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The Bloodgen Project of the European Union, 2003–2009

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Key Words
BLOODchip · Blood groups · Blood group antigens

Summary
The Bloodgen project was funded by the European Commission between 2003 and 2006, and involved academic blood centres, universities, and Progenika Biopharma S.A., a commercial supplier of genotyping platforms that incorporate glass arrays. The project has led to the development of a commercially available product, BLOODchip, that can be used to comprehensively genotype an individual for all clinically significant blood groups. The intention of making this system available is that blood services and perhaps even hospital blood banks would be able to obtain extended information concerning the blood group of routine blood donors and vulnerable patient groups. This may be of significant use in the current management of multi-transfused patients who become alloimmunised due to incomplete matching of blood groups. In the future it can be envisaged that better matching of donor-patient blood could be achieved by comprehensive genotyping of every blood donor, especially regular ones. This situation could even be extended to genotyping every individual at birth, which may prove to have significant long-term health economic benefits as it may be coupled with detection of inborn errors of metabolism.

Schlüsselwörter
BLOODchip · Blutgruppen · Blutgruppenantigene

Zusammenfassung
Das Bloodgen-Projekt wurde zwischen 2003 und 2006 von der Europäischen Kommission gefördert und bestand aus akademischen Blutzentren, Universitäten und der Progenika Biopharma S.A., einem kommerziellen Anbieter von Genotypisierungsplattformen, die Glas-Arrays beinhalten. Das Projekt führte zur Entwicklung eines kommerziell erwerbbaren Produkt – BLOODchip –, mit dem eine Person für alle klinisch signifikanten Blutgruppen umfassend genotypisiert werden kann. Das System wurde kommerziell verfügbar gemacht, um Blutspendeeinrichtungen und möglicherweise sogar Krankenhausblutbanken in die Lage zu versetzen, umfangreiche Informationen bezüglich der Blutgruppe von Dauerspendern und gefährdeten Patientengruppen zu generieren. Dies könnte von besonderer Bedeutung für das aktuelle Management von multitransfundierten Patienten sein, die aufgrund eines unvollständigen Blutgruppen-Matching alloimmunisiert wurden. Möglicherweise lässt sich zukünftig durch umfassende Genotypisierung aller Blutspender, besonders aber der Dauerspender, ein besseres Matching zwischen Spender- und Patientenblut erzielen. Möglicherweise wird es sogar eine Genotypisierung jeder Person bei seiner Geburt geben, durch die eine erhebliche Reduzierung der langfristigen Gesundheitskosten erzielt werden könnte, da sie mit der frühen Detektion von angeborenen Stoffwechseldefekten einhergeht.

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Introduction

Throughout the 1990s all major blood group systems were defined at the level of the gene, and polymorphisms were deciphered for most blood group alleles [reviewed in 1–5]. These polymorphisms were mainly caused by single nucleotide polymorphisms (SNPs), but also, notably in the diverse *RH* and *MNS* systems, by gene conversions, duplications and, in the case of the Caucasian D-negative genotype, deletions. Most blood groups are dependent on polymorphic variation within protein structures (for example the K/k (Kell) polymorphism is a SNP altering codon 183 methionine (K) to threonine (k)) [6]. Throughout most of this decade this information remained largely an academic exercise with little direct application in transfusion medicine. There was one very important exception to this statement: genotyping for fetal RhD blood group for the management of haemolytic disease of the fetus and newborn (HDFN) was the first real clinical application of this information [7–11]. Later this methodology was applied to a number of other blood groups that are implicated in HDFN [12, 13], and these assays were initially applied to fetal material obtained by amniocentesis or chorionic villus (CV) sampling, which was spare material normally disposed of by the now obsolete Liley technique for the prediction of severity of HDFN [14]. In the late 1990s / early 2000s the emphasis was switched to using maternal plasma instead of invasively sampled fetal material [15–17], which eliminated any procedurally related risk (approximately 1% of fetuses spontaneously abort during the amniocentesis procedure). Non-invasive testing for fetal *RHD* blood group genotype is now widespread and has led to the elimination of amniocentesis and CV sampling for assessment of HDFN [18–20].

