66. Flegel WA, Gottschall JL, Denomme GA. Implementing mass-scale red cell genotyping at a blood center. *Transfusion* 2015; *55* : 2610-5.

67. Boccoz SA, Le Goff G, Blum LJ, Marquette CA. Microarrays in blood group genotyping. Molecular typing of blood cell antigens. *Methods Mol Biol* 2015; *1310* : 105-13.

68. Drago F, Karpasitou K, Spinardi L, Crespiatico L, Scalamogna M, Poli F. A microsphere-based suspension array for blood group molecular typing: An update. *Transfus Med Hemother* 2010; *37* : 336-8.
69. Ferri G, Bini C, Ceccardi S, Pelotti S. ABO blood group genotyping of multiple single nucleotide polymorphisms using SNaPshot. *Int Congr Ser* 2004; *1261* : 556-8.
70. Palacajornsuk P, Halter C, Isakova V, Tarnawski M, Farmar J, Reid ME, *et al*. Detection of blood group genes using multiplex SNaPshot method. *Transfusion* 2009; *49* : 740-9.
71. Latini FR, Gazito D, Arnoni CP, Muniz JG, de Medeiros Person R, Carvalho FO, *et al*. A new strategy to identify rare blood donors: Single polymerase chain reaction multiplex SNaPshot reaction for detection of 16 blood group alleles. *Blood Transfus* 2014; *12* (Suppl 1) : s256-63.
72. Wagner FF, Bittner R, Döscher A, Petershofen EK, Müller TH. Mid-throughput blood group phenotype prediction by pooled capillary electrophoresis. *Transfusion* 2007; *47* : 172A-3A.
73. Meyer S, Vollmert C, Trost N, Brönnimann C, Gottschalk J, Buser A, *et al*. High-throughput Kell, Kidd, and Duffy matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry-based blood group genotyping of 4000 donors shows close to full concordance with serotyping and detects new alleles. *Transfusion* 2014; *54* : 3198-207.
74. McBean RS, Hyland CA, Flower RL. Approaches to determination of a full profile of blood group genotypes: Single nucleotide variant mapping and massively parallel sequencing. *Comput Struct Biotechnol J* 2014; *11* : 147-51.
75. van der Schoot CE, de Haas M, Engelfriet CP, Reesink HW, Panzer S, Jungbauer C, *et al*. Genotyping for red blood cell polymorphisms. *Vox Sang* 2009; *96* : 167-79.
76. Kaur R, Jain A. Rare blood donor program in the country: Right time to start. *Asian J Transfus Sci* 2012; *6* : 1-2.
77. Chaudhary RK, Shukla JS, Ray V. Minor red cell antigens in North Indian blood donor population. *Indian J Hematol Blood Transfus* 2003; *21* : 34-5.
78. Thakral B, Saluja K, Sharma RR, Marwaha N. Phenotype frequencies of blood group systems (Rh, Kell, Kidd, Duffy, MNS, P, Lewis, and Lutheran) in North Indian blood donors. *Transfus Apher Sci* 2010; *43* : 17-22.
79. Agarwal N, Thapliyal RM, Chatterjee K. Blood group phenotype frequencies in blood donors from a tertiary care hospital in North India. *Blood Res* 2013; *48* : 51-4.
80. Lamba DS, Kaur R, Basu S. Clinically significant minor blood group antigens amongst North Indian Donor population. *Adv Hematol* 2013; *2013* : 215454.
81. Kahar MA, Patel RD. Phenotype frequencies of blood group systems (Rh, Kell, Kidd, Duffy, MNS, P, Lewis, and Lutheran) in blood donors of south Gujarat, India. *Asian J Transfus Sci* 2014; *8* : 51-5.
82. Makroo RN, Bhatia A, Gupta R, Phillip J. Prevalence of Rh, Duffy, Kell, Kidd & MNSs blood group antigens in the Indian blood donor population. *Indian J Med Res* 2013; *137* : 521-6.
83. Pahuja S, Pujani M, Gupta SK, Chandra J, Jain M. Alloimmunization and red cell autoimmunization in multitransfused thalassemics of Indian origin. *Hematology* 2010; *15* : 174-7.
84. Gupta R, Singh DK, Singh B, Rusia U. Alloimmunization to red cells in thalassemics: Emerging problem and future strategies. *Transfus Apher Sci* 2011; *45* : 167-70.
85. Chaudhari CN. Red cell alloantibodies in multiple transfused thalassaemia patients. *Med J Armed Forces India* 2011; *67* : 34-7.
86. Thakral B, Saluja K, Sharma RR, Marwaha N. Red cell alloimmunization in a transfused patient population: A study from a tertiary care hospital in North India. *Hematology* 2008; *13* : 313-8.
87. Varghese J, Chacko MP, Rajaiah M, Daniel D. Red cell alloimmunization among antenatal women attending a tertiary care hospital in South India. *Indian J Med Res* 2013; *138* : 68-71.
88. Shukla JS, Chaudhary RK. Red cell alloimmunization in multi-transfused chronic renal failure patients undergoing hemodialysis. *Indian J Pathol Microbiol* 1999; *42* : 299-302.
89. Gogri H, Kulkarni S, Vasantha K, Jadhav S, Ghosh K, Gorakshakar A. Partial matching of blood group antigens to reduce alloimmunization in Western India. *Transfus Apher Sci* 2016; *54* : 390-5.
90. Makroo RN, Bhatia A, Hegde V, Chowdhry M, Thakur UK, Rosamma NL. Antibody screening & identification in the general patient population at a tertiary care hospital in New Delhi, India. *Indian J Med Res* 2014; *140* : 401-5.
91. Kulkarni S, Vasantha K, Ghosh K. Antigen negative red blood cell inventory of Indian blood donors. *Transfus Apher Sci* 2016; *55* : 131-5.
92. Kulkarni S, Choudhary B, Gogri H, Vasantha K, Ghosh K. Quality assurance of reagent red blood cells by DNA analysis. *Asian J Transfus Sci* 2014; *8* : 6-25.
93. Gogri H, Kulkarni S, Ghosh K. Molecular genotyping of Indian blood group system. *Asian J Transfus Sci* 2015; *9* : 11-44.
94. Parchure D, Kulkarni S, Ghosh K. Noninvasive fetal RHD genotyping using cell-free fetal DNA from maternal plasma. *Asian J Transfus Sci* 2016; *10* : 8-33.

Reprint requests: Dr Ajit Gorakshakar, ICMR-National Institute of Immunohaematology, 13th Floor, New Multistoreyed Building, K. E. M. Hospital Campus, Parel, Mumbai 400 012, Maharashtra, India
e-mail: ajit5678@yahoo.com