Fetal genotyping for blood group status has remained the main usage of DNA-based typing in Europe although donor genotyping is becoming more commonplace in North America. With the evolution of high-throughput genotyping platforms, especially glass and bead array approaches, the feasibility of utilising such systems to enable closer matching of donors and patients was quickly realised [21]. Several projects were initiated in the early 2000s as feasibility studies using these genotyping systems [reviewed in 22]. This review describes the efforts of the Bloodgen consortium (*www.bloodgen.com*) which developed a glass array capable to genotyping over 116 blood group-specific SNPs (BLOODchip version 1.0). Since completion of the project in September 2006 BLOODchip version 1.0 has been CE-marked for RhCE, Kell as required in current directives from the European Commission (EC). Version 1.0 has the capacity to detect all major *ABO*, *RHD*, *RHCE*, *MNS, KEL, FY, JK, DO, DI,* and *CO* alleles. Further developments of BLOODchip include version 2 (addition of all clinically relevant *HPA* alleles) and version 3 (new *RHCE* and *RHD* alleles, plus *LW*, *LU* alleles, and additional *JK* alleles). BLOODchip will shortly be CE-marked for RhD diagnostic use, but at the present time there are no plans to CE mark for ABO diagnostic use, remaining as a research tool. This is because of the genetic complexity of *ABO* alleles, and the potential risk that genotyping may mis-score an ABO blood unit. Nevertheless, we are confident that with extensive use and resultant determination of the majority of *ABO* alleles, blood group genotyping may prove as robust as ABO serological testing and may replace it in routine use.

Technical Background

It is not the intention of this review to give a thorough review of the technology that supports the BLOODchip platform as these have been described in some detail before [22, 23]. In brief, the BLOODchip platform requires a standard approach for DNA extraction, followed by PCR amplification of DNA containing the SNPs responsible for blood group polymorphisms by a dedicated series of three multiplex (MPX) PCRs. The PCR products are then fragmented, labelled, and hybridised to a glass array containing multiple copies of probes corresponding to each paired allele. Detection of binding to each probe is then achieved using a standard laser array scanner. Then a comparison of strength of binding of the labelled PCR products to each probe is made using bespoke software. The BLOODchip system software then provides an output of genotype and predicted serological phenotype. The predicted phenotype is especially sensitive when considering variant Rh phenotypes, especially partial D. This was a major activity within the Bloodgen project – to produce a viable genotyping platform that is able to correctly predict unusual Rh phenotypes, the most complex of blood groups. After DNA extraction, approximately 6–8 h processing (PCR, labelling, fragmentation, hybridisation, and data interpretation) is required before the genotype is determined. For this reason, BLOODchip is not intended for use in emergency situations although in the future large banks of genotyped blood may significantly aid electronic cross-matching.

Bloodgen Workpackage Structure

The workload of the project was carried out in designated workpackages. *Workpackage (WP) 1* concerned the fabrication of arrays and involved the design of probes complementary to the target DNAs containing the blood group-specific SNPs.

WP2 involved the development of fluoro-single-sequence primer assays. This WP was subsequently discontinued due to technical issues.

WP3 involved standardisation of DNA extraction and optimisation of MPX PCR assays. The MPX PCRs were designed

following the generation of a list of required blood group SNPs that should populate the array. The labelling and fragmentation protocols were also optimised in this WP.

WP4 was a small-scale clinical trial. A biobank of extremely rare genomic DNA derived from individuals with Rh blood group phenotypes. These samples were then analysed with a prototype version of BLOODchip.

WP5 was a multi-centre clinical trial. Most Bloodgen participants then conducted a large-scale analysis of a cohort of genomic DNA samples obtained from blood donors, patients, newborns, and known weak D phenotype individuals.

Bloodgen formally finished with a dissemination event held at the International Society Blood Transfusion (ISBT) congress in Cape Town in September 2006. Progenika Biopharma (Derio, Spain) then continued to develop BLOODchip and completed the CE marking exercise with DNA samples provided by the consortium.

Current Use Of Blood Group Genotyping and How BLOODchip May Assist These Situations

Taking aside the aforementioned applications in fetal blood group genotyping, there are several instances where genotyping for blood group status has been applied. One major application has been in defining the blood group genotype in multi-transfused patients where the presence of transfused blood makes it very difficult to ascertain the blood group of an individual by serology. DNA-based typing had been utilised for over a decade in the management of multi-transfused patients [24–26]. Conventional PCR-SSP (PCR with sequence-specific priming) approaches to type the recipient have been found to have low false-positive results, probably due to the fact that donor blood will be predominantly enucleated red blood cells devoid of DNA. One would anticipate that use of BLOODchip in such circumstances would also be unlikely to generate false-positive results. Clinical trials with BLOODchip and such samples are underway. Other studies have included the quality assurance of D-negative red cell units, to detect RhD variants that may not have been detected using conventional serological techniques. Molecular typing for RhD variants is much simpler than a serological investigation, which in theory would require a large battery of monoclonal anti-D and rare human polyclonal antisera to low-frequency antigens associated with partial D antigen (for example, BARC expressed in DVI; Tar on DVII red cells). Commercially available sequence-specific primer kits have been described [27], which test for a number of partial and weak D alleles, but by no means all. However, a major focus of the Bloodgen project was to produce a genotyping platform that could genotype the vast majority of RhD variants that are caused by either hybrid *RHCE-RHD* genes (for example DVI) [28–31] or point mutations (e.g. partial D, DNU [32] and weak D types [33, 34]). This is achieved by a combination of exon scanning, which is a process that entails amplifying every *RHD* exon and BLOODchip detecting *RHD* exons by having a specific probe set for each. Drop-out of *RHD* exons can then be readily detected, and a characteristic pattern corresponding to hybrid *RHD-RHCE* genes can be deciphered and predicted. A probe set for each SNP causative of the vast majority of RhD variants (namely partial, weak, D-elute and D-negative) is present on BLOODchip version 1.0 and will be significantly extended on BLOODchip version 3.0.

Weakened Duffy b antigen expression was deciphered at the molecular level independently by three groups in 1998 [35–37]. This has allowed the determination of the Fy^{bweak} (or Fy^{x}) allele at the DNA level. Weakened Fy^{b} expression can cause issues in the provision of reagent red cells that are of presumed genotype *FY*B*/*FY*B,* i.e. they are phenotypically Fy(a– b+). If an individual has the relatively common genotype *FY*B*/*FY*X* then they would have substantially less Fy^{b} antigen on their red cells than a homozygous *FY*B*/*FY*B* individual. If the individual with weakened Fy^{b} (namely presumed incorrectly to be Fy^{b} homozygous) is selected as one of those for a red cell panel used to detect anti-Fy^{b} in patient sera, then there is suboptimal detection of this antibody. Such a scenario has been proven in a clinical situation [38]. True *FY*B* homozygosity can be defined by genotyping to aid quality assurance of red cell panels.

Performance of BLOODchip 1.0 in Clinical Trials

Tables 1 and 2 review the performance of BLOODchip in CE-marking clinical trails and reveal the high degree of accuracy that can be achieved using this platform being significantly more accurate than serology. There are several examples of mis-typings by serology identified by BLOODchip that may lead to alloimmunisations which of course should be avoided, especially in vulnerable patient groups and individuals (for example multi-transfused patients and women of child-bearing age). Analysis of the 'non-Rh' blood groups reveals the accuracy of BLOODchip especially in FY and MNS blood groups. Rare MNS variants that are difficult to detect by serology and Fy^{bweak} antigen expression are major contributors to the better performance of BLOODchip. However, the performance of BLOODchip with RhD typing is outstanding and reveals several scenarios, which will be reviewed here, that could be avoided if genotyping replaces conventional serology.

Mis-Typing Partial D Phenotype Samples as D-Positive by Serology

BLOODchip identifies five such samples in the cohort of 3,000: 2 DVI, 1 DHMi, 1 DNU, and 1 DV individual. If these individuals were mis-typed as patients they could become alloimmunised by being transfused D-positive blood, or failing to receive prophylactic anti-D at the end of the pregnancy carrying a D-positive child.

Table 1. CE marking exercise of BLOODchip, Rh CcEe, Kell, Duffy, Kidd, Dombrock, Diego and Colton antigens, versus serological typings, 1,000 genomic DNA samples (workpackage 5)

Blood group antigen (system)	Serological testing	BLOODchip	Comments
Rh C/c (RH)	998/1,000	999/1,000	
Rh E/e (RH)	997/1,000	*1,000/1,000*	includes one typographical error
C^{w+} (RH)	18*	28	*serological confirmation of genotype
C^{x+} (RH)	0*	2	*serological confirmation of genotype
VS+ (RH)	9*	15	*serological confirmation of genotype
K/k (KEL)	*1,000/1,000*	*1,000/1,000*	
Kp^a/Kp^b (KEL)	*358/358*	357/358	
Js^a/Js^b (KEL)	122/123	*123/123*	
Jk^a/Jk^b (JK)	596/597	597/597	
$Fy^a/Fy^b/Fy(a– b–)$ (FY)	498/506	506/506	
MN (MNS)	425/455	455/455	
Ss (MNS)	479/483	483/483	
Di^a/Di^b (DI)		120/120	confirmed by DNA sequencing
Do^a/Do^b/ (DO)		120/120	confirmed by DNA sequencing
Co^a/Co^b (CO)	169/170	170/170	

Mis-Typing Partial D Phenotype Samples as Weak D by Serology

Almost all (but definitely *not* all) [39] partial D phenotype samples have weakened expression of D antigen. Thus these may be typed serologically as weak D. However, if they are of known partial D type, then transfusion of D-positive blood to such individuals should be avoided, and in theory at least they should receive prophylactic anti-D during pregnancy as above. There has been some debate as to the classification of partial D and weak D [40], but any resultant nomenclature should communicate the risk of alloimmunisation to D antigen in each RhD variant as on the whole 'classical' weak D phenotypes are less prone to RhD alloimmunisation, whereas partial D are.

Mis-Typing Partial D Phenotype as D-Negative by Serology

One such sample was found in the cohort of 3,000: a DIIIc phenotype individual. If such a sample is used for transfusion to a D-negative recipient, then there is the chance of producing anti-D. DIIIc phenotype individuals have relatively high D antigen site numbers [39], so its unclear why this was mistyped serologically but its identification within the donor cohort reveals it a risk that can be eliminated by mass-scale application of genotyping.

Mis-Typing Weak D and D-Elute Phenotypes as D-Positive by Serology

There were several examples of this detected by BLOODchip including weak D type 2, 3, 4, and 11. Particular weak D (and D-elute) phenotypes have known to become alloimmunised when transfused with D-positive blood [33, 41, 42], (weak D type 4.2 and 15 and D-elute RHD (IVS3+1g>a)) hence genotyping is able to identify such individuals, and their transfusion management can be adjusted accordingly. Interestingly, no samples amongst the cohort studied here were identified as weak D by BLOODchip but were RhD– by serology, indicating the effectiveness of monoclonal anti-D reagents used by the consortium. In one study, a D-negative recipient received a unit of weak D type 2 red cells that had been mis-typed serologically as D-negative and as a consequence had become alloimmunised [41]

Mis-Ttypings by BLOODchip

There were two examples of BLOODchip scoring an individual as D-positive but was in fact detected as weak D by serology. The most likely outcome in this situation is that the individual carries an unknown *RHD* allele. BLOODchip has sufficient capacity to add all new RhD variants as they become described – indeed BLOODchip version 3.0 has 18 new *RHD* alleles added to it. It is hoped that these types of mis-scoring will be diminished if not eliminated when more widespread application of genotyping takes place.

Future Use of Blood Group Genotyping

The Bloodgen consortium have for many years been advocating the mass-scale application of genotyping as an alternative to blood group serology. Small-scale trials have already proven the effective superiority of genotyping-based approaches in a head-to-head comparison with serological investigations (see table 1). Where genotyping completely outperforms serology is in the analysis of variant RhD and RhCE individuals, the detection of Fy^{bweak}, and unusual MNS variants. All of these situations are clinically relevant – partial D phenotype individuals can readily become immunised producing anti-D by either transfusion or pregnancy. *RHCE* (and *MNS*) variant alleles can cause significant problems in multi-transfused sickle cell patients [43. 44]. However, it must be stressed that at present BLOODchip typing has focussed largely on blood donors and to be truly effective typing of both patients and donors should be done to achieve most effective matching.

Table 2. Performance of BLOODchip version 1.0 versus serology; RhD investigationsa

Discordant results BLOODchip version 1.0 versus serology			
Number	genotype (defined by BLOODchip)	BLOODchip	serology
a) Data entry errors in serology (3)			
613	DVI type II	partial D	RhD+
3693	DVI type II	partial D	RhD+
3527	weak D type 1	weak D	RhD–
b) Partial D by BLOODchip version 1.0 and RhD+ by serology (5)			
1993	DVI type IV	partial D	RhD+
2824	DNU	partial D	RhD+
4154	DVI type II	partial D	RhD+
4571	DVa type 2 (Hus) / DV type 7	partial D	RhD+
4676	DHMi	partial D	RhD+
c) Partial D by BLOODchip version 1.0 and weak D by serology (7)			
926	DCS	partial D	weak D
1054	DVI type II	partial D	weak D
1106	DVI type II	partial D	weak D
1996	DVI type IV	partial D	weak D
4654	DVI type II	partial D	weak D
5317	DVI type II	partial D	weak D
7632	DVI type II	partial D	weak D
d) Partial D by BLOODchip version 1.0 and RhD– by serology (1)			
2253	DIIIc	partial D	RhD–
e) Weak D by BLOODchip version 1.0 and RhD+ by serology (6)			
615	weak D type 2	weak D	RhD+
3277	RHD (M295I)_possible ht Del / weak D type 11	weak D	RhD+
3586	weak D type 2	weak D	RhD+
4450	weak D type 3	weak D	RhD+
4978	weak D type 4	weak D	RhD+
7284	weak D type 4	weak D	RhD+
f) Del by BLOODchip version 1.0 and RhD+ by serology (1)			
2874	RHD (L153P)	Del	RhD+
g) Del by BLOODchip version 1.0 and weak D by serology (1)			
6769	RHD (M295I)_ Del	Del	weak D

Discrepancies against BLOODchip version 1.0			
Number	genotype (defined by BLOODchip)	BLOODchip	serology
RhD+ by BLOODchip version 1.0 and weak D by serology (2)			
3694	'Apparently non-negative'	RHD	weak D
3695	'Apparently non-negative'	RHD	weak D

[a]The table shows the results of analysing 3,000 samples with BLOODchip version 1.0 and comparing the results directly with serology. The only two discrepant samples against BLOODchip (bottom section of table) are most likely novel RhD variants and are currently undergoing DNA sequence analysis. Data from Shinnapapa et al. (manuscript in preparation).

Eventually it may be economic to genotype every individual at birth. In that circumstance screening for inborn errors of metabolism (as suggested by many health authorities) could be coupled with both HLA and blood group genotyping. This information could then be highly useful to an individual as either a future patient or blood/organ donor during their lifetime.

Several genotyping platforms are now commercially available that gives the opportunity for blood banks to apply this technology for routine testing of their donors. This has a cost implication, and it is imperative that this is considered alongside the life-threatening circumstances of becoming alloimmunised as either a multi-transfused patient or an expectant mother. For this reason, studies involving the health-economic evaluation of the impact of mass-scale blood group genotyp-

ing need to be conducted. There is no doubt that molecular genotyping has a significant edge over serology – several studies in addition to the Bloodgen project have proved this. The ball is now in the court of transfusion services to take these improvements and translate them into safer blood supplies and transfusion policies.

Disclosure

A. Martinez, D, Tejedor, M. López and E. Jiménez are employees of Progenika Biopharma S.A.

